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Gangliosides as bimodal regulators of cell growth

(mitogen/cholera toxin/transformed cells)

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ABSTRACT **The B subunit of cholera toxin, which binds specifically to several molecules of ganglioside galactosyl-(β1→3)-*N*-acetylgalactosyminyl(β1→4)-[*N*-acetylneuraminyl(α2→3)]-galactosyl(β1→4)glucosyl(β1→1)ceramide (GM1) on the cell surface, stimulated DNA synthesis and cell division in quiescent, nontransformed mouse 3T3 cells in a dose-dependent manner. In addition, the B subunit potentiated the response of the 3T3 cells to other mitogens, such as epidermal growth factor, platelet-derived growth factor, and insulin. This synergistic effect indicates that the B subunit does not act identically to any of these growth factors but probably modulates a common effector system crucial for cell proliferation. In distinct contrast, the B subunit inhibited the growth of *ras*-transformed 3T3 cells as well as rapidly dividing normal 3T3 cells. Thus, the same cells, depending on their state of growth, exhibited a bimodal response to the B subunit. We conclude that endogenous gangliosides may be bimodal regulators of positive and negative signals for cell growth.**

Gangliosides, sialic acid-containing glycosphingolipids, have long been recognized as characteristic constituents of the plasma membrane of mammalian cells (1, 2). Although gangliosides have been identified as receptors for bacterial toxins (2) and viruses (3), relatively little is known about their normal function in membranes. In recent years, interest in these membrane constituents has increased due to the discovery that many monoclonal antibodies raised against tumor cells recognize carbohydrate sequences present on gangliosides and these determinants appear to be tumor-specific antigens (reviewed in refs. 4–6). Furthermore, the alterations in ganglioside metabolism and organization related to oncogenic transformation, cell cycle, and density-dependent growth inhibition (1, 6) raised the possibility that gangliosides play an important role in the regulation of cell growth. Such a possibility was reinforced by observations that exogenously added gangliosides altered the growth of a variety of cell types (7–13). In particular, exogenous gangliosides inhibited the action of several growth factors as well as the tyrosine kinase activity associated with the growth factor receptors (8, 9), suppressed the proliferation of lymphocytes induced by lectins, antigens, and interleukin 2 (4, 10), and sensitized tumor cells to growth inhibitors (11). In contrast, exogenous gangliosides stimulated the proliferation of astroglial (12) and neuroblastoma cells (13).

Thus, exogenous gangliosides have been reported to cause opposite effects on cell growth. This apparently conflicting aspect of ganglioside action has made it difficult to determine whether gangliosides play a role as membrane transducers of positive or negative growth signals. In addition, little progress has been made in clarifying the significance of these observations with respect to the function of endogenous gangliosides in the process of cellular proliferation. To directly examine potential functions for endogenous gangliosides, we have developed a different approach (14). The B subunit of cholera toxin, which is pentavalent and binds only to ganglioside galactosyl(β1→3)-*N*-acetylgalactosyminyl(β1→4)-[*N*-acetylneuraminyl(α2→3)]-galactosyl-(β1→4)glucosyl(β1→1)ceramide (GM1) on the cell surface (2), was used as a ganglioside-specific probe to induce proliferation of thymocytes (14). We have now extended this approach to other types of cells, such as normal and transformed murine 3T3 cells, to evaluate the role of membrane gangliosides in the regulation of cell growth in general.

MATERIALS AND METHODS

Materials. The B subunit of cholera toxin was purchased from List Biologicals (Campbell, CA). [^{3}H]Thymidine (55 Ci/mmol; 1 Ci = 37 GBq) was purchased from ICN. Epidermal growth factor (EGF), insulin, and platelet-derived growth factor (PDGF) were obtained from Collaborative Research (Waltham, MA). Other materials and chemicals were obtained from commercial sources.

Cell Lines and Cell Cultures. Swiss 3T3, BALB/c 3T3, NIH 3T3, and all of the *ras*-transformed NIH 3T3 cell lines were generous gifts of Stuart Aaronson (National Cancer Institute, Bethesda, MD). All of the latter were established from clones of NIH 3T3 cells transfected with human cellular Ha-*ras* (15), Ki-*ras* (16) and N-*ras* (17) oncogenes. Stock cultures of murine 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (vol/vol) fetal bovine serum in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Measurement of DNA Synthesis in Quiescent 3T3 Cells. Swiss, NIH, and BALB/c 3T3 cells were subcultured into 24-well tissue culture plates (Costar, Cambridge, MA) at a density of 2.5×10^4 cells per well in 1 ml of DMEM containing 10% fetal bovine serum and refed with the same medium after 2 days. Such cultures were used at least 5 days after the last change of medium when the cells were confluent and quiescent (18). To the quiescent cultures, 1 ml of DMEM was added without or with the B subunit and the various growth factors as indicated in the figure and table legends. After 20 hr, the cells were pulsed with 0.5 μCi of [^{3}H]thymidine for 4 hr and then washed two times with 1 ml of ice-cold phosphate-buffered saline and two times with ice-cold 5% trichloroacetic acid. The insoluble material was dissolved in 0.5 ml of 0.25 M NaOH, which was transferred to glass scintillation vials containing 10 ml of Ready-Solv HP (Beckman) and analyzed for radioactivity.

Measurement of DNA Synthesis in Exponentially Growing 3T3 Cells. Cells were seeded at a density of 3×10^3 cells per well as described above and grown in DMEM containing 10% fetal bovine serum. After 2 days (NIH 3T3 and Swiss 3T3) or

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Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; GM1, galactosyl(β1→3)-*N*-acetylgalactosyminyl(β1→4)-[*N*-acetylneuraminyl(α2→3)]-galactosyl(β1→4)glucosyl(β1→1)ceramide.

3 days (*ras*-transformed NIH 3T3), the cells were treated with the B subunit or with growth factors and were pulsed 40 hr later with [^{3}H]thymidine for 1 hr. The incorporation of ^{3}H into trichloroacetic acid-insoluble material was determined as described above.

RESULTS

The B Subunit of Cholera Toxin Stimulates DNA Synthesis and Cell Division in Quiescent Nontransformed Mouse 3T3 Cells. Quiescent cells can be stimulated to synthesize DNA and proliferate by the addition of fresh serum or combinations of growth factors (19). To investigate the potential functions of endogenous gangliosides in cell growth, we exposed quiescent mouse cells (NIH, BALB/c, and Swiss 3T3 cells) to the B subunit of cholera toxin. The B subunit stimulated incorporation of [^{3}H]thymidine by all three cell lines in a concentration-dependent fashion (Fig. 1*A*). A significant effect was observed at 50 ng/ml (≈1 nM) and maximum mitogenic stimulation was achieved at 500 ng/ml. Maximal [^{3}H]thymidine incorporation occurred at 20–24 hr (data not shown). The B subunit not only stimulated DNA synthesis but also caused cell division (Fig. 1*B*). A maximal increase in cell numbers was observed after a 48-hr exposure to the B subunit and was comparable to the increase mediated by 2% fetal bovine serum. Thus, the kinetics of the response of quiescent 3T3 cells to the B subunit of cholera toxin appear to be similar to other mitogens.

Synergistic Effects Between the B Subunit of Cholera Toxin and Other Growth-Promoting Factors. As shown in Table 1, stimulation of [^{3}H]thymidine incorporation in response to the B subunit was comparable to that caused by other known mitogens, such as unfractionated serum, EGF, and PDGF. Addition of the B subunit in the presence of EGF, PDGF, or fetal bovine serum further enhanced the stimulation of DNA synthesis due to the growth factors alone (Table 1). There were some differences among the three mouse 3T3 cell lines. The response to PDGF was potentiated the most by the B subunit in NIH 3T3 cells, whereas EGF stimulation was most enhanced by the B subunit in Swiss 3T3 cells. In contrast, the

Table 1. Stimulation of DNA synthesis in quiescent normal mouse 3T3 cells by the B subunit of cholera toxin and growth factors

Cell line	B subunit (1 μg/ml)	Other additions: None	EGF (4 ng/ml)	PDGF (2 units/ml)	FBS (2.5%)	FBS (5%)
NIH	−	1.9	25.0	3.8	13.5	17.8
	+	13.5	30.1	17.5	36.3	33.1
Swiss	−	4.8	19.5	13.6	35.8	51.5
	+	13.0	75.4	23.9	89.7	96.8
BALB/c	−	3.9	19.8	8.5	ND	19.3
	+	19.3	40.4	22.9	ND	48.1

Quiescent cultures were exposed to the indicated mitogens in the absence (−) and presence (+) of the B subunit and assayed for incorporation of [^{3}H]thymidine; data are expressed as cpm of [^{3}H]thymidine incorporated per well × 10^{-3}. Results are the means of triplicate determinations that varied <10% and are from a representative experiment. Similar results were obtained with each cell line in at least five additional experiments. FBS, fetal bovine serum; ND, not determined.

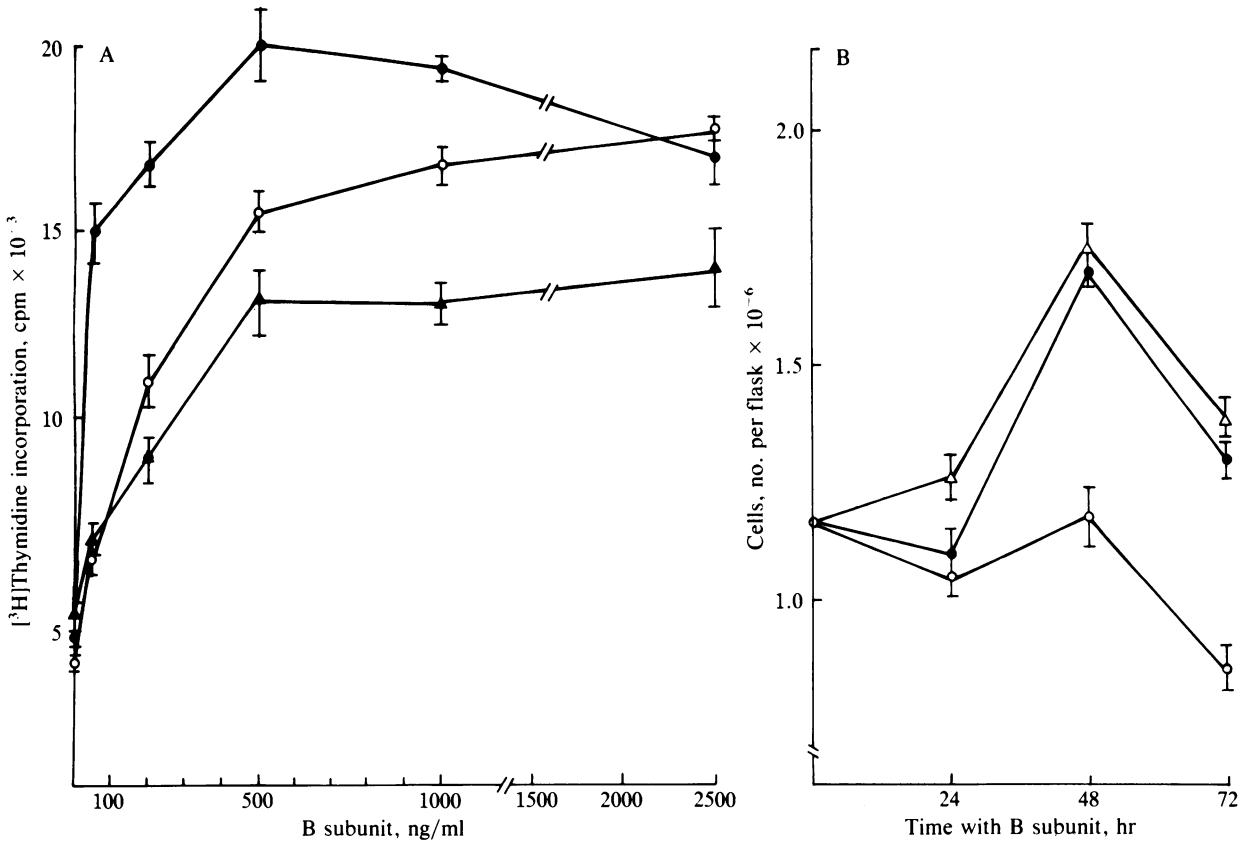

FIG. 1. Stimulation of cell growth by the B subunit of cholera toxin. (*A*) Quiescent cultures of BALB/c 3T3 cells (●), Swiss 3T3 cells (▲), and NIH 3T3 cells (○) were exposed to various concentrations of the B subunit and assayed for [^{3}H]thymidine incorporation. Each value is the mean ± SD of triplicate determinations from a representative experiment. Similar results were obtained in at least five additional experiments with each cell line. (*B*) NIH 3T3 cells were grown in 25-cm^2 flasks until confluent and quiescent and then incubated with no addition (○), 1 μg of the B subunit per ml (△), or 2% fetal bovine serum (●). At the indicated times, the cells were removed from the flasks and counted on a Coulter model ZBI cell counter. Data represent the mean ± SD of three independent cultures treated identically.

B subunit only slightly enhanced the response of NIH 3T3 cells to EGF. This potentiating effect between the B subunit and the growth-promoting agents can be clearly seen in Fig. 2*A* where we determined the dose–response of Swiss 3T3 cells to EGF in the absence and presence of the B subunit. The synergistic interaction between the B subunit and growth factors was observed even at optimal concentrations of the mitogens (Fig. 2*B*). Thus, the response of the cells to the B subunit with EGF or insulin was greater than the sum of the responses to each of the effectors alone. This nonadditive response even was observed when EGF and insulin were tested together; while the two growth factors synergized with each other, addition of the B subunit caused a further potentiation of [^{3}H]thymidine incorporation (24.7- vs. 12.5-fold).

The B Subunit Inhibits Growth of Transformed Cell Lines. In contrast to our earlier finding that an interaction of the B subunit with endogenous gangliosides results in lymphocyte stimulation (14) and our present observation that it induced proliferation of confluent, nondividing cells, others have suggested that gangliosides are associated with the inhibition of cell growth (7–9). In view of the growth-inhibitory effect of some antibodies directed to certain gangliosides present on the plasma membranes of tumor cells (20, 21), we examined the effect of the B subunit on the proliferation of transformed mouse cells. Initial experiments with Ha-*ras*-transformed NIH 3T3 cells demonstrated that the B subunit caused a dose-dependent inhibition of DNA synthesis. A concentration as low as 50 ng/ml inhibited cell proliferation by 24% and 1 μg/ml caused a maximal inhibition of 58% (Fig. 3). The inhibition evoked by the B subunit was also time-dependent (Fig. 3 *Inset*). Whereas the B subunit inhibited DNA synthesis by 40% at 24 hr, this was increased to 76% at 72 hr. The antiproliferative effect of the B subunit was confirmed by direct cell counting. The increase in cell numbers was reduced by 40% when the cells were cultured in the presence of 1 μg of the B subunit per ml for 24 hr.

To exclude the possibility that the inhibitory action of the B subunit on the Ha-*ras*-transformed cells was specific to that cell line or oncogene, the effects of the B subunit on two other cell lines transformed by different *ras* oncogenes were examined. As shown in Table 2, the proliferation of Ki- and N-*ras*-transformed cells also was inhibited by the B subunit. Although the three transformed cell lines exhibit different degrees of sensitivity to 1 μg of the B subunit per ml, they all were significantly affected (≈25%) by a very low concentration (50 ng/ml) of the B subunit. In this regard, the degree of sensitivity to the B subunit appeared to correspond to the doubling times of the cells, the more rapidly dividing cells being more inhibited by the B subunit. The antiproliferative activity of the B subunit was not associated with any cytotoxic effects, as the number of viable cells, determined by trypan blue exclusion, was always >95%. The inhibitory effect of the B subunit was observed regardless of whether EGF or fetal bovine serum was added to the medium (Table 2). In contrast to the Ha- and Ki-*ras*-transformed cells, the proliferation of the N-*ras*-transformed cells was significantly increased by the addition of 5% fetal bovine serum. The inhibition by the B subunit was reduced in this case. The inhibitory effect of the B subunit appears to be specific as

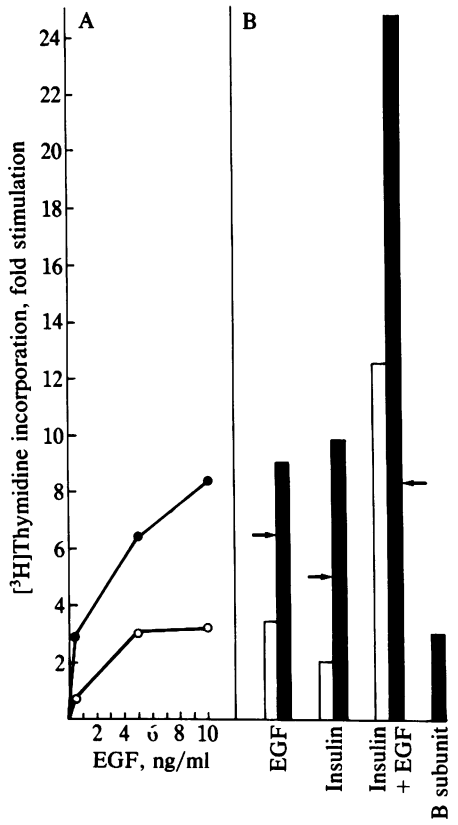

FIG. 2. Synergistic effects on cell growth by the B subunit and growth factors. (*A*) Quiescent cultures of Swiss 3T3 cells were exposed to the indicated concentrations of EGF in the absence (○) and presence (●) of 1 μg of the B subunit per ml and assayed for [^{3}H]thymidine incorporation. (*B*) Same as in *A* except the cells were exposed to EGF (10 ng/ml) and/or insulin (1 μg/ml) in the absence (open bars) or presence (solid bars) of the B subunit. Arrows indicate the expected stimulation if the effects of the growth factors and the B subunit were strictly additive. Values are from a representative experiment in which triplicate determinations varied <10%.

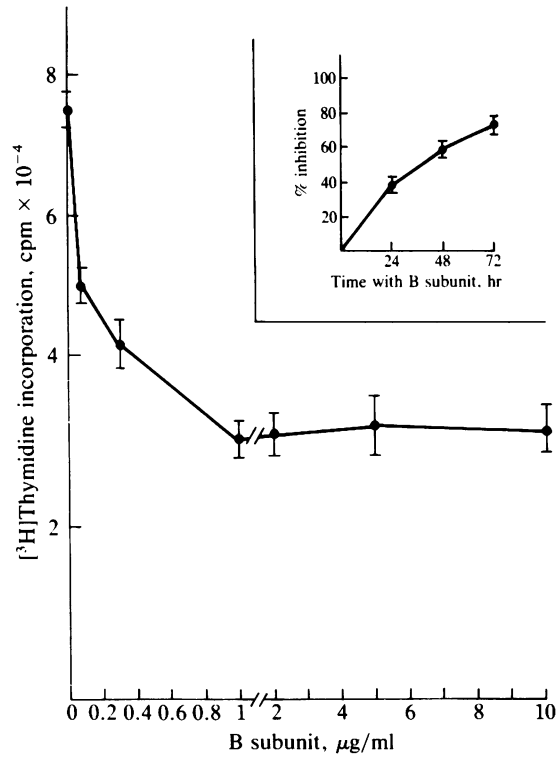

FIG. 3. Inhibition of Ha-*ras*-transformed NIH 3T3 cell growth by the B subunit of cholera toxin. Cells transformed by Ha-*ras* were exposed to the various concentrations of B subunit and assayed for [^{3}H]thymidine incorporation. (*Inset*) Time dependence of the inhibition of DNA synthesis induced by the B subunit. The cells were treated without (control) and with 1 μg of the B subunit for various time periods. The results are from a representative experiment and are expressed as the % inhibition ± SD compared to the control cells at each time.

Table 2. Inhibition by the B subunit of growth of *ras*-transformed NIH 3T3 cells

Cell line	B subunit (1 μg/ml)	Other additions: None	Other additions: EGF (40 ng/ml)	Other additions: FBS (5%)
Ha-*ras*	−	100	113	99
	+	40	53	49
Ki-*ras*	−	100	101	120
	+	49	51	72
N-*ras*	−	100	109	142
	+	59	63	115

Details are the same as those described in the legend to Fig. 3. NIH 3T3 cells were transformed with the indicated *ras* oncogene. [^{3}H]Thymidine incorporation is expressed as % of control. Results are from a representative experiment. The amounts of [^{3}H]thymidine incorporated into control cells (mean ± SD of triplicate wells in cpm × 10^{-3}) were 204 ± 18.9 (Ha-*ras*), 162 ± 3.8 (Ki-*ras*), and 83.5 ± 0.8 (N-*ras*). Similar results were obtained in at least five additional experiments with each cell line. FBS, fetal bovine serum.

concanavalin A, a lectin that can bind to cell-surface glycoconjugates and is a known mitogen for lymphocytes, had no effect on the growth of the transformed cells (data not shown).

Bimodal Effects of the B Subunit on Growth of Nontransformed Mouse Cells. The opposite effects of the B subunit on confluent, quiescent 3T3 cells and on the rapidly dividing transformed cells suggested that the difference might be due either to inherent differences in response patterns of normal and transformed cells or to differences between quiescent and proliferating cells. Experiments with growing, normal 3T3 cells demonstrated that a bifunctional response pattern to the B subunit can be observed in a single cell line (Table 3). Thus, in contrast to the observation that the B subunit induced proliferation of quiescent, nondividing cells (Fig. 1, Table 1), when the cells were exponentially growing, the B subunit inhibited their proliferation. These differences in responses to the B subunit were not due to differences in the assay conditions. Thus, the B subunit inhibited [^{3}H]thymidine incorporation into the growing cells even when the labeling pulse was increased to 4 hr or the exposure time to the B subunit was reduced to 24 hr. In addition, when confluent but not yet quiescent cultures of 3T3 cells were exposed to the B subunit, their proliferation was not stimulated but was slightly inhibited. Similar to the result shown for the transformed cells, the inhibitory effects of the B subunit on growing 3T3 cells were observed regardless of whether EGF or fetal bovine serum was added to the cells even though these factors by themselves increased [^{3}H]thymidine incorporation.

Table 3. Inhibition of DNA synthesis in exponentially growing mouse 3T3 cells by the B subunit of cholera toxin

Cell line	B subunit (1 μg/ml)	Other additions: None	Other additions: EGF (40 ng/ml)	Other additions: FBS (5%)
NIH 3T3	−	57.9 ± 3.2	63.8 ± 5.3	120 ± 10
	+	33.4 ± 2.8	40.2 ± 3.6	91.3 ± 5.4
Swiss 3T3	−	61.6 ± 3.9	78.9 ± 0.6	83.9 ± 4.4
	+	41.5 ± 0.6	55.7 ± 1.4	60.7 ± 6.7

Mouse 3T3 cells were seeded at 3 × 10^3 cells per well in 24-well tissue culture plates in 1 ml of DMEM containing 10% fetal bovine serum. After 2 days, 1 ml of DMEM was added with the indicated mitogens. Incorporation of [^{3}H]thymidine was determined; data are expressed as cpm × 10^{-3}. Values are the mean ± SD of triplicate wells from a representative experiment. Similar results were obtained in at least four additional experiments with each cell line. FBS, fetal bovine serum.

DISCUSSION

The B subunit of cholera toxin is a potent growth stimulator for quiescent mouse 3T3 cells comparable to other known mitogens such as EGF, PDGF, and insulin. We have shown previously that the B subunit activates resting thymocytes (14). Thus, the ability of the B subunit to stimulate resting cells to divide appears to be a general phenomenon. The only known receptor for the B subunit is the ganglioside GM1 (2). This has been clearly demonstrated in thymocytes (14) and in BALB/c 3T3 cells (22). We have also shown that the B subunit used in our studies is devoid of any adenylate cyclase activating A subunit (14). It is well known that cyclic AMP analogues and cholera toxin stimulate the growth of some types of cells, including 3T3 cells (23, 24). Cholera toxin also potentiates the action of PDGF in competent induction as well as abrogates the requirement for EGF in cell progression (25). Our present results raise the possibility that some of the effects of cholera toxin may be due to the binding of its B subunit to cell-surface GM1 and not wholly to its ability to activate adenylate cyclase.

In addition to stimulating the cells, the B subunit potentiated the effects of other mitogens that stimulated quiescent 3T3 cells. These included EGF, PDGF, serum, and insulin. Based on preliminary experiments in defined medium, we believe that the B subunit by itself is a weak mitogen that can potentiate the action of other mitogens. The ability of the B subunit to act synergistically with other mitogens may indicate that it does not act identically to any of these growth factors. The B subunit may mediate its effects in a fundamentally different way than other mitogens through a specific, not yet defined, pathway. Alternatively, the B subunit may enhance the sensitivity of the cells to other mitogens by directly modulating a common effector system crucial for cell proliferation.

The mechanism by which mitogens stimulate cells to divide is not yet understood. Activation of Na^+/H^+ exchange (26), stimulation of polyphosphatidylinositol phosphate breakdown (27), Ca^{2+} mobilization (28), and activation of protein kinase C (29) have been suggested as possible early essential events in the mitogenic response. More recent experiments, however, have shown that some mitogens can cause proliferation in the absence of polyphosphatidylinositol phosphate breakdown (30) or protein kinase activation (31). An increase in cytosolic free Ca^{2+} appears to be an early and general response to mitogenic stimulation. Whereas some mitogens (i.e., PDGF and concanavalin A) mobilize intracellular stores of Ca^{2+} through generation of inositol trisphosphate (32, 33), EGF, in contrast, induces a rapid rise in intracellular Ca^{2+} through an influx of extracellular Ca^{2+} (34).

In studies in collaboration with Sergio Grinstein, the B subunit induced a pronounced increase in intracellular Ca^{2+} in lymphocytes (35) as well as quiescent 3T3 cells (unpublished observations). Reduction of extracellular Ca^{2+} below 1 μM by addition of 1 mM EGTA immediately abolished the transient rise in Ca^{2+} caused by the B subunit. Thus, in analogy with EGF, the B subunit may transiently elevate intracellular Ca^{2+} through an influx of extracellular Ca^{2+}.

In this study, we also found that the B subunit can inhibit the growth of transformed 3T3 cells as well as rapidly dividing normal 3T3 cells. Although the underlying basis for this inhibitory effect is unclear, we were able to demonstrate a bimodal response to the B subunit by the same cells just by varying their state of growth. Whether the same or a different signal transduction mechanism is involved in the stimulatory and inhibitory responses to the B subunit remains to be determined. Although it is unusual for the same effector to stimulate and inhibit the growth of cells, it is not unprecedented. Transforming growth factor β is a bifunctional regulator of cell growth, as it inhibits the growth of many

tumor cells yet stimulates the growth of non-neoplastic fibroblasts (36, 37). In addition, transforming growth factor β acts synergistically with PDGF to stimulate the growth of Fisher rat 3T3 cells transfected with a cellular *myc* gene, but with EGF, it inhibits their growth (37). Thus, the response of a cell to a growth factor may depend not only on the growth factor itself but also on the total set of stimulatory and inhibitory agents that are operating on the cell at that time.

The mechanism(s) by which the B subunit of cholera toxin is able to modulate the growth response of cells positively and negatively is unknown. It is clear, however, that the only known function of the B subunit is to bind to ganglioside GM1 on the cell surface. Transformed 3T3 cells have less GM1 than normal 3T3 cells (1, 6) and it has been reported that levels of cell-surface gangliosides increase as normal 3T3 cells reach confluency (6). Thus, the bimodal response to the B subunit may be related to the amount or distribution of surface GM1 being occupied. From our findings, we propose that endogenous gangliosides may be "biomodulators" of positive and negative growth signals.

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