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OVEREXPRESSION OF A β-GALACTOSIDE BINDING PROTEIN CAUSES TRANSFORMATION OF BALB3T3 FIBROBLAST CELLS

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Overexpression of an animal lectin, rat β-galactoside binding protein (GBP) in mouse BALB3T3 fibroblast cells by stable introduction of a GBP cDNA expression plasmid results in the acquisition of transformed phenotype which includes a loss of anchorage dependence, reduced contact inhibition, colony formation in soft agar, and tumor formation in nude mice. The transformation depends on the level of the expression of both GBP and TGFγ2 activities confirming that both activities are ascribable to a single bifunctional protein TGFγ2/GBP. The results indicate that GBP acts as a growth regulator and is directly involved in regulation of cell proliferation.

Many vertebrate tissues contain β-galactoside binding lectins (1-4). A 14kDa β-galactoside binding protein (GBP) is concentrated in cytoplasm and extracellular compartments of many embryonic and adult tissues and has a strict sugar-binding specificity (5-8). Ubiquitous and relatively abundant distribution of GBP suggests its involvement in fundamental functions in the development or maintenance of cells in tissues, although its physiological function remains totally unidentified.

We have previously identified a growth factor, TGFγ2, from an avian sarcoma virus transformed rat NRK cell line, 77N1, which shows stimulation of DNA synthesis and the promotion of anchorage independent growth of BALB3T3 fibroblast cells (9,10). Purification and determination of the partial amino acid sequence of TGFγ2 led us to an unexpected finding that TGFγ2 and rat GBP (11) are identical or closely related proteins. In fact, the TGFγ2 purified from rat 77N1 cells showed GBP activity and, in turn, GBP purified from rat lung showed TGFγ2 activity (manuscript in preparation). In this report, we show that overexpression of GBP in BALB3T3 fibroblast cells by stable introduction of a rat NRK GBP cDNA resulted in the acquisition of transformed phenotype quite similar to that caused by treatment of cells with exogenous TGFγ2.

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Methods

Transformation of BALB3T3 cells by transfection of a GBP cDNA expression plasmid: Rat GBP cDNA was isolated by a PCR cloning method (12). cDNA was synthesized using poly (A)$^+$RNA from 77N1 cells as a template and oligo (dT) as a primer, followed by amplification by a polymerase chain reaction (PCR) using oligonucleotides PR1 (5'-TCTCTAGA AATCTCTTCGCTTCAATCATGGC-3') and PR2 (5'-TCTCTAGATTCACTCAAAGGCCACACACTTA-3') as primers. PR1 and PR2 contain the sequences -18 ~ +5 and that complementary to +387 ~ +409 of rat lung GBP cDNA, respectively, in addition to the XbaI recognition sequence in their 5'-portions. The amplified fragment cloned into the XbaI site of pUC18 was then sequenced and confirmed to encode a full-length GBP cDNA. The GBP cDNA was inserted into the EcoRI site of the expression vector pSRD (13) which contains the SV40 early promoter/enhancer and an HTLV-IR sequence to construct a GBP cDNA expression plasmid pSRD-GBP. Transfection of BALB3T3 cells was carried out by the standard calcium phosphate coprecipitation procedure (14). BALB3T3 cells ($5x10^5$) in 60 mm dish were transfected with pSV2neo (1μg), which carries a neomycin resistance gene, and the GBP cDNA expression plasmid pSRD-GBP (15 μg). The transfected cells were cultured in a selection medium containing 400 μg/ml G418 (Geneticin) and G418-resistant clones (1 to 6) were isolated after 2 weeks. Transfection of cells with pSV2neo alone resulted in the isolation of a G418-resistant clone 20.

Immunoblot analyses: Samples were electrophoresed, transferred to a polyvinylidene difluoride sheet (Immobilon; Millipore) by electrophoresis. The filter was blocked by incubation in milk-TBS (20 mM Tris-HCl, pH7.4, containing 0.9% NaCl and 0.1% Tween 20) and then, treated with anti-TGFγ2 rabbit serum (15) for one hour, and treated with a "Blotting Detection Kit" (Amersham). Samples were electrophoresed on 15% polyacrylamide gels under reducing conditions by the method of Laemmli (16). Mr values were determined by comparison with prestained protein standards (Rainbow markers RPN755, Amersham).

Assay for DNA synthesis and colony formation: Methods were described previously (10).

Results

Introduction of GBP cDNA in BALB3T3 cells results in the expression of both TGFγ2 and GBP activities

In order to confirm the identity of TGFγ2 to GBP, we isolated cDNA clones from 77N1 cells by a PCR cloning procedure on the basis of the published sequence information of rat lung GBP cDNA (11). The cloned nucleotide sequence showed the complete identity to that of rat lung GBP and, thus, the deduced amino acid sequence contained those of peptides derived from TGFγ2 isolated from the rat NRK 77N1 cells (manuscript in preparation). A GBP cDNA expression plasmid was then introduced into BALB3T3 cells by cotransfection with pSV2neo and six independent G418 resistant cell clones (clones 1-6) were isolated for further characterization. To ascertain the expression of introduced GBP cDNA in these transfectants, we analyzed the cell extracts by immunoblotting using an antiserum raised against TGFγ2 purified from 77N1 cells (15). This antiserum reacts with a 14kDa GBP isolated from rat lung as well as the 14kDa TGFγ2 which was used as an antigen (Fig. 1. A). When examined for cell extracts, this antiserum recognized a 14kDa band for 77N1 cells, and cell clones 1, 3, and 6 (Fig. 1. J, C, E, and H).

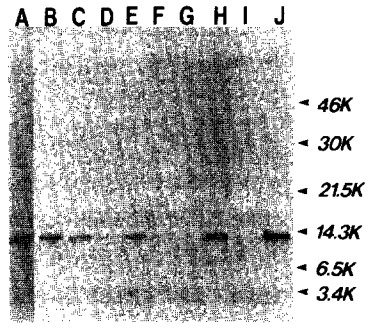

Fig.1. Immunoblot detection of overexpressed TGFγ2/GBP in the GBP cDNA transfected cell clones. Cell extracts from each clone (20μg) were electrophoresed and analyzed by immunoblotting with anti-TGFγ2 antibody. A, purified lung GBP (10ng); B, TGFγ2 (10ng); C-H, clones 1-6, respectively; I, parental BALB3T3; J, 77N1.

A very weak band was detected for BALB3T3 cells, and cell clones 2, 4, and 5 (Fig. 1. I, D, F, and G).

Then, we analyzed both GBP and mitogenic activities of the cell extracts. As shown in Table 1, the cell extracts from clones 1, 3, and 6 showed GBP activities measured by means of hemagglutination assay. Further, they showed a dose-dependent stimulation of DNA synthesis on BALB3T3 cells. The DNA synthesis activity was abolished by heating at 100°C for 3 minutes or by treatment with 1 M acetic acid or 65 mM dithiothreitol, typical feature of TGFγ2 activity distinguishable from other known growth factors (18-22). Thus, the expression of both of the two distinct activities, GBP and TGFγ2 activities, are associated with the appearance of the 14kDa band immunoreactive with the antiserum for TGFγ2. The results are quite consistent with the notion that TGFγ2 and GBP are an identical protein. In other words, a single protein TGFγ2/GBP posseses both TGF and GBP activities.

Overexpression of a GBP cDNA in BALB3T3 cells leads to the expression of the transformed phenotype

The transfected BALB3T3 cell clones (clones 1-6) were examined for growth properties. We first examined their morphology in monolayer

Table 1. DNA Synthesis and Hemagglutination Activities in Cell Extract from Each Stable Clone

	^{125}[I]Iododeoxyuridine Incorporation (x10^{3}cpm)[a]				Hemagglutination[b]
	Dose (μg)				Activity
Cell	4	12	20	40	
BALB3T3	0.6	0.6	0.3	0.3	-
77N1	4.8	18.0	35.3	47.7	+
BALB3T3 Clone 1	0.7	2.5	4.0	7.4	+
2	0.5	0.1	0.2	0.1	-
3	1.4	1.3	3.6	6.9	+
4	0.5	0.1	0.1	0.6	-
5	0.4	0.2	0.1	0.1	-
6	2.3	9.1	19.4	39.1	+

[a]DNA synthesis activity was determined on parental BALB3T3 cells. The incorporation of labeled materials by a 5% fetal bovine serum stimulated well was 40.1 x 10^3 cpm .

[b]Cell extract (20 μg) was used to determine hemagglutination activity on rabbit red blood cells.

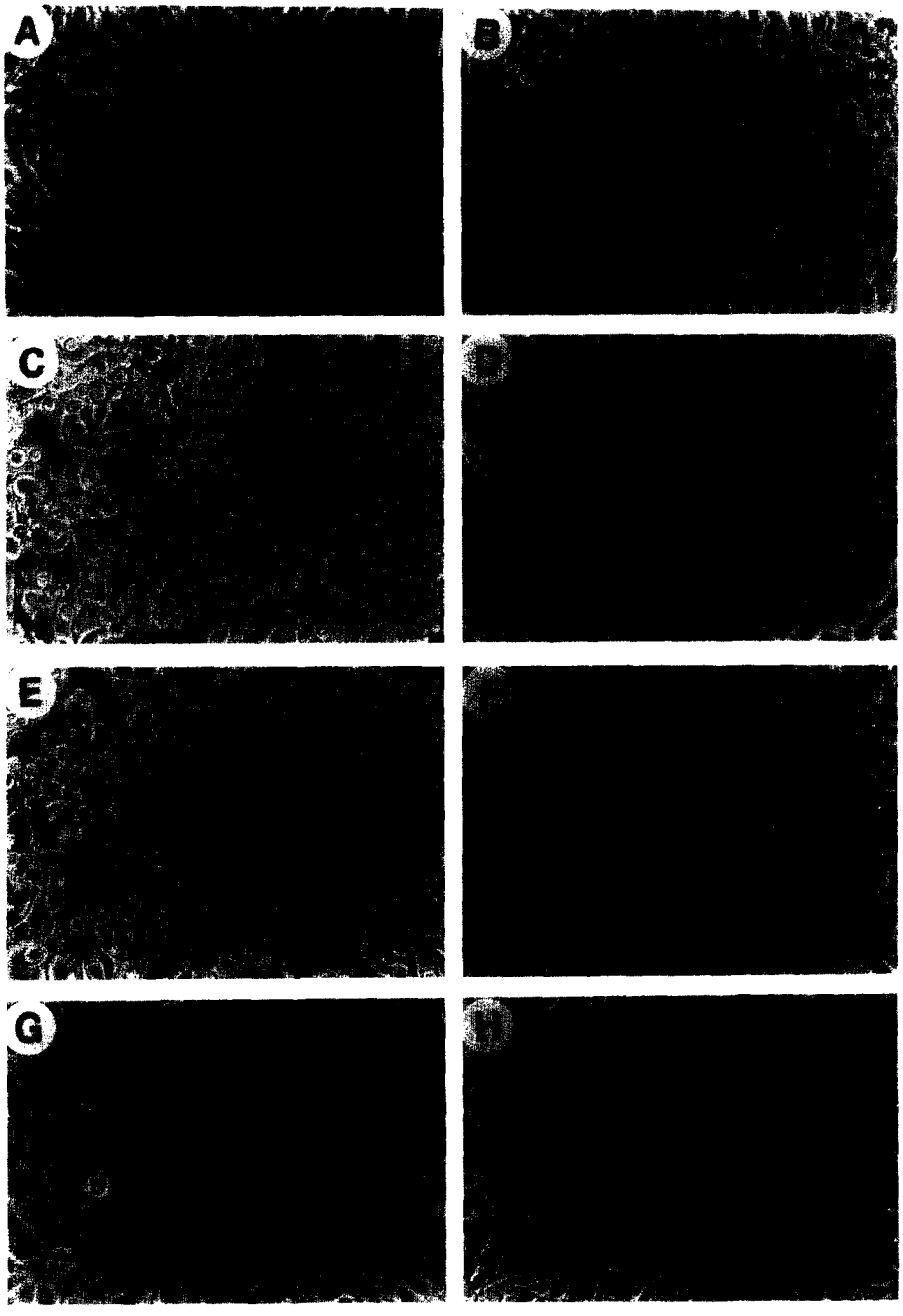

Fig.2. Morphology of stable transfectants of BALB3T3 cells. Cells ($2x10^5$) plated with 5 ml of DMEM containing 10% fetal bovine serum in 60 mm dish were cultured for 4 days and photographed. A, parental BALB3T3; B, clone 20; C-H, clones 1-6, respectively.

culture in comparison with that of the parental cell line BALB3T3. As shown in Fig. 2, three clones (clones 2, 4, and 5) showed the typical cobble stone morphology (Fig. 2D, F, and G) and contact inhibition of cell growth like the parental BALB3T3 cells (Fig. 2A). Clones 1, 3, and 6, which expressed a 14kDa protein and DNA synthesis activity, showed a

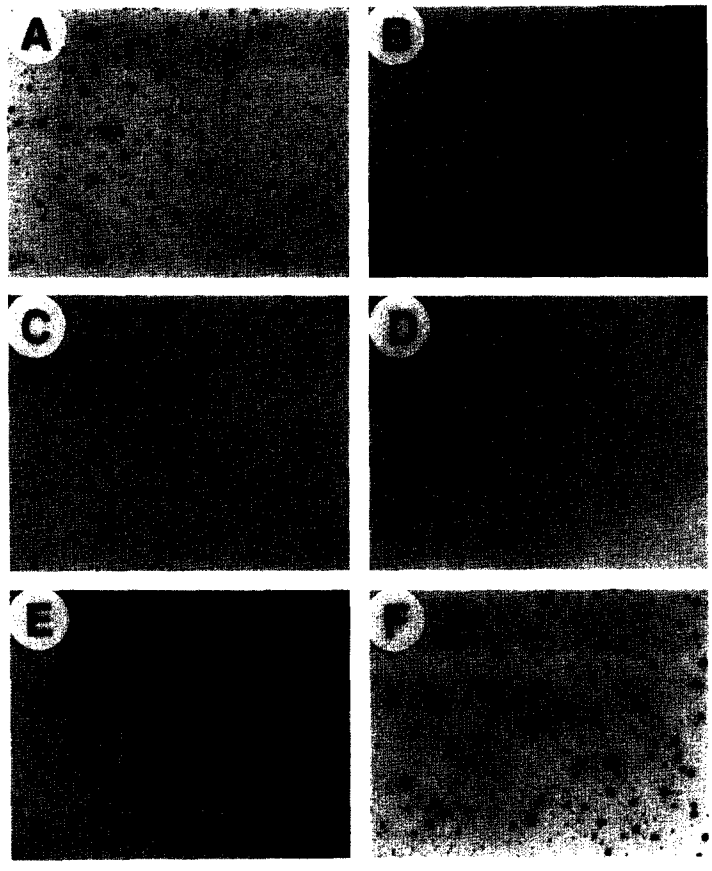

Fig.3. Colony formation of TGFγ 2/GBP cDNA transfected cell clones in soft agar. Each transfected cell clone was seeded in 0.3% soft agar in DMEM containing 5% fetal bovine serum and photographed. A-F , clones 1-6, respectively.

criss-cross pattern (Fig. 2C, E, and H). We also examined these clones for their ability to form colonies in soft agar, because the acquisition of anchorage independent growth correlates with in vivo tumorigenicity. The morphologically transformed cells in monolayer culture grew well and showed anchorage independent growth in soft agar, with colony efficiencies of 18.5%, 7.5%, and 49.8%, respectively (Fig. 3A, C, and F). Clones 2, 4, and 5 did not show these activities (Fig. 3B, D, and E). Thus the expression of the 14kDa protein correlates with morphological transformation and anchorage independent growth in soft agar.

Furthermore, clone 6 cells induced tumor formation in nude mice within two weeks after injection. On the other hand, mice injected with parental BALB3T3 cells failed to form tumors for more than 4 month (data not shown). These results indicate that GBP expressing BALB3T3 cells are transformed.

Growth stimulatory activity of TGFγ 2/GBP from transformed BALB3T3 cells is independent of GBP activity

The identity of TGFγ 2 to GBP raised an intriguing question whether GBP activity is involved in the TGF activity. As described above, the

Table 2. The Effect of Saccharides on DNA Synthesis and Hemagglutination Activities

Saccharide	DNA Synthesis Activity concentration (mM) 1	5	25	50	Inhibitory Activity of Hemagglutination
α-D(+)-Melibiose	110.0	102.7	120.3	97.2	-[a]
D(+)Galactose	111.3	106.3	128.4	100.7	-[a]
Thiodigalactoside	97.4	99.6	105.0	96.0	+[b]
Lactose	96.6	99.7	95.6	98.0	+[c]

DNA synthesis activity of cell extract (20μg) from clone 6 with each dose of saccharides was determined on parental BALB3T3 cells. Results are expressed as the percentage of activity compared with that without saccharides. [a]No inhibitory effect at a dose 50 mM. [b]Effective at a dose more than 0.5 mM. [c]Effective at a dose more than 5 mM.

extract of clone 6 caused stimulation of DNA synthesis under the conditions where the extracts of parental cells do not cause such stimulation. Thus, we next examined the effects of the addition of excess amount of various saccharides on the activity to stimulate DNA synthesis. As shown in Table 2, the activity was not affected by any saccharides examined, although the GBP activity was completely affected in a sugar-dependent manner when examined by hemagglutination assay.

Discussion

In the present study, we presented evidence indicating that overexpression of GBP by stable introduction of GBP cDNA caused transformation of BALB3T3 fibroblast cells. This implies that GBP is directly involved in regulation of cell proliferation. Furthermore, expression of both the GBP activity and transformed phenotype was associated with the expression of TGFγ2 determined on the basis of immuno-blotting using anti-TGFγ2 antiserum and the activity to stimulate DNA synthesis. This strongly supports the notion that both TGFγ2 and GBP are identical protein and that the expression of the transformed phenotype is the results of the autocrine action of a single bifunctional protein TGFγ2/GBP. Although there remains a possibility that the increase of the expression of TGFγ2 in cell clones overexpressing GBP is merely a secondary effect of the overexpression of GBP, this is quite unlikely considering our independent observation that TGFγ2 and GBP purified from different sources by different procedures show both TGFγ2 and GBP activities (manuscript in preparation). Thus, it is quite likely that both TGFγ2 and GBP activities are ascribable to a single bifunctional protein TGFγ2/GBP.

Recently Wells and Mallucci have shown that an autocrine negative growth regulator, secreted from mouse embryonal fibroblasts (MEFs) and inhibits the growth of MEFs in a cytostatic manner, contained the sequence of GBP and that recombinant GBP showed both GBP and the growth regulator activities (23). This is quite consistent with our results that GBP shows TGFγ2 activity. Furthermore, this also suggests an interesting possibility that GBP acts as both positive and negative

growth regulators depending on the cell type examined. In fact, the negative growth regulator isolated from MEF had no inhibitory effect on Swiss 3T3 and rat-1 fibroblast cells (24). Further, we have observed TGFγ 2 inhibited growth of human thyroid epithelial cells in serum and thyroxine dependent growth conditions, although it showed mitogenic activation of rodent fibroblasts such as BALB3T3 (manuscripts in preparation). Thus it is quite likely that GBP acts as both positive and negative growth regulators as demonstrated for TGFβ (25).

The observation that growth stimulatory activity of TGFγ 2/GBP is not inhibited by excess amount of β -galactoside indicates that both TGF and GBP activities share distinct molecular determinants. Although the precise roles of the two distinct activities on cell proliferation and transformation remain to be determined, it is tempting to speculate that GBP is stored as a latent TGFγ 2 molecule with the GBP activity in normal cells where contact inhibition is observed and some physiological alterations in the cells lead to the "release" of the TGF activity. These alterations may include changes in cell-cell interaction. Such a model can explain the relatively abundant expression of GBP in a variety of tissues.

The regulation of the growth factor activity of TGFβ by interaction with another protein is also known. In this case, TGFβ is complexed with an inhibitory masking protein (26,27). In contrast, TGFγ 2/GBP contains another functional unit with GBP activity that may determine the spatial position of the protein itself. The GBP activity of TGFγ 2/GBP may thus serve as a regulatory switch for the TGF activity making a unique mechanism of growth factor action. The ubiquitous and rather abundant distribution of TGFγ 2/GBP in various animal tissues makes a unique situation among the transforming proteins so far identified. It is possible that TGFγ 2/GBP functions as a regulator not only of cell proliferation, but also of cell differentiation and development where the significance of cell-cell interaction is suggested.

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