Growth Factors, Signaling Pathways, and the Regulation of Proliferation and Differentiation in BC3H1 Muscle Cells.

I. A Pertussis Toxin-sensitive Pathway Is Involved

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Abstract. Cells of the nonfusing muscle cell line BC3H1 stop proliferating and express a family of muscle-specific proteins when the FBS concentration is reduced from 20 to 0.5% (Munson, R., K. L. Caldwell, and L. Glaser. 1982. J. Cell Biol. 92:350-356). Several growth factors have been shown to block differentiation in this cell line. To begin to investigate the potential role of G proteins in signal transducing pathways from these receptors, we have examined the effects of cholera toxin (CT) and pertussis toxin (PT) on proliferation and differentiation in BC3H1 cells. PT specifically ADP ribosylates a protein with an apparent molecular mass of 40 kD in BC3H1 cell membranes, whereas CT specifically ADP ribosylates three proteins of 35-43 kD. When added to exponentially growing cells in 20% FBS, CT and PT inhibited [3H]thymidine incorporation by up to 75% in a dose-dependent fashion. We found the synthesis of creatine kinase (CK) and skeletal muscle myosin light chain was reversibly induced in cells in 20% FBS treated with PT, but no increased synthesis was seen in cells treated with CT or in control cells; Northern analysis indicated this induction was at the level of mRNA. In cells shifted to 0.5% FBS, CT inhibited the normally induced synthesis of CK whereas PT potentiated it by ~50%. Forskolin also inhibited growth in 20% FBS and differentiation in 0.5% FBS medium in a dose-dependent fashion. Both forskolin and CT elevated cAMP levels compared with control or PT-treated cells, suggesting that CT is blocking proliferation and differentiation by elevating cAMP levels. These results establish that a PT-sensitive pathway is involved in regulating proliferation and differentiation in BC3H1 cells, and we postulate that PT functions by ADP ribosylating a G protein that transduces signals from growth factor receptors in these cells.

BC3H1 cells are a continuous, nonfusing muscle cell line (Schubert et al., 1974). In response to reduced serum concentrations, these cells stop proliferation and begin to express a family of proteins including creatine kinase (CK), myokinase, nicotinic acetylcholine receptors, and skeletal and smooth muscle actin (Schubert et al., 1974; Munson et al., 1982; Olson et al., 1983a,b, 1984; Strauch and Rubenstein, 1984; Lathrop et al., 1985a; Strauch et al., 1986; Wice et al., 1987). This process can be reversed by the addition of high concentrations of serum, and quiescent, differentiated cells will shut down the synthesis of these muscle proteins and reenter the cell cycle (Munson et al., 1982; Strauch and Rubenstein, 1984; Lathrop et al., 1985a).

Because these cells can transit from a "proliferative" to a "differentiated" phenotype and back to the proliferative phenotype by manipulating the growth conditions, they provide an excellent model system to study how signals are transduced from cell surface receptors that ultimately result in determining whether a cell will proliferate or differentiate. For example, it has been demonstrated that the addition of acidic or basic fibroblast growth factor (FGF) to differentiated BC3H1 cells will cause them to shut down the synthesis of CK or actin mRNA (Lathrop et al., 1985b; Wice et al., 1987). It has also been shown that purified epidermal growth factor (EGF) will shut down the synthesis of CK or actin in differentiated cells (Wang and Rubenstein, 1988).

G proteins may play a key role in such signal transduction mechanisms. The G proteins comprise a family of membrane-associated, GTP-binding proteins that are believed to transduce signals from extracellular receptors to intracellular effector molecules (for reviews see Gilman, 1984, 1987). Two of these proteins, Gs and Gi, have been extensively characterized as linking stimulatory and inhibitory hormone receptors, respectively, to adenylate cyclase (for reviews see Smigel et al., 1984; Gilman, 1984, 1987; Lefkowitz and...

1. Abbreviations used in this paper: CK, creatine kinase; CT, cholera toxin; EGF, epidermal growth factor; FGF, fibroblast growth factor; PT, pertussis toxin; TBST, TBS, pH 8.0, containing 0.05% Tween 20.
Proliferation

Materials and Methods

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once with PBS and 1 ml of 0.1% crystal violet in 0.1 M citric acid was added.
as indicated in Fig. 2. At daily intervals, triplicate test dishes were rinsed
tometer.

vin et al., 1986, 1988). For cell counts, cells plated at 2 x 10⁴/35-mm tis-
to the same medium containing 0.5% FBS.

BC3H1 mouse muscle cells (Schubert et al., 1974), obtained from the
American Type Culture Collection (Rockville, MD), were cultured in DME

Roles of G proteins in skeletal muscle cell development although both PT and cholera toxin (CT) substrates have been
identified in skeletal muscle sarcolemmal and T-tubule fractions (Scherer et al., 1987). It has also been reported that both PT and CT substrates decrease in amount as LGE9 myo-
blasts fuse and differentiate (Morris and Bilezikian, 1986).

Little is known about the regulation of G proteins or the roles of G proteins in skeletal muscle cell development although both PT and cholera toxin (CT) substrates have been
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blasts fuse and differentiate (Morris and Bilezikian, 1986).

In addition, the ras oncogene, a GTP-binding protein with significant homology to the alpha subunit of transducin and Go (Hurley et al., 1984; Lochrie et al., 1985; Tanabe et al., 1985), has been implicated in the regulation of myogenesis. Transfection of C2 muscle cells with oncogenic Harvey ras or N ras completely blocked fusion and myogenic differentia-
gation (Olson et al., 1987; Gossett et al., 1988). We have also
found that oncogenic Harvey ras would block differentiation in transfected BC3H1 cells (Kelvin et al., 1987; Kelvin et al., 1989), and similar results have also been reported by Payne et al. (1987). These results implicate the GTP-binding ras protein as being potentially involved in signaling pathways that regulate muscle cell proliferation and differentiation. To further investigate the potential role of G proteins in myogen-
esis, we report here on the effects of CT and PT on prolifer-
ration and differentiation in BC3H1 muscle cells. While both toxins inhibit proliferation, PT induces differentiation where-
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Materials and Methods

Cell Culture

BC3H1 mouse muscle cells (Schubert et al., 1974), obtained from the
American Type Culture Collection (Rockville, MD), were cultured in DME
with 1 glitc glucose supplemented with 20% FBS, penicillin (100 U/ml),
and streptomycin (100 μg/ml). To induce differentiation, cells were switched
to the same medium containing 0.5% FBS.

Proliferation

[3H]thymidine incorporation was determined as previously described (Kel-
vin et al., 1988, 1988). For cell counts, cells plated at 2 x 10⁵/35-mm tis-
sue culture dish were switched 24 h later to fresh medium containing drugs
as indicated in Fig. 2. At daily intervals, triplicate test dishes were rinsed
once with PBS and 1 ml of 0.1% crystal violet in 0.1 M citric acid was added.
Cells were removed with a rubber policeman and counted in a hemacy-

tometer.

ADP Ribosylation

A "membrane-rich fraction" was prepared from proliferating BC3H1 cells
by a modification of the protocol of Imboden et al. (1986). Cells were plated
at 2 x 10⁵/100-mm dish; 24 h later cells were preincubated (if necessary)
in varying concentrations of CT or PT for 4 h at 37°C in a total vol of 3
ml. Each plate was rinsed with 5 ml cold PBS, and cells were removed with
0.25% (trypsin, pelleted for 2 min in an Eppendorf microfuge, (made by
Brinkman Instruments, Inc., Westbury, NY) and resuspended in 1 ml cold
lysis buffer containing 150 mM Tris-HCl, pH 7.5, 5 mM EDTA, 6 mM
dithiothreitol (DTT), and 0.2 μg/ml PMSF. The lysis buffer was made up
fresh each time from separate stock solutions. Cells were incubated on ice
for 2 min, sonicated at 100 W for 5 s on a Braunsonic 1510 sonicator
(B-Braun Instruments, Bethlehem, PA) equipped with a microprobe, and
centrifuged at 500 g for 10 min. The pellet was discarded and the superna-
tant centrifuged at 100,000 g for 60 min (SW 55 Ti rotor; Beckman Instru-
ments, Inc., Palo Alto, CA). The final pellet ("membrane-rich fraction") was
resuspended in 75 μl of 200 mM PO₄ buffer, pH 7.5, and protein deter-
mined by a colorimetric protein assay method (Bio-Rad Laboratories, Rich-
mond, CA).

For the ADP ribosylation assay (adapted from Ribeiro-Neto et al.,
1985), 100 μg of total protein from the membrane-rich fraction was diluted
with an equal volume of ribosylation buffer in a microfuge tube (final con-
centration, 20 mM thymidine, 2 mM ATP, 2 mM GTP, 2.5 mM MgCl₂,
2 mM EDTA, 2 mM DTT, and 0.2 μg/ml of DNase I). Ribosylation buffer
was made fresh each time from separate stocks. CT and PT were activated
by incubation with 25 mM DTT in PO₄ buffer, pH 7.4, at 37°C for 30 min
(Hildebrandt et al., 1982, 1983; Moss et al., 1983) and added at a concen-
tration of 10 μg/ml for CT and 20 μg/ml for PT. At 1 μg/Ct/sample [32P]NAD+
used, 289 Ci/mmol; ICN Radiochemicals, Div. ICN Biomedicals Inc.
(Irvine, CA) was added (0.07 μM NAD⁺) and tubes were incubated at 37°C
for 30 min. To stop the reaction, 1.8 ml of 10% TCA was added, and tubes
were placed at −20°C for 15 min. Tubes were centrifuged for 1 min, the
supernatant was gently removed, and remaining liquid removed with tissue.
Polied samples were resuspended in 50 μl of 50 mM Tris-HCl, pH 8.0,
30 μl electrophoresis sample buffer containing 10% 2-mercaptoethanol was
added, and tubes was treated to neutralty with 1 N HCl, tubes were placed in
a boiling water bath for 5 min, and samples loaded onto a 10% acrylamide
gel. Proteins were electrophoretically separated in the presence of SDS
(Laemmli, 1970), and the gel was stained with Coomassie Brilliant Blue
R-250, destained, dried, and autoradiographed. A low molecular mass
marker kit (Bio-Rad Laboratories) was used to establish a standard curve.
Kodak X-OMAT AR film was exposed at −70°C and developed in Kodak
GBX-2 developer.

Differentiation

For the measurement of CK, cells were gently rinsed with cold PBS, pH
7.4, removed with 0.25% trypsin, and CK activity was determined as previously
described (Simard and Connolly, 1987).

Myosin was examined in cell extracts after electrophoresis in 10% acryl-
amide gels (Laemmli, 1970) and electrophoretic transfer to nitrocellulose
(Towbin et al., 1979). Blots were rehydrated in TBS, pH 8.0, containing
0.05% Tween 20 (TBST), and incubated for 30 min in TBST containing
10% normal goat serum. Blots were then incubated with myosoma culture
supernatants (designated QBM-2) diluted 1:75 in TBST. The QBM-2 anti-
body (Merrifield et al., 1983) reacts with myosin light chains. After over-
night incubation, blots were washed three times for 5 min each in TBST,
and incubated in alkaline phosphatase-conjugated rabbit anti-mouse IgG
(1:7,500 dilution; Promega Biotec, Madison, WI) for 30 min. Blots were
washed three times for 5 min each in TBST, the color reaction was initiated
by the addition of fresh nitro blue tetrazolium and 5-bromo-4-chloro-3-
indoly phosphate substrate, and the reaction terminated by the addition of
20 mM Tris, pH 8.0, containing 5 mM EDTA. For myosin heavy chain, blots
were incubated with a polyclonal chicken anti-fetal calf myosin heavy chain
serum (Jandreski and Liew, 1984) followed by rabbit anti–chicken IgG,
and alkaline phosphatase-conjugated goat anti–rabbit IgG (1:7,500 dilution;
Promega Biotec, Madison, WI).

cAMP Measurements

Cells were grown in 6 x 35-mm cluster well plates and incubated with drugs
or control medium as indicated in Fig. 7. To determine cAMP levels, cells
were washed once in ice cold PBS, 1.0 ml/well 0.01 N HCl containing iso-
butyl methyl xanthine at 1 mM was added for 60 min. The HC1 extract
was stored at −20°C until cAMP was determined by RIA (Teitelbaum and
Berl, 1986). This assay was done using a cAMP RIA kit from New England
Nuclear (Boston, MA) or Biomedical Technologies, Inc. (Stoughton, MA).
We used the acetylated assay. At each timepoint, four independent cAMP
determinations were made and two wells were used for protein determina-

Northern Analysis

Cytoplasmic RNA was isolated by the method of Rasmussen et al. (1987)
from proliferating (20% FBS medium) or differentiated (0.5% FBS medi-
pH 6.5, 0.5% skim milk powder, 1% SDS, 1% glycine, 5 mM EDTA, and at 42°C in 50% formamide containing 0.75 M NaCl, 50 mM Na phosphate, probe, and cross-linked by UV exposure. Blots were prehydridized for 6 h under high stringency conditions at 50°C, and autoradiographed.

Results

When BC3H1 membranes were incubated with [32p]NAD in the presence of activated CT, three proteins with apparent molecular masses of 35-43 kD were ADP ribosylated (Fig. 1 A). This ribosylation is CT specific and intracellular: it is inhibited in a dose-dependent fashion by preincubation of intact cells with CT although the two lower molecular mass species appear to be more sensitive to preincubation than the 43-kD peptide (Fig. 1 A). Three high molecular mass proteins are also present on this autoradiogram although these are not specifically ribosylated by CT as they are also seen to be labeled in its absence. In cells treated with PT, a single species at 40 kD is seen to be ribosylated (Fig. 1 B), and this protein may represent the α subunit of Gi (Ui, 1984). This ribosylation is also inhibited in a dose-dependent fashion by preincubation of intact cells with PT (Fig. 1 B).

Treatment of exponentially growing BC3H1 cells with CT (Fig. 2 a) and PT (Fig. 2 b) inhibits [3H]thymidine incorporation in a dose-dependent fashion. In this experiment, we saw a 73% inhibition of thymidine incorporation with PT at a dose of 10 ng/ml after 48 h. Higher doses did not depress thymidine incorporation further (data not shown). In a series of eight separate experiments, [3H]thymidine incorporation was inhibited by 67 ± 9.7% at 10 ng/ml of toxin. With CT, we obtained an inhibition of 54% at 10 μg/ml after 48 h. In a series of eight separate experiments, the mean reduction in thymidine incorporation was 61 ± 12%. This reduction in [3H]thymidine incorporation is not due to an inhibitory effect of either toxin on thymidine uptake but rather reflects a true reduction in the rate of proliferation of these cells (Fig. 2 c). Furthermore, these toxins are not merely killing the cells; no significant number of cells was detached from the culture dish by either toxin over 3-4 d, and >95% of either control, PT- or CT-treated cells would exclude trypan blue (data not shown). That these agents are not toxic is also demonstrated by [3H]thymidine rescue by specific growth factors (Kelvin et al., 1989).

BC3H1 cells proliferate in 20% FBS (growth medium) and have a fibroblast-like morphology; when switched to 0.5% FBS (differentiation medium), cells stop proliferating, have a more elongated, myoblast-like appearance, and synthesize muscle-specific proteins (Munson et al., 1982). Both CT and PT have reduced proliferation of BC3H1 cells. Do these cells differentiate? PT-treated BC3H1 cells growing in 20% FBS medium (growth medium) have a myoblast-like morphology. In contrast, CT-treated cells retain a fibroblast-like appearance (data not shown). We monitored biochemical differentia-
Figure 2. Cell growth of toxin-treated BC3H1 cells. Exponentially growing cells in 20% FBS were treated with varying doses of PT (a) or CT (b) for 48 h and [3H]thymidine incorporation was determined. Both PT and CT treatment resulted in significant inhibition of thymidine incorporation. In both of these experiments and in all subsequent figures showing thymidine incorporation data, the values used to calculate percentages were the mean of quadruplicate samples. In all cases, the control value (no drug) was taken as 100% and samples were treated with PT or CT expressed as a percentage of this control value. (c) Both CT and PT result in a significant reduction in the number of cells with time.

seen with 20% control cells (Fig. 4 a). The appearance of CK in 0.5% FBS in the presence of PT was very similar to that seen in control cells for the first 3 d of the experiment, but after 4 and 5 d there was significantly higher enzyme activity in toxin-treated cells (Fig. 4 a). In CT (10 µg/ml), no CK was detectable in 20% cells (data not shown); in 0.5% FBS, a pronounced inhibition of CK production was seen over the 5 d of the experiment, and this was also most apparent at 4 and 5 d after the shift to 0.5% FBS medium (Fig. 4 b).

To investigate the possibility that CK synthesis could be uniquely affected by these drug treatments, we assessed myosin expression in toxin-treated cells using an immunoblot assay with the QBH-2 monoclonal antibody. This antibody reacts with skeletal muscle myosin but not smooth muscle or nonmuscle myosin (Merrifield et al., 1983). We found that PT treatment for 4 d in 20% FBS induced significant levels of myosin light chain expression (Fig. 5). No signal is detectable in cells growing in 20% FBS (Fig. 5), or in cells treated with CT (data not shown). A similar result was observed with a polyclonal antibody against cardiac myosin heavy chain (Jandreski and Liew, 1984). Significant levels of myosin heavy chain synthesis were detected in BC3H1 cells grown in 20% FBS and treated with PT but not in 20% control cells (data not shown).

These results indicate that both PT and CT inhibit proliferation, but only PT induces muscle-specific protein synthesis. How could PT and CT be exerting these differential effects? CT treatment increases cAMP levels in a variety of cell types (Gill, 1977). Therefore, we tested the effect of other agents, known to affect cAMP levels, on proliferation and differentiation in BC3H1 cells. 10⁻⁴ M forskolin inhibited [3H]thymidine incorporation in 20% FBS by 81% (Fig. 6 a). CK levels in forskolin-treated cultures in 0.5% FBS were dramatically lower than control cells in 0.5% FBS and almost identical to those seen in control cells in 20% FBS (Fig. 6 b). Treatment of cells with 0.5% ethanol (final level of ethanol in forskolin-treated cultures at 10⁻⁴ M) had no significant effect on CK or [3H]thymidine incorporation (data not shown). Dibutyryl cAMP also inhibited [3H]thymidine incorporation (in 20% FBS) and CK expression (in 0.5% FBS) in a dose-dependent fashion (data not shown).

Both CT and forskolin rapidly elevated cAMP levels in these cells, and an approximate fourfold increase in cellular levels was seen with these two drugs by 60 min (Fig. 7). However, there was no significant difference between control and PT-treated cells. Over 4 d, the total level of cAMP dropped in both control and treated cultures (in particular between 2 and 4 d in CT- and forskolin-treated cultures) although these cells continued to express much higher levels of cAMP than did control cells. There was no significant difference between control and PT-treated cells over the course of the experiment. These results suggest that elevated cAMP levels are inhibitory to both proliferation and differentiation in BC3H1 cells.

How then does PT inhibit proliferation but allow for differentiation? If cells in 20% FBS are treated with PT, and the PT is removed, there is a rapid reversal in the steady-state levels of CK (Fig. 8). This induction of differentiation is at the mRNA level. When cells are incubated in high serum (20% FBS) in the presence of PT (10 ng/ml), analysis of RNA isolated from BC3H1 cells indicates that significant
levels of CK mRNA are seen in PT-treated cells. Little or no message was detected in control cells grown in 20% FBS or in 20% FBS plus CT, but this mRNA was clearly induced in control cells switched to 0.5% FBS (Fig. 9). These results suggest that a PT-sensitive pathway conducts signals from some factor in the serum that affects proliferation and differentiation.

Discussion
In this study we have demonstrated that both PT and CT, which ADP ribosylate the α subunits of Gi and Gs respectively, inhibit proliferation in BC3H1 muscle cells in a dose-dependent fashion. However, PT induces differentiation of these cells, whereas CT inhibits it. This induction of differentiation by PT is time and dose dependent. CK as well as both myosin light and heavy chains are induced by PT. Previous work on this cell line has indicated that very little or no muscle-specific protein synthesis or mRNA could be detected in BC3H1 cells growing in 20% FBS (Munson et al., 1982; Olson et al., 1983a, b, 1984; Strauch and Rubenstein, 1984; Strauch et al., 1986; Spizz et al., 1986; Wice et al., 1987). Thus, the fact that we detect significant levels of myosin and CK protein, and CK mRNA in 20% FBS cells treated with PT indicates that there is a specific PT induced initiation of muscle cell differentiation.

CT ADP ribosylates a series of at least three proteins in the 35–43-kD molecular mass range in BC3H1 membranes.
Figure 4. Timecourse of CK expression in toxin-treated cultures. Cells were plated at $1 \times 10^5$ in a 35-mm dish in 20% FBS medium and 24 h later switched to fresh 20% or 0.5% FBS containing (a) PT at 10 ng/ml or (b) CT at 10 μg/ml. (a) Compared with control cells, there was a significant induction of CK expression by PT in both 20% FBS and 0.5% FBS. (b) In cultures switched to 0.5% FBS, CT inhibited the increase in CK expression seen in control cells by ~50%.

Figure 5. Myosin light chain expression in PT-treated BC3-H1 cells. Lanes 1 and 2, control cells in 20% FBS; lanes 3 and 4, cells in 20% FBS + PT (10 ng/ml for 4 d); lane 5, rat skeletal muscle myosin (RM). Lanes 1 and 3 contain 40 μg and lanes 2 and 4 contain 10 μg of cell extract. Immunoblots probed with the QBM-2 antibody demonstrate that myosin light chain expression has been induced in 20% FBS medium by treatment with PT, whereas no expression is seen in control cells.

Figure 6. Forskolin effects on proliferation and differentiation. (a) Forskolin inhibits $[\text{H}]$thymidine incorporation in a dose-dependent fashion in 20% FBS medium. (b) In cells switched to 0.5% FBS to induce differentiation, the expression of CK was inhibited in a dose-dependent fashion by forskolin.

CT has been shown to ADP ribosylate the α subunit of Gs, which has an apparent molecular mass of ~45 kD in several different systems (Gilman, 1984, 1987). Little is known about the G proteins in skeletal muscle; however, similar results were reported for the L6E9 myoblast line (Morris and Bilezikian, 1986) where CT was shown to ADP ribosylate three bands in the 39–45-kD range. Scherer et al. (1987) have reported two proteins of 42 and 62 kD ribosylated by CT in skeletal muscle sarcolemmal membranes. The proteins we have described may represent the muscle equivalent(s) of Gs, although biochemical purification and characterization of the G protein family from this cell line will be required to establish this point. However, it is clear that substrates ADP ribosylated by CT in BC3H1 cells are intracellular, associated with the membrane, and specifically

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We have presented data that elevated cAMP levels are inhibitory to both proliferation and differentiation in this cell line based on the use of CT, dibutyl cAMP, and forskolin as well as direct measurements of cytoplasmic cAMP. There are a variety of different reports with respect to a role for cAMP in muscle cell differentiation. Stygall and Mirsky (1980) reported that CT would induce differentiation in primary rat myoblast cultures. They found this treatment also resulted in elevated cAMP levels and reported that dibutyl cAMP would also stimulate fusion. In contrast, several reports have found that high levels of dibutyl cAMP or elevated cAMP would inhibit fusion (Zalin, 1973; Wahrmann et al., 1983; Entwistle et al., 1986) but did not block CK induction (Zalin, 1973). It has been suggested that a transient rise in cAMP levels is an inductive factor in fusion (Zalin and Montague, 1974) although this has been disputed, and these workers reported that cAMP levels rise after fusion and before the initiation of CK synthesis in primary chick myoblasts (Schutze et al., 1984). In our hands, CT did not block the fusion of primary chick myoblasts or of the mouse C2 muscle cell line (data not shown), but we have shown in this report that CT clearly inhibits differentiation in BC3H1 cells. This discrepancy may reflect differences between the cell lines, and/or may reflect the fact that signaling pathways differ in primary muscle cells, which permanently differentiate, versus BC3H1 cells, which retain the capacity to reenter the cell cycle.

Preliminary experiments have indicated that FGF can restore control levels of [3H]thymidine incorporation in CT-treated BC3H1 cells (data not shown) suggesting that CT is not capable of blocking all growth factor receptor–mediated signals. Thus, we believe that CT is exerting its effect on BC3H1 cells by ADP ribosylating Gs and raising cAMP levels. We are currently examining the relationship between cAMP levels and growth factor regulation of proliferation and differentiation.

PT ADP ribosylates a very prominent 40-kD species in BC3H1 cell membranes, and this labeling is eliminated by preincubation of intact cells with toxin. We are currently examining any potential role for the PT-ribosylated substrate(s) will

**Figure 7.** cAMP determinations in BC3H1 cells. 24 h after plating, cells were switched to fresh 20% FBS medium containing either forskolin (10⁻⁴ M) or CT (10 μg/ml). Both forskolin and CT induced a significant rise in cAMP levels within 60 min, whereas PT-treated cells were indistinguishable from control cells. Values represent mean of four separate samples.

**Figure 8.** Reversal of CK induction by PT. BC3H1 cells were plated at 1 × 10⁴/35-mm dish in 20% FBS. 24 h later, cells were switched to fresh medium containing 10 ng/ml PT. By 5 d after PT addition, cells were expressing high levels of CK in 20% FBS. If cells were left in PT-containing medium, they continued to express high levels of CK. However, removal of PT and transfer to fresh 20% FBS medium resulted in a rapid drop in the steady-state levels of CK.

**Figure 9.** Northern analysis of creatine kinase mRNA in toxin-treated BC3H1 cells. Cytoplasmic RNA was isolated from cells grown in 20% FBS or from cells switched to 0.5% FBS for 4 d. Cells were incubated with PT or CT for 96 h in 20% FBS. 10 μg of cytoplasmic RNA per lane was subjected to electrophoresis in a formaldehyde-agarose gel and transferred to Zeta probe. The blot was hybridized with the R21 CK³²P-labeled probe and autoradiographed.
require their purification and analysis during myogenic differentiation.

PT will block hormone activation of adenylyl cyclase (Gilman, 1984, 1987; Anand-Srivastava et al., 1987; Kassis et al., 1987). In addition, PT will block proliferative responses stimulated by growth factors that presumably work through phospholipase C and inositol phosphate turnover (Paris and Pouyssegur, 1986; Chambard et al., 1987; Paris et al., 1987; Leterrio et al., 1987). Although the PT-ribosylated substrate in both the adenylyl cyclase system and the inositol phosphate systems are very similar in size and structure (for examples, Brass et al., 1986; Wojcikiewicz et al., 1986; Aub et al., 1986; Kikuchi et al., 1986; Chambard et al., 1987), they are not identical. We speculate that PT works in BC3H1 cells by blocking a signal from serum since cells in 20% FBS plus PT will differentiate even though all the necessary factors to promote proliferation and inhibit differentiation are present, and these cells do not have elevated levels of cAMP. Thus, we predict that the PT substrate in these cells will be associated with a growth factor receptor that does not work through activation of adenylyl cyclase.

An alternate explanation is that the ADP ribosylation event mediated by PT is actually a positive signal to differentiate. This has been proposed from studies on hematopoietic differentiation in which inhibitors of ADP ribosylation could block the commitment and/or differentiation of stem cells (Dexter et al., 1985). However, as outlined in Kelvin et al. (1989), the fact that specific growth factors can overcome the PT effects argues against the ADP ribosylation event itself being an important signal.

Pouyssegur and co-workers have recently shown that two growth factors, FGF and thrombin, acting through independent pathways can induce 3Hthymidine incorporation in lung fibroblasts (Paris and Pouyssegur, 1986; Magnaldo et al., 1986; Chambard et al., 1987; Paris et al., 1987). However, only one of these pathways is blocked by PT. Similar results were also reported for bombesin and PDGF stimulation in 3T3 cells (Letterio et al., 1987). Thus, in BC3H1 cells, there may be more than one pathway to regulate proliferation and/or differentiation and PT may be exerting its effect by blocking signals in one of these pathways. In the accompanying report we examine the effect of PT on growth factor–mediated signaling pathways. Based on the present data, we conclude that there is a PT-sensitive signaling pathway in these cells and this pathway is involved in the transduction of signals that regulate proliferation and differentiation in BC3H1 cells.

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