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Expression of the Cholera Toxin B Subunit in the Golgi Apparatus of Swiss 3T3 Cells Inhibits DNA Synthesis Induced by Basic Fibroblast Growth Factor[1]

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We attempted to express the cholera toxin B subunit (CTXB) in the Golgi apparatus of cultured mammalian cells by means of gene transfection. Complementary DNA of CTXB was ligated with the Golgi-retention signal sequence of human β1,4 galactosyltransferase cDNA, and the chimeric gene yielded was inserted into a mammalian expression vector. The resultant construct was transfected into COS-1 cells for transient expression and into Swiss 3T3 cells for stable expression. The expression of a fusion protein encoded by the chimeric gene was demonstrated according to the following criteria: first, detection of a protein exhibiting the expected molecular mass on Western blot analysis using an anti-CTXB antibody; second, detection of the protein located in the Golgi area by indirect immunofluoresence microscopy; and third, detection of GM1 binding activity in cell lysates. Stable transformants satisfying the above criteria were subjected to an assay for mitogen-induced DNA synthesis. These transformants exhibited significantly lower DNA synthesis than mock transfection cells on stimulation with basic fibroblast growth factor (bFGF), whereas the two types of cells exhibited similar responses to 10% fetal calf serum and other mitogens, such as epidermal growth factor, 12-*O*-tetradecanoylphorbol-13-acetate, calcium ionophore A23187, and platelet-derived growth factor. Analysis of the binding of radio-iodinated bFGF to the cells revealed that the transformants did not exhibit a significant decrease in the binding affinity or the number of high affinity sites. These results suggest that the fusion protein specifically inhibits the bFGF signaling not at the binding step but rather at a later step(s) triggered by the binding.

Key words: basic fibroblast growth factor, cDNA transfection, cholera toxin B subunit, DNA synthesis, ganglioside.

The cholera toxin B subunit (CTXB) binds to GM1 ganglioside, Galβ1-3GalNAcβ1-4[Siaα2-3]Galβ1-4Glcβ1-1ceramide, and is used as a probe for investigations on GM1-mediated cellular responses. Spiegel and Fishman reported that CTXB had bimodal effects on cellular growth depending upon the growth state, *i.e.* it induced the proliferation of confluent and quiescent Swiss 3T3 cells, but inhibited that of sparse and rapidly growing cells (*1*). We attempted to determine whether or not CTXB can modulate cellular growth *in vivo* as well as *in vitro*. A possible means of addressing this question would be a transgene experiment. This type of experiment has several potential advantages: first, CTXB can be expressed *in vivo* by transfecting its cDNA into an embryo; second, tissue-specific expression is possible through the use of specific promoters; and third, expression can be induced at a specific stage of development. In this context we first transfected cDNA of CTXB into cultured mammalian cells and then analyzed the effect of the expressed CTXB on cellular growth.

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Abbreviations: bFGF, basic fibroblast growth factor; CTXB, cholera toxin B subunit; EGF, epidermal growth factor; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; SDS, sodium dodecyl sulfate; Sia, sialic acid; TPA, 12-*O*-tetradecanoylphorbol-13-acetate. The nomenclature for gangliosides is based on the system of Svennerholm (1963) *J. Neurochem.* 10, 613-623: GM3, Siaα2-3Galβ1-4Glcβ1-1ceramide; GM2, GalNAcβ1-4[Siaα2-3]Galβ1-4Glcβ1-1ceramide; GM1, Galβ1-3GalNAcβ1-4[Siaα2-3]Galβ1-4Glcβ1-1ceramide; GD1a, Siaα2-3Galβ1-3GalNAcβ1-4[Siaα2-3]-Galβ1-4Glcβ1-1ceramide.

MATERIALS AND METHODS

Materials—The materials used in this work were as follows: restriction endonucleases from New England Biolabs (Beverly, MA, USA); the pUC18 and pUC19 vectors from Pharmacia (Uppsala, Sweden); the Klenow fragment of DNA polymerase and *Eco*RI linker from Takara Shuzo (Kyoto); Waymouth, HAM's F10 and Dulbecco's modified Eagle's media (DMEM), G-418, peni-

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cillin, and streptomycin from Life Technologies (Grand Island, NY, USA); fetal calf serum (FCS) from Boehringer Mannheim Yamanouchi (Tokyo); Immobilon membranes and Multiscreen-GV plates from Millipore (Bedford, MA); prestained molecular mass marker proteins from Bio-Rad Laboratories (Richmond, CA, USA); Triton X-100 and NP-40 from Nacalai Tesque (Kyoto); cholera toxin B subunit (CTXB) and an antibody against CTXB from List Biological Laboratories (Campbell, CA, USA); biotinylated or fluorescein isothiocyanate (FITC)-conjugated second antibodies and a Vectastain ABC kit from Vector Laboratories (Burlingame, CA, USA); basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), calcium ionophore A23187, insulin, platelet-derived growth factor (PDGF), and GM1 from Funakoshi (Tokyo); and [^{125}I]bFGF, and [^{125}I]iododeoxyuridine from Daiichi Pure Chemicals (Tokyo). GM1 was tritiated according to Novak *et al.* (*2*), and its specific radioactivity was 205 Ci/mol. A monoclonal antibody against human β1,4 galactosyltransferase was prepared in our laboratory (*3, 4*).

Plasmid Construction—A chimeric gene between cDNA of CTXB and the 5′ portion of human β1,4 galactosyltransferase cDNA was constructed as follows. Complementary DNA of CTXB was cut out from pCTBR2 (*5*) by digestion with *Bam*HI and *Pst*I, and then subcloned into the pUC18 vector. This construct was digested with *Eco*RI and *Ava*I to obtain a 3.0 kbp fragment, which contained the full-length cDNA of CTXB and most of the pUC18 vector. For the insertion of a Golgi-retention signal sequence upstream of CTXB cDNA, the 3.0 kbp fragment was ligated with a *Eco*RI–*Acc*III fragment (0.3 kbp) derived from human galactosyltransferase cDNA. Complementary DNA of the human galactosyltransferase had been isolated and sequenced in our laboratory, and its sequence had been registered under accession number D29805 in the EMBL/Gen Bank/DDBJ Data Bank.

For transfection into cultured mammalian cells, the chimeric gene was introduced into a mammalian expression vector, pCXN2 (*6*), as follows. The chimeric gene in pUC18 was digested with *Hin*dIII and the resultant *Hin*dIII site was blunt-ended with the Klenow fragment of DNA polymerase. The blunt-ended fragment was ligated with the *Eco*RI linker and then digested with *Eco*RI. The resultant fragment (0.6 kbp) was introduced into the *Eco*RI site of pCXN2 (pCXN2GB), and the construct was transfected into cultured mammalian cells as described below. The cDNA of human galactosyltransferase in pUC19 was also digested with *Eco*RI and the resultant fragment (1.4 kbp) was introduced into the *Eco*RI site of pCXN2 (pCXN2hGT).

Transfection of the Chimeric Gene into Cultured Mammalian Cells COS-1 cells were maintained in HAM's F10 medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Subconfluent COS-1 cells were harvested and transfected with either pCXN2, pCXN2GB, or pCXN2hGT by electroporation using a Gene Pulser (Bio-Rad Laboratories). The voltage and time constant were 220 V and 16 ms, respectively. The transfected cells were cultured for 48 or 96 h, and then subjected to Western blot analysis and immunocytochemistry.

Swiss 3T3 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Subconfluent cells were subjected to electroporation as described above. The voltage and time constant were 220 V and 2 ms, respectively. The transfected cells were subjected to G-418 selection (1 mg/ml) and the resultant colonies were isolated with cloning cylinders.

Western Blot Analysis—The cells were washed three times with phosphate-buffered saline (PBS) prior to solubilization with a buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), and 20% glycerol. The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), followed by semi-dry electroblotting using a BE-500 blotter (Bio Craft, Tokyo). Protein bands were transferred onto an Immobilon membrane and then detected with an appropriate first antibody, followed by Vectastain ABC staining using a biotinylated second antibody.

Immunocytochemistry—The cells were cultivated on a chamber slide glass (Nunc, Naperville, IL, USA), washed with PBS, fixed with 4% paraformaldehyde in PBS, and then treated with 0.05% Triton X-100 in PBS. The cells were incubated with an appropriate first antibody, followed by staining with a FITC-conjugated second antibody.

Assay for GM1 Binding Activity of the Fusion Protein—The cells were washed three times with PBS and then solubilized with 2% NP-40 in PBS on ice. After centrifugation at $10{,}000\times g$ for 20 min, the supernatant was collected. An aliquot of the supernatant (less than 150 μl) was mixed with nine volumes of butanol and the resultant mixture was incubated for 10 min on ice. After centrifugation at $10{,}000\times g$ for 20 min, the resultant precipitate was washed two times with 90% butanol in PBS. The precipitate was then suspended in the original volume of 2% NP-40 in PBS, and 90 nCi of tritiated GM1 ([^{3}H]GM1) was added to the suspension. After incubation at 37°C for 10 min, the suspension was subjected to butanol precipitation as described above. The precipitate was washed four times by successive butanol precipitation (W1–W4). The resultant precipitate was again suspended in 2% NP-40 in PBS, and 3 μg of non-tritiated GM1 was added to the suspension. After incubation at 37°C for 10 min, the suspension was subjected to butanol precipitation. This process was repeated twice (E1 and E2), which was regarded as specific elution. Each supernatant obtained on butanol precipitation was placed in a vial and its radioactivity was counted with a liquid scintillation counter.

DNA Synthesis Assay—Assay for DNA synthesis was performed by the method of Hirai *et al.* (*7*). Briefly, subconfluent cells were harvested, suspended in 0.5% FCS in DMEM, and then seeded onto a Multiscreen-GV plate at the cell density of 5×10^3 cells per well. After the cells had been cultivated for 24 h, they were stimulated with 10% FCS or various mitogens, such as bFGF (0.3 ng/ml), EGF (10 ng/ml), TPA (10 ng/ml), calcium ionophore A23187 (0.1 μM), insulin (2 μg/ml), and PDGF (5 μg/ml), in serum-free medium, which consisted of 50% each of DMEM and Waymouth medium supplemented with transferrin (2 μg/ml) and bovine serum albumin (20 μg/ml). After stimulation for 16 h, 135 nCi of [^{125}I]iododeoxyuridine was added to each well and the cells were further cultivated for 6 h. The cells were washed three times with saline and then treated with 5% trichloroacetic acid at 4°C

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for 15 min. The resultant precipitate was washed three times with 5% of trichloroacetic acid. The radioactivity incorporated into the precipitate was counted with a gamma counter.

Assay for [^{125}I]bFGF Binding to the Transformants—Assay for [^{125}I]bFGF binding to the cells was performed as follows. Subconfluent cells grown on a 6-well plate were washed three times with PBS, and then with a buffer consisting of 50 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, and 5 mg/ml bovine serum albumin. Serial dilutions of [^{125}I]bFGF in the same buffer were then added to the wells. After incubation on ice for 4 h, the cells were washed three times with the same buffer containing 250 μg/ml heparin, and then solubilized with a buffer consisting of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, and 1% Triton X-100, on ice for 40 min. The radioactivity recovered in this buffer was counted with a gamma counter. The K_d value was calculated by the method of Scatchard (*8*).

Ganglioside Analysis—Cells on a culture dish were washed three times with PBS, treated with 1 mM EDTA in PBS, and then harvested with a rubber policeman without trypsin treatment. The harvested cells were homogenized with 10 volumes of water. After lyophilizing the homogenate, cellular lipids were extracted successively with mixtures of chloroform/methanol (2 : 1, v/v), (1 : 1, v/v), and chloroform/methanol/water (30 : 60 : 8, v/v/v). The extracted lipids were subjected to DEAE Sephadex A25 column chromatography, mild alkaline treatment and reversed-phase column chromatography as described previously (*9*). The ganglioside fraction thus obtained was analyzed by thin-layer chromatography. The developing solvent used was a mixture of chloroform/methanol/0.4% $CaCl_2$ in water (55 : 45 : 10, v/v/v) or chloroform/methanol/5 N ammonium hydroxide/0.4% $CaCl_2$ in water (55 : 45 : 4 : 6, v/v/v/v). Gangliosides were detected with resorcinol reagent.

Protein Content—Protein concentrations were determined by either the dye-binding assay (Bio-Rad Laboratories) or the bicinchoninic acid assay (Pierce, Rockford, IL, USA), with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Expression of the Fusion Protein Encoded by the Chimeric Gene in COS-1 Cells—COS-1 cells transfected with pCXN2GB or pCXN2 were subjected to Western blot analysis using the anti-CTXB antibody. A positive band was only detected for the cells transfected with pCXN2GB, *i.e.* not for those transfected with pCXN2, mock transfection cells. The band corresponded to a molecular mass of about 100 kDa under non-reducing conditions on SDS-PAGE (Fig. 1), and about 22 kDa under reducing conditions (data not shown), suggesting that the fusion protein, with a predicted molecular mass of 20.5 kDa, forms a pentamer in COS-1 cells. The fusion protein thus expressed in COS-1 cells was located by immunocytochemistry. Positive signals were observed mainly in the perinuclear region as massive areas of staining together with a few granular ones in the cell body (Fig. 2a), suggesting that the fusion protein is mainly expressed in the "Golgi area." COS-1 cells were also transfected with pCXN2hGT containing cDNA of human galactosyltransferase and then subjected to immunocytochemistry using the anti-human galactosyltransferase antibody. The staining pattern was essentially identical with that described above (Fig. 2b). Thus we concluded that the fusion protein is expressed in the Golgi apparatus of COS-1 cells.

Expression of the Fusion Protein in Swiss 3T3 Cells—Sixty clones of transformants carrying pCXN2GB were isolated and each clone was subjected to Western blot analysis. Four of the sixty clones were found to express the fusion protein at a high level (GB cells). GB cells were subjected to immunocytochemistry and the fusion protein was detected in the "Golgi area" (Fig. 2e). Their staining patterns were essentially identical with that of transformants carrying pCXN2hGT (hGT cells).

We examined the GM1 binding activity in a detergent extract of GB cells. The cell extract was subjected to butanol precipitation to recover the fusion protein, and then the fusion protein was incubated with [^{3}H]GM1. The fusion protein was expected to bind [^{3}H]GM1 to form a complex. After washing the complex (W1-W4), [^{3}H]GM1 in the complex was eluted by adding non-tritiated GM1 (E1 and E2). The amount of radioactivity thus eluted was regarded as reflecting GM1 binding activity. When an extract of GB cells was subjected to the assay, a significant increase in radioactivity was detected in fraction E1 (Fig. 3), suggesting that the fusion protein in GB cells exhibits the GM1 binding activity. When the extract of Swiss 3T3 cells was used, the radioactivity gradually decreased, indicating a background level. The binding activities of the four clones of GB cells were found to be in the range of 0.3-1 pmol/mg protein, but those of hGT and mock transfection cells were within the background level.

bFGF-Dependent DNA Synthesis in the Transformants—The transformants were subjected to the DNA synthesis

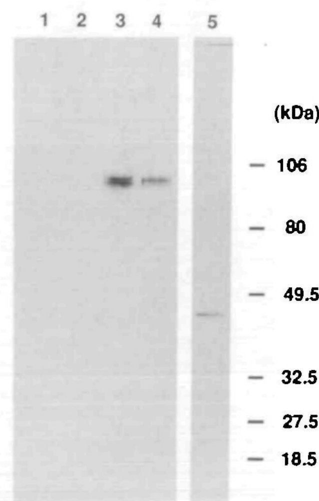

Fig. 1. **Expression of the fusion protein in COS-1 cells.** Cos-1 cells were transfected with pCXN2 (lanes 1 and 2) or pCXN2GB (lanes 3 and 4), and then harvested (after 48 and 96 h, respectively). Cellular proteins and authentic CTXB (lane 5) were separated by SDS-PAGE under non-reducing conditions and then subjected to Western blot analysis using the anti-CTXB antibody Bars indicate the positions of prestained molecular mass standards: rabbit muscle phosphorylase *b* (106 kDa), bovine serum albumin (80 kDa), hen egg ovalbumin (49.5 kDa), bovine carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa), and hen egg lysozyme (18.5 kDa).

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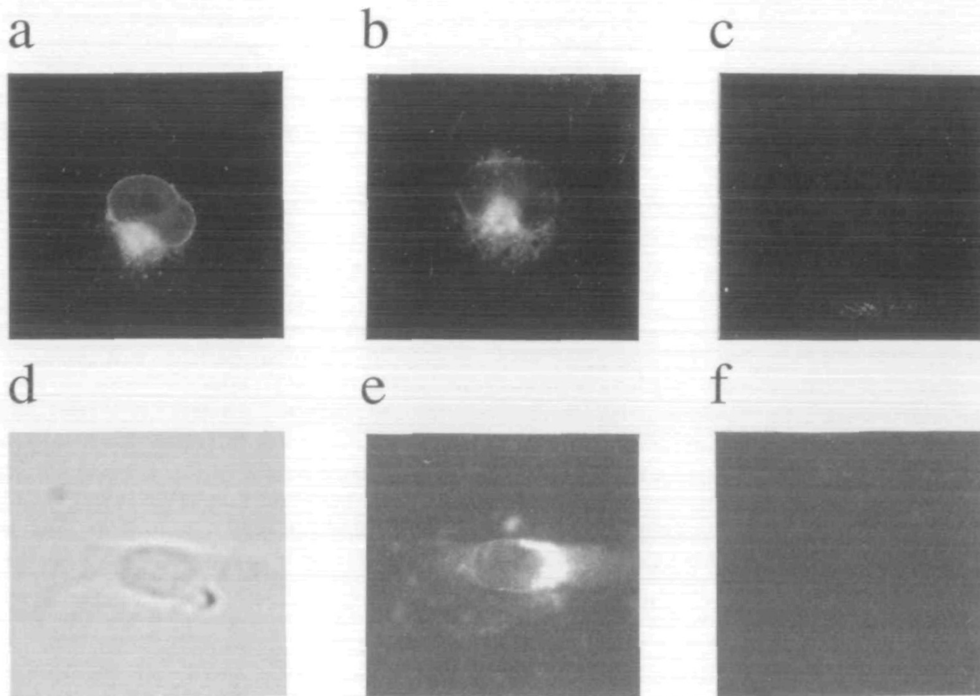

Fig. 2. **Golgi localization of the fusion protein.** COS-1 cells were transfected with pCXN-2GB (a), pCXN2hGT (b), or pCXN2(c), and then subjected to immunocytochemistry using the anti-CTXB antibody (a and c) or the anti-human galactosyltransferase antibody (b). Stable transformant cells, which carried pCXN2GB (d and e) or pCXN2(f), were also subjected to immunocytochemistry using the anti-CTXB antibody.

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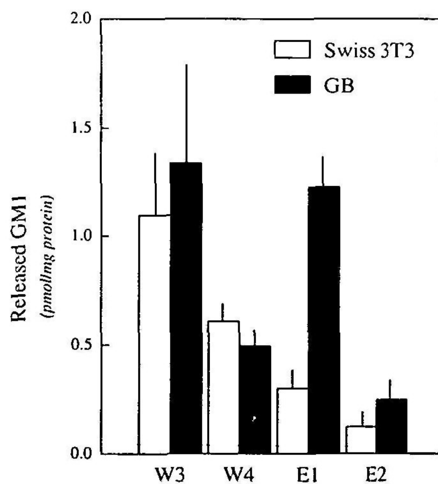

Fig. 3. **GM1 binding activity of GB cells.** Detergent extracts of Swiss 3T3 and GB cells were subjected to butanol precipitation. The resultant precipitates were suspended and incubated with [^{3}H]GM1, and then the suspensions were subjected to successive butanol precipitation (W1-W4). Finally, the resultant precipitates were incubated with 3 μg of non-tritiated GM1 and then subjected to butanol precipitation (E1 and E2). The radioactivity recovered in each supernatant was counted. Note that the level of radioactivity gradually decreased in the supernatants of Swiss 3T3 cells (open columns), whereas that in GB cells (shadowed columns) significantly increased in fraction E1 (*$p<0.01$, Student's t test). Each assay was performed in triplicate, and the standard deviation is indicated with error bars.

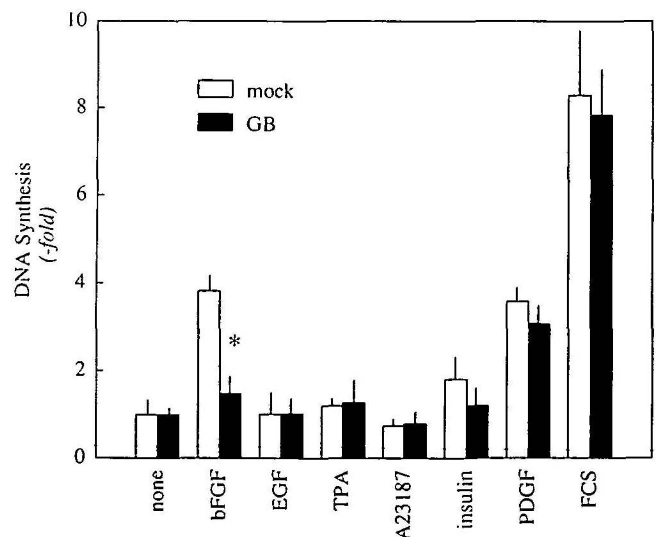

Fig. 4. **Effects of various mitogens on DNA synthesis by GB cells.** GB and mock transfection cells were cultivated with 0.5% FCS in DMEM for 24 h, and then stimulated with 10% FCS and various mitogens, such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), calcium ionophore A23187 (A23187), insulin and platelet-derived growth factor (PDGF). DNA synthesis was monitored as the incorporation of ^{125}I-labeled deoxyuridine. Note that the incorporation of radioactivity into GB cells was significantly lower than that into mock transfection cells when they were stimulated with bFGF (*$p<0.01$, Student's t test). Each assay was performed in triplicate, and the standard deviation is indicated with error bars.

assay, the cells being stimulated with 10% FCS and various mitogens, such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), calcium ionophore A23187, insulin, and platelet-derived growth factor (PDGF). When bFGF was used as the mitogen, the GB cells exhibited significantly lower DNA synthesis than mock transfection cells (Fig. 4). This lower response of GB cells was not due to a potential inability of DNA synthesis because their response

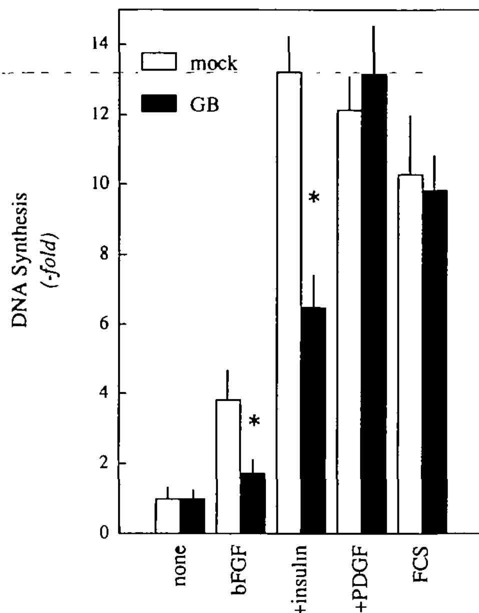

Fig. 5. **Effect of the combination of bFGF and insulin or PDGF on the DNA synthesis by GB cells.** Note that mock transfection cells exhibited a 13-fold increase in DNA synthesis but GB cells only exhibited a 6-fold one when the combination of bFGF and insulin (+insulin) was employed (* $p<0.01$, Student's t test). Both types of cells exhibited a more than 12-fold increase with the combination of bFGF and PDGF (+PDGF). Each assay was performed in triplicate, and the standard deviation is indicated with error bars.

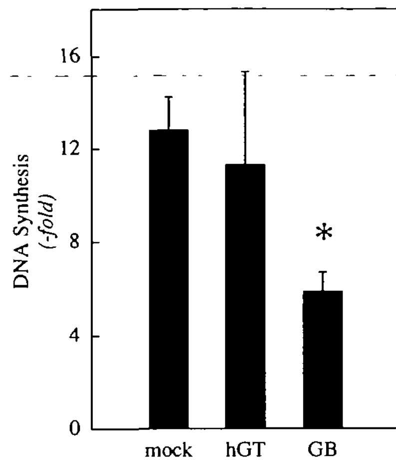

Fig. 6. **DNA synthesis by the transformants on stimulation with bFGF and insulin.** The clones of GB ($n=4$), hGT ($n=3$), and mock transfection cells ($n=3$) were stimulated with the combination of bFGF and insulin, and then their DNA synthesis was monitored. Note that GB cells exhibited significantly lower DNA synthesis than that of hGT or mock transfection cells (* $p<0.01$, Student's t test).

was almost the same as that of mock transfection cells when PDGF or 10% FCS was used. Combinations of bFGF and other mitogens were also examined. The combination of bFGF and PDGF resulted in a more than 12-fold increase in DNA synthesis in both types of cells, again indicating the potential ability of DNA synthesis of GB cells. When the combination of bFGF and insulin was used, the mock transfection cells exhibited a 13-fold increase but the GB cells only a 6-fold one (Fig. 5). This indicates that the effects of bFGF and insulin are more than additive, and that the phenotypes of the two types of cells were clearly distinguishable with this combination. The four GB cell clones were stimulated by the combination of bFGF and insulin, and were demonstrated to exhibit significantly lower responses than not only mock transfection cells but also hGT cells (Fig. 6). These results suggest that the fusion protein reduces bFGF-induced DNA synthesis by inhibiting a specific step(s) of bFGF signaling in GB cells.

Assay for [^{125}I] bFGF Binding to the Transformants—It has been established that bFGF forms a complex with heparansulfate proteoglycan on the cell surface and that this complex is then presented to a high affinity receptor to initiate the signal transduction (*10*). These processes might be inhibited by the fusion protein. We therefore examined the binding of [^{125}I]bFGF to GB cells. Scatchard analysis revealed that the K_d value and number of binding sites for GB cells were 805 ± 160 pM and $8.5\pm0.92\times10^4$ sites per cell, respectively, whereas these values for mock transfection cells were 725 ± 132 pM and $9.4\pm0.46\times10^4$ sites per cell, respectively. An experiment on the affinity cross-linking of [^{125}I]bFGF to the receptor did not reveal any difference in the cross-linking pattern between the above two cell types (data not shown). The lack of a significant difference in the binding affinity, the number of binding sites or the cross-linking pattern suggests that the fusion protein does not inhibit the interaction of bFGF with heparansulfate proteoglycan or with high affinity receptors, but does inhibit the signaling at a later step(s) in GB cells.

In the signal transduction pathway, the binding of bFGF to high affinity receptors leads to dimerization and autophosphorylation of the receptors for activation of the receptor kinases. The activated receptor kinases then phosphorylate several substrates such as phospholipase Cγ (*11*). These membrane associated events are followed by activation of mitogen-activated protein (MAP) kinases and end up enhancing the transcription of various cellular genes, such as those of dihydrofolate reductase, DNA polymerase α and thymidine kinase, which are involved in initiation of DNA synthesis (*12, 13*). In fact, mitogen-induced DNA synthesis was well correlated with the induced phosphorylation of MAP kinases in Swiss 3T3 cells. In addition, when protein kinase C was depleted in Swiss 3T3 cells, these responses were inhibited to the same extent on stimulation with various mitogens, such as TPA, bombesin, thrombin, EGF and PDGF (*14*). On the other hand, bFGF-induced responses were not affected by the depletion of protein kinase C. This raises the possibilities that there is a unique pathway for bFGF signaling upstream of MAP kinases, and that this pathway may be inhibited by the fusion protein. Further analysis is required to identify which step of the signaling is inhibited in GB cells.

Ganglioside Analysis of the Transformants—It has been documented that gangliosides and their metabolites, such as sphingosine and ceramide derivatives, modulate signal transduction induced by various mitogens (*15, 16*). These compounds might also modulate the signal transduction of bFGF and be involved in the inhibition of the signaling in GB cells. We therefore examined the effect of exogenous GM1 on the signaling induced by bFGF and insulin, expecting that the exogenous GM1 might reverse the reduced DNA synthesis of GB cells. On the addition of GM1 to

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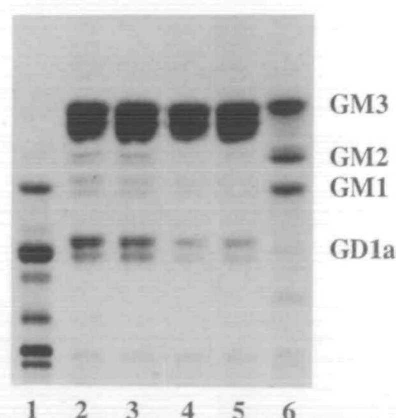

Fig 7. **Ganglioside patterns of stable transformants.** Ganglioside fractions prepared from Swiss 3T3, mock transfection, GB, and hGT cells (lanes 2, 3, 4, and 5, respectively) were analyzed by thin-layer chromatography using a solvent system of chloroform/methanol/0.4% $CaCl_2$ in water (55 : 45 : 10, v/v/v), followed by chemical detection with resorcinol reagent. Lanes 1 and 6 are mouse brain gangliosides, and a standard mixture of GM3, GM2, and GM1, respectively.

culture media at the final concentration of 5 μM, however, DNA synthesis by GB cells was inhibited by 30%, and the same extent of inhibition was observed with mock transfection cells. A lower concentration of GM1 did not have any effect on either type of cells. Thus we could not find any difference in the effect of exogenous GM1 between GB and mock transfection cells. It is possible, however, that endogenous gangliosides but not exogenous ones are involved in the inhibition of the signaling. Thus, endogenous gangliosides were analyzed as described below.

Ganglioside analysis revealed that the parent cells expressed Siaα2-3Galβ1-4Glcβ1-1ceramide (GM3) and Siaα2-3Galβ1-3GalNAcβ1-4[Siaα2-3]Galβ1-4Glcβ1-1ceramide (GD1a) as major gangliosides, with trace amounts of GalNAcβ1-4[Siaα2-3]Galβ1-4Glcβ1-1ceramide (GM2) and GM1 (*17*). Mock transfection cells exhibited an identical ganglioside pattern to the parent cells, whereas GB cells had less GD1a than the other two types (Fig. 7). The initial interpretation was that the fusion protein trapped cellular GM1 and thereby inhibited the biosynthesis of GD1a from GM1. This was not the case, however, because hGT cells carrying cDNA of human galactosyltransferase exhibited a similar ganglioside pattern to that of GB cells. This suggests that the ganglioside patterns of the two transformants are dependent upon the sorting signal portion, not the CTXB portion of the fusion protein. We could not find a specific change in the ganglioside pattern which was correlated with the inhibition of the signaling. It is still possible, however, that the fusion protein induced a specific change in ganglioside metabolism, such as in the turnover rates and subcellular localization of gangliosides. Further analysis is required to detect a specific change in the ganglioside metabolism in GB cells.

The results together suggest that CTXB can be expressed in an intact multicellular organism as a negative modulator of bFGF signaling. It has been established that bFGF is not merely a growth factor but also an inducer of cellular differentiation *in vivo*, *i.e.* it induced mesoderm formation in an early *Xenopus* embryo (*18*). The transfection of the chimeric gene used in the present experiment or other CTXB-related constructs to an embryo would be one of the possible means of modulating the inducer activity as well as the mitogenic activity of bFGF *in vivo*.

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