

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

II. Two Signaling Pathways Distinguished by Pertussis Toxin and a Potential Role for the *ras* Oncogene

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Abstract. In the preceding report (Kelvin, D. J., G. Simard, H. H. Tai, T. P. Yamaguchi, and J. A. Connolly. 1989. *J. Cell Biol.* 108:159–167) we demonstrated that pertussis toxin (PT) blocked proliferation and induced differentiation in BC3H1 muscle cells. In the present study, we have used PT to examine specific growth factor signaling pathways that may regulate these processes. Inhibition of [³H]thymidine by PT in 20% FBS was reversed in a dose-dependent fashion by purified fibroblast growth factor (FGF). In 0.5% FBS, the normally induced increase in creatine kinase (CK) activity was blocked by FGF in both the presence and absence of PT. Similar results were obtained with purified epidermal growth factor (EGF). We subsequently examined the effect of a family of growth factors linked to inositol lipid hydrolysis and found that thrombin, like FGF, would increase [³H]thymidine incorporation and block CK synthesis. However, PT blocked thymidine incorporation induced by thrombin, and blocked the inhibition of CK turn-on in 0.5% FBS by thrombin. The *ras* oncogene, a G protein homologue, has previously been shown to block muscle cell

differentiation in C2 muscle cells (Olson, E. N., G. Spizz, and M. A. Tainsky. 1987. *Mol. Cell. Biol.* 7:2104–2111); we have characterized a BC3H1 cell line, BCT31, which we transfected with the *val*¹² oncogenic Harvey *ras* gene. This cell line did not express CK in response to serum deprivation. Whereas [³H]-thymidine incorporation was inhibited by 70–80% by increasing doses of PT in control cells, BCT31 cells were only inhibited by 15–20%. ADP ribosylation studies indicate this PT-insensitivity is not because of the lack of a PT substrate in this cell line. Furthermore, PT could not induce CK expression in BCT31 cells as it did in parental cells. We conclude that there are at least two distinct growth factor pathways that play a key role in regulating proliferation and differentiation in BC3H1 muscle cells, one of which is PT sensitive, and postulate that a G protein is involved in transducing signals from the thrombin receptor. We believe that *ras* functions in the transduction of growth factor signals in the nonPT-sensitive pathway or downstream from the PT substrate in the second pathway.

EVIDENCE from a number of muscle cell systems indicates that the control of myoblast proliferation/differentiation is stochastic and can be regulated by extrinsic factors (Konigsberg, 1971, 1976; Linkhart et al., 1981, 1982; Compton et al., 1986; Clegg et al., 1987). More specifically, addition of the mitogen fibroblast growth factor (FGF)¹ was shown to prolong proliferation and delay differentiation in primary muscle cells (Gospadarowicz et al., 1976) and the MM14 clonal mouse muscle cell line

(Linkhart et al., 1981, 1982; Clegg et al., 1987), and its removal for even short periods of time (2–3 h) triggers terminal differentiation in the MM14 line.

The role of FGF in inducing muscle-specific gene expression has been examined in detail in BC3H1 muscle cells. If purified FGF is added to differentiated cells in 0.5% FBS, the level of CK declines (Lathrop et al., 1985a), and the level of creatine kinase (CK) mRNA synthesized is reduced by at least 10-fold within 24 h (Spizz et al., 1986). However, purified FGF has been reported not to induce thymidine incorporation or proliferation (Lathrop et al., 1985b; Spizz et al., 1986; Wice et al., 1987). Rather, cells move from G0 to a point 4–6 h into the G1 portion of the cell cycle, and this has been reported with both acidic (Lathrop et al., 1985b) and basic FGF (Wice et al., 1987).

How are signals transduced from the FGF receptor to

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1. *Abbreviations used in this paper:* CK, creatine kinase; FGF, fibroblast growth factor; PT, pertussis toxin.

regulate events at the mRNA level in BC3H1 cells? Tsuda et al. (1985) reported that FGF could stimulate protein kinase C and Ca^{2+} mobilization in fibroblasts. However, Magnaldo et al. (1986) have concluded that early mitogenic events mediated by FGF in hamster lung fibroblasts are not coupled to phospholipase C activation or inositol lipid breakdown, and more recently FGF has been shown to stimulate tyrosine kinase activity in 3T3 cells (Coughlin et al., 1988). Moreover, partially purified FGF receptors have been reported to have an associated tyrosine kinase activity (Huang and Huang, 1986). Consistent with this evidence, Wice et al. (1987) found that vanadate, an inhibitor of tyrosine-specific protein phosphatases, would mimic the effects of FGF in BC3H1 cells, and cause these cells to repress α -actin and CK synthesis and to exit the G0 portion of the cell cycle (Wice et al., 1987). These authors conclude that FGF is most likely working by the activation of tyrosine-specific protein kinases.

As muscle differentiation may be regulated by multiple growth factors, GTP binding G proteins are also obvious candidates to serve a signal transduction role; they have been shown to be involved in transducing signals from a variety of receptors, including growth factor receptors (Taylor, 1986; Taylor and Merritt, 1986; Cockroft, 1987; Gilman, 1987; Lefkowitz and Caron, 1988). In the preceding manuscript (Kelvin et al., 1989), we demonstrated that a PT-sensitive site is involved in this regulation in BC3H1 muscle cells. PT will block thrombin-induced hydrolysis of phosphatidyl-inositol 4,5-bisphosphate by phospholipase C as well as the stimulatory effects of thrombin on cell proliferation in hamster lung fibroblasts (Magnaldo et al., 1986; Paris and Pouyssegur, 1986, 1987; Paris et al., 1987; Chambard et al., 1987). Since PT will ADP-ribosylate a G protein in these cells, it has been proposed that PT exerts its effects by blocking signals transduced by a G protein coupled to growth factor receptors.

Another member of the class of G proteins that has been linked to the transduction of signals from cell surface receptors is p21 *ras* (for reviews see Cooper and Lane, 1984; Levinson, 1986; Barbacid, 1987). The *ras* protein has significant homology to the α -subunit of transducin and the α -subunit of Go (Hurley et al., 1984; Lochrie et al., 1985; Tanabe et al., 1985). The homologous regions correspond most closely with regions believed to be involved in guanine nucleotide binding and GTPase activity of *ras* suggesting that the *ras* protein may function as a signal transducing G protein. This idea has been supported by the demonstration that microinjection of antibodies to *ras* will block nerve growth factor-induced neurite extension in PC-12 cells (Hagag et al., 1986), the serum-induced expression of *c-fos* in quiescent fibroblasts (Stacey et al., 1987), and serum- or growth factor-induced thymidine incorporation in quiescent fibroblasts (Mulcahey et al., 1985).

Olson and co-workers have demonstrated that oncogenic H-*ras* or N-*ras* would completely block myogenic differentiation in transfected C2 myoblasts (Olson et al., 1987; Gossett et al., 1988). We have previously shown that transfection of BC3H1 cells with the oncogenic form of Harvey *ras* could modulate differentiation in BC3H1 cells (Kelvin et al., 1987); similar results have also been reported by Payne et al. (1987) for transfected BC3H1 cells. Furthermore, it was shown that *ras*-transfected BC3H1 cells did not express

Ca^{2+} and Na^{+} channels, membrane ion channels that appear after prolonged mitogen withdrawal (Caffrey et al., 1987).

In this study we have used PT to examine growth factor signaling pathways in BC3H1 muscle cells. We now report that both FGF and thrombin will stimulate proliferation and block differentiation in BC3H1 cells. However, the FGF effect is not blocked by PT, but the thrombin effect is. BC3H1 cells transfected with the *ras* oncogene do not differentiate. Moreover, these cells are much less sensitive to the growth inhibitory effects of PT, and it will not induce differentiation in these cells. We believe that the *ras* protein may be involved in regulating proliferation and differentiation signals in BC3H1 cells but at a site downstream from the PT site, or in a different pathway.

Materials and Methods

Cell Culture

BC3H1 mouse muscle cells (Schubert et al., 1974) were cultured as previously described (Kelvin et al., 1989). In some experiments, cells were switched to a serum-free medium (designated ITS) consisting of DME supplemented with antibiotics and insulin (15 μ g/ml), transferrin (15 μ g/ml), and selenium (5 ng/ml). The detailed growth characteristics of this cell line in ITS medium has been reported previously (Kelvin et al., 1988).

Proliferation and Differentiation

3H incorporation, cell counts, and CK assays were all performed as previously described (Kelvin et al., 1986; Simard and Connolly, 1987; Kelvin et al., 1989).

Transfection

BC3H1 cells in the logarithmic phase of growth were transfected by the CaPO₄ method with pHO plasmid DNA (Spandidos and Wilkie, 1984; Graham and Van der Eb, 1973). The pHO6T1 plasmid contains the activated T24 Ha-*ras* gene (val¹²) as well as the aph gene which confers resistance to the neomycin analog G418. In addition, a control plasmid with no *ras* gene, designated pHO6, was constructed by removal of the *ras* gene. Cell lines were selected by treatment with 200 μ g/ml of G418. After ~3–4 wk, stable cell lines, capable of growing in G418, were obtained. These lines were maintained in the absence of G418 although periodic growth in G418 indicated the cell lines used retained the resistance phenotype (data not shown).

Northern and Southern Blot Analysis

For Southern blot analysis total genomic DNA was isolated from 10⁷ cells (Maniatis et al., 1982). Aliquots of DNA were digested with Eco RI, subjected to electrophoresis on an agarose gel, transferred to a nitrocellulose filter, hybridized with a Bam HI to Bam HI EJ *ras* fragment isolated from the pHO6T1 plasmid (labeled by nick translation with ³²P dCTP), and washed under low stringency conditions (Maniatis et al., 1982).

For Northern analysis, cytoplasmic RNA was isolated, subjected to electrophoresis, and transferred as described (Kelvin et al., 1989). Blots were hybridized with a Kpn I to Sac I EJ *ras* fragment isolated from the pHO6T1 plasmid (labeled by nick translation with ³²P dCTP), and washed under high stringency conditions (Maniatis et al., 1982).

Materials

Acidic FGF and EGF were obtained from R & D Systems, Inc. (Minneapolis, MN) PT was from List Biological Laboratories, Inc. (Campbell, CA) and human thrombin from Calbiochem Behring Corp. (San Diego, CA). Purified human α -thrombin was a generous gift of Dr. J. Ofozu, McMaster University (Hamilton, Canada).

Results

In the preceding paper (Kelvin et al., 1989), we showed that

if cells are treated with PT in high serum (20% FBS) medium, a medium which normally promotes proliferation and prevents differentiation, not only do the cells stop growing but the synthesis of CK is induced. In this study, we have used PT in an attempt to elucidate signaling pathways that regulate proliferation and differentiation in BC3H1 cells.

FGF blocks differentiation in BC3H1 cells. Does PT work by blocking signals from the FGF receptor? In all experiments, we used PT at 10 ng/ml, a dose that produced maximal inhibition of [³H]thymidine incorporation and maximal stimulation of CK activity. If exponentially growing cells in 20% FBS growth medium were incubated with varying doses of purified acidic FGF and PT simultaneously, the PT depression of [³H]thymidine incorporation was eliminated in a dose-dependent fashion with a half-maximal effect at ~1 ng/ml FGF (Fig. 1 a). At high doses of FGF, thymidine incorporation was stimulated to greater than control values, and this has also been consistently seen in other experiments with high doses of FGF in the absence of PT (data not shown).

Similar results were also seen if cells rendered quiescent by serum starvation were incubated with FGF in ITS serum-free medium (Fig. 1 b). In contrast to previous reports in which BC3H1 cells switched to 0.5% medium supplemented with FGF showed no appreciable [³H]thymidine incorporation (Lathrop et al., 1985a, b; Wice et al., 1987), we have found significant [³H]thymidine incorporation at higher doses of FGF in this serum-free medium. Half-maximal stimulation was seen at ~3 ng/ml of FGF, and this stimulation was not inhibited by incubation with 10 ng/ml PT (Fig. 1 b).

To test the possibility that FGF somehow blocked the PT effect extracellularly, we first treated BC3H1 cells with PT for 24 h in 20% FBS. This dramatically reduced the level of [³H]thymidine incorporation. If FGF was added to these "quiescent" cells, a single high dose (30 ng/ml) stimulated DNA synthesis severalfold in the continued presence of PT (data not shown).

Acidic FGF (30 ng/ml) almost completely blocked the appearance of CK activity in cells shifted to 0.5% FBS. On the other hand, PT potentiated this response by ~50% (Fig. 1 c). When PT and FGF were added at the same time, the cells expressed very low levels of CK after 4 d (Fig. 1 c), and these were not significantly different from cells treated with FGF alone. When varying doses of FGF were added in either the presence or absence of PT, half-maximal inhibition was seen between 1 and 3 ng/ml FGF (data not shown). Taken together, these results provide strong evidence that FGF is acting via a PT-insensitive pathway to stimulate proliferation and inhibit differentiation in BC3H1 muscle cells.

EGF has also been reported to stimulate cell proliferation and to block CK and actin synthesis in BC3H1 cells (Wang and Rubenstein, 1988). Cells rendered quiescent by serum starvation were incubated with varying doses of EGF in ITS medium (Fig. 2 a). [³H]Thymidine incorporation was stimulated by EGF (half-maximal stimulation between 1 and 3 ng/ml), and this effect was insensitive to PT. Like FGF, EGF would also inhibit CK activation if added when cells were switched to differentiation medium, and this inhibition was largely PT insensitive (Fig. 2 b). Half-maximal inhibition of differentiation was seen between 1 and 3 ng/ml of EGF (data not shown).

The fact that PT induced differentiation in 20% FBS suggested it was blocking a mitogenic signal in serum that nor-

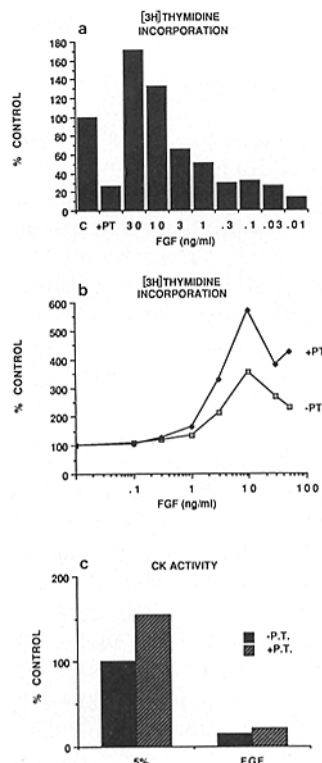


Figure 1. FGF effects on thymidine incorporation and CK expression in BC3H1 cells. (a) Exponentially growing cells in 20% FBS were incubated simultaneously with PT (except control [C] cells) and varying doses of acidic FGF. Thymidine incorporation was measured 48 h later. (b) Cells were rendered quiescent by transfer to serum-free ITS medium. Increasing doses of acidic FGF were added in the presence or absence of PT and thymidine incorporation was measured 48 h later. (c) Cells were switched to 0.5% FBS medium to induce differentiation, and CK was activity measured 4 d later. Pair of bars (left), cells ± PT. Pair of bars (right), cells + FGF (30 ng/ml) ± PT. CK activity was the mean of three separate determinations; in this and succeeding histograms representing CK activity, it is expressed as a percentage of control activity, with the CK activity in control cells in 0.5% FBS medium taken as 100%.

mally represses muscle protein synthesis. The present data indicate this signal is not FGF or EGF. A family of growth factors stimulate the formation of inositol phosphates in response to factor binding to cell surface receptors (e.g., Berridge et al., 1984; Brown et al., 1984; Carney et al., 1985; Heslop et al., 1986; Takuwa et al., 1987), and this can be blocked by PT (e.g., Paris and Pouyssegur, 1986). Thus, we tested the ability of varying doses of several of these growth

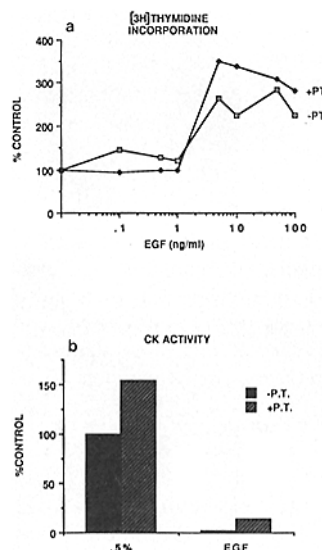


Figure 2. EGF effects on thymidine incorporation and CK expression in BC3H1 cells. (a) Cells were rendered quiescent by switching to serum-free ITS medium. Varying doses of EGF were added in the presence or absence of PT and thymidine incorporation was measured 48 h later. (b) Cells were switched to 0.5% FBS medium to induce differentiation, and CK activity was measured 4 d later. Pair of bars (left), cells ± PT. Pair of bars (right), cells + EGF (30 ng/ml) ± PT.

factors on [^3H]thymidine incorporation in quiescent BC3H1 cells in serum-free medium. Little or no stimulation was seen with gastrin releasing peptide or vasopressin and variable results were obtained with bombesin and bradykinin. However, thrombin consistently stimulated [^3H]thymidine incorporation in quiescent cells in a dose-dependent fashion (Fig. 3 *a*). Furthermore, this stimulation was inhibited by simultaneous incubation with 10 ng/ml PT (Fig. 3 *b*). In 20% FBS, when cells were treated simultaneously with PT and thrombin, even very high doses of thrombin (100 nM) could not overcome the inhibitory effect of PT (Fig. 3 *c*). In this particular experiment, thymidine incorporation was only inhibited by $\sim 30\text{--}40\%$ by PT; however, similar results have been seen in other experiments in which greater inhibition was seen with PT (data not shown). If cells were first treated with PT for 24 h, and then thrombin was added, a high dose of thrombin (50 nM) could not increase thymidine incorporation in the continued presence of PT although FGF could (data not shown).

Like FGF and EGF, thrombin (10 nM) also inhibited CK induction in cells switched to differentiation medium (0.5% FBS; Fig. 3 *d*). In this experiment, $\sim 37\%$ of the amount of CK in control cells was present in thrombin-treated cells. However, if PT was added with thrombin, this inhibitory effect was abolished (Fig. 3 *d*). We have subsequently repeated these experiments with purified α -thrombin, and found it will stimulate thymidine incorporation and completely block CK activation in BC3H1 cells. These effects were completely reversed by PT (A. Sue-A-Quan, G. Simard, J. A. Connolly, unpublished results).

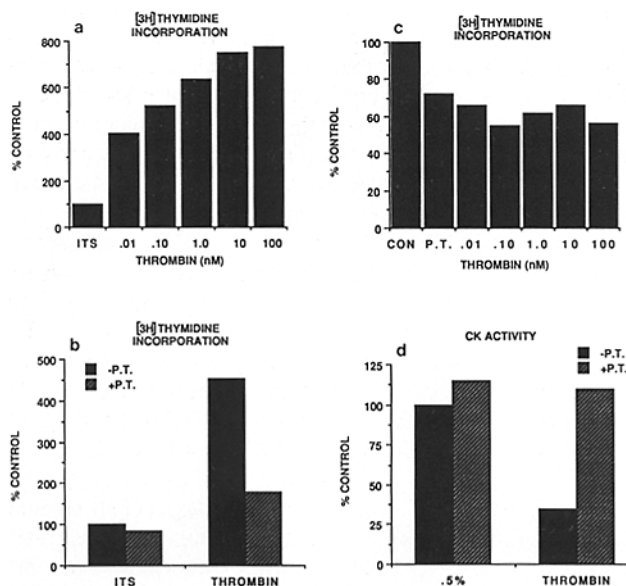


Figure 3. Thrombin effects on thymidine incorporation and CK expression in BC3H1 cells. (*a*) Cells were rendered quiescent by a switch to ITS medium, varying doses of thrombin added, and thymidine incorporation was determined 28 h later. (*b*) Quiescent cells were incubated with thrombin (10 nM) in the presence or absence of PT, and thymidine incorporation was determined. (*c*) Exponentially growing cells in 20% FBS were incubated with PT (10 ng/ml) and increasing doses of thrombin. (*d*) Cells were switched to 0.5% FBS to induce differentiation, and CK activity was measured 4 d later. Pair of bars (*left*), cells \pm PT. Pair of bars (*right*), cells + thrombin (10 nM) \pm PT.

We have found that FGF and thrombin will stimulate thymidine incorporation in serum-starved BC3H1 cells. Similar results were obtained if labeled nuclei were counted after tritiated thymidine autoradiography (data not shown). Thus, a significant proportion of these cells have transited the G1 phase of the cell cycle and entered into S phase. To determine if these cells were capable of proliferating in response to either of these growth factors alone, we measured cell number over time. In cells switched to 0.5% FBS or ITS, and supplemented with either FGF (30 ng/ml) or thrombin (10 nM), there was no significant increase in cell number over 5 d (Fig. 4). Thus, it appears these cells require more than one factor to transit through the cell cycle. Furthermore, the amount of thymidine incorporation or the percentage of labeled nuclei seen with a single growth factor was much lower than that seen with 20% FBS stimulation. In a separate study, we have found that the sequential exposure of cells to FGF and EGF gave thymidine incorporation values similar to 20% FBS medium and induced cell proliferation (Kelvin et al., 1988). These results suggest that thrombin is exerting effects on proliferation and differentiation in BC3H1 cells in a PT-sensitive pathway, and that G proteins may play a crucial role in transmitting such signals.

As the *ras* oncogene, another member of the G protein family, has previously been shown to block myogenic differentiation in C2 cells (Olson et al., 1987; Gossett et al., 1988), we examined the effects of PT on *ras*-transfected BC3H1 cells. Cells were transfected with the pHO6T1 plasmid containing the *val*¹² Harvey *ras* oncogene, and we have characterized one of these transfected cell lines, designated BCT31. Southern analysis indicated that this cell line stably incorporated the *ras* oncogene (Fig. 5 *a*), and Northern analysis with a human *ras* probe indicated that significant levels of mRNA were expressed from this transfected gene (Fig. 5 *b*). BCT31 cells did not express significant levels of CK, even after 4 d in 0.5% FBS (Fig. 5 *c*). In addition, these cells retained a myoblast-like morphology and failed to express significant levels of acetylcholine receptors, another marker of muscle differentiation (data not shown). Cells transfected with the pHO6 plasmid, which does not contain a *ras* gene, were not significantly different from control cells in their expression of muscle proteins (data not shown).

We examined the ability of PT to inhibit thymidine incorporation in BC3H1 and BCT31 cells in 20% FBS (Fig. 6 *a*). Although PT would inhibit incorporation by up to 80% in a dose-dependent fashion in control cells, it only reduced incorporation by $\sim 20\%$ in BCT31 cells (Fig. 6 *a*). Furthermore, when we examined [^3H]thymidine incorporation in BCT31 cells and control BC3H1 cells in the presence of PT (10 ng/ml), the amount of thymidine incorporation induced by EGF (50 ng/ml) in serum-free ITS medium was found to

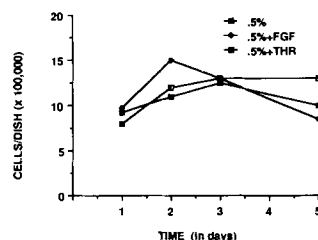


Figure 4. Effect of growth factors on proliferation of BC3H1 cells. Cells were switched to 0.5% FBS medium in the presence or absence of FGF (30 ng/ml) or thrombin (10 nM) and cell number was measured at 24-h intervals.

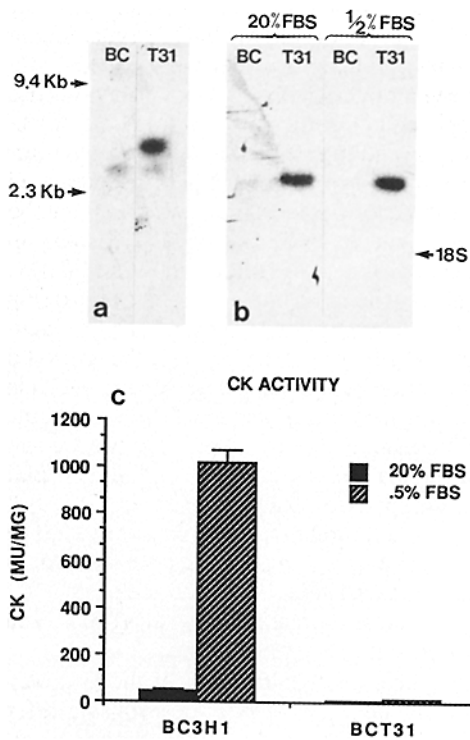


Figure 5. Characterization of *ras*-transfected BCT31 cells. (a) Southern blot analysis. DNA was probed with a ^{32}P -labeled EJ *ras* fragment that hybridizes to both mouse and human genomic *ras*. (b) Northern blot analysis. Cytoplasmic RNA was probed with a ^{32}P -labeled EJ *ras* fragment that hybridizes predominantly to human EJ *ras* RNA. (c) CK activity was determined 4 d after switching BCT31 or parental BC3H1 cells to fresh 20% or 0.5 FBS.

be 88% (for BC3H1) and 93% (for BCT31) of the values obtained in the absence of PT. These data indicate that BCT31 cells were largely insensitive to the effects of PT on ^3H thymidine incorporation.

BCT31 EJ *ras*-transfected cells do not express detectable levels of CK in 0.5% FBS (Fig. 5 b). Incubation of BCT31

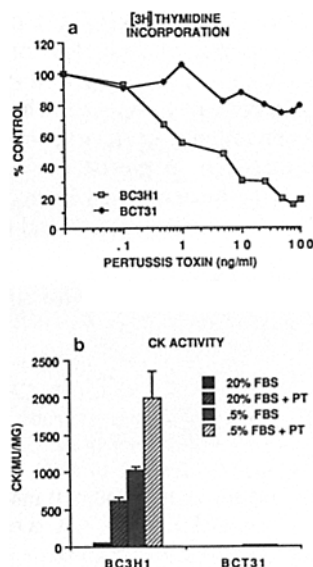


Figure 6. PT sensitivity of proliferation and differentiation in BCT31 cells. Cells were plated in 20% FBS. (a) 24 h later, varying doses of PT were added, and 24 h after PT addition, ^3H thymidine incorporation was determined. Each point represents the average of two separate samples. (b) 24 h later, cells were switched to fresh 20% FBS or 0.5% FBS in the presence or absence of PT. 96 h later, CK activity was determined. Each point represents the mean of triplicate determinations.

cells with 10 ng/ml PT did not induce any detectable CK activity in 20% FBS or 0.5% FBS although PT induced higher levels of CK activity in both serum conditions in BC3H1 cells (Fig. 6 b). The inability of PT to block proliferation or induce differentiation in BCT31 cells was not the result of a lack of substrate for this toxin. A similar, 40 kD substrate was ADP ribosylated in both BCT31 and BC3H1 cells (Fig. 7 a). Furthermore, this ribosylation in BCT31 cells was blocked by preincubation of these cells with increasing doses of PT (Fig. 7 b).

Discussion

These results demonstrate that both FGF and EGF are capable of stimulating thymidine incorporation and inhibiting differentiation in BC3H1 muscle cells in the presence of PT. Since PT can inhibit serum-induced proliferation and ADP ribosylates a membrane-associated, intracellular substrate, presumably a G protein, these results suggest that there is a second pathway that is PT sensitive. The present data also suggest that thrombin is acting in this PT-sensitive pathway to promote proliferation and block differentiation in BC3H1 cells.

Several growth factors including FGF, EGF, and TGF- β have been shown to modulate muscle cell differentiation (for review see Florini and Magri, 1988). Recently, it has been proposed that FGF can act as a morphogen to induce mesodermal lineages in *Xenopus* animal pole cells (Slack et al., 1987) and this can be potentiated by TGF- β (Kimelman and Kirschner, 1987). Intriguingly, Seed and Hauschka (1988) recently reported that FGF induced differentiation in one population of chick limb bud myoblasts but blocked it in a second. As far as we know, this current study is the first demonstration that thrombin can modulate muscle cell proliferation or differentiation. Thrombin could work alone or in conjunction with other growth factors during commitment or the regulation of differentiation in muscle. We are currently examining thrombin effects on skeletal muscle cell lines and primary muscle cell cultures.

It is interesting to note that PT inhibits BC3H1 cell growth in high serum (20% FBS) medium but does not block signals from FGF or EGF receptors. PT will also block thymidine incorporation by 30–50% and decrease proliferation of 3T3 cells stimulated with serum-containing medium (Hildebrandt et al., 1986) although this cell line has receptors for a wide variety of growth factors (Heslop et al., 1986; Berridge et al., 1984; Brown and Blakeley, 1983; Tsuda et al., 1985; Macphee et al., 1984; Zachary et al., 1986). Similarly, Chambard et al. (1987) found that EGF and FGF stimulated thymidine incorporation in lung fibroblasts in a PT-insensitive manner, but PT reduced thymidine incorporation by 85% in serum-containing medium. One possible explanation for this paradox is that the levels of EGF and FGF in the serum may be quite low, and growth factors working in a PT-sensitive pathway account for the majority of serum-stimulated growth in BC3H1 cells. Consistent with this interpretation is our observation that cells growing in 20% FBS medium supplemented with FGF have significantly higher levels of thymidine incorporation than cells in 20% FBS medium alone.

Our results are consistent with two independent signaling pathways regulating proliferation and differentiation in

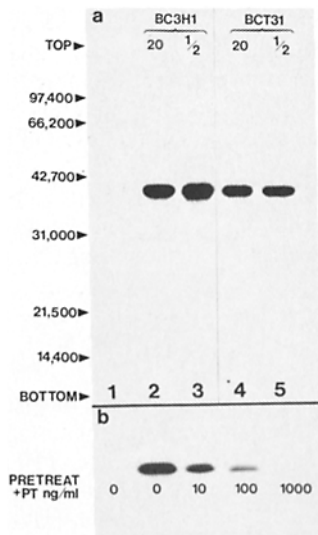


Figure 7. ADP ribosylation by PT in BCT31 cells. (a) Cell membranes were prepared from exponentially growing cells in 20% FBS or differentiated cells (4 d in 0.5% FBS) from EJ *ras*-transfected BCT31 cells or parental BC3H1 cells. (b) Preincubation of intact BCT31 cells with increasing doses of PT prevented the PT-induced ADP ribosylation of this substrate in membranes.

BC3H1 muscle cells. Similar results have been reported in other cell systems. Letterio et al. (1986) found that the bombesin-induced expression of *c-myc* and DNA synthesis in 3T3 cells could be blocked by PT, but PT had no effect on PDGF-induced proliferative responses. Pouyssegur and co-workers have shown that α -thrombin induces the hydrolysis of phosphatidyl-inositol 4,5-bisphosphate in cells made quiescent by serum starvation (Paris and Pouyssegur, 1986; L'Allemain et al., 1986), and that this hydrolysis is blocked by PT (Paris and Pouyssegur, 1986, 1987). However, these cells are also stimulated to proliferate by EGF or FGF in a second, PT-independent pathway (Paris and Pouyssegur, 1986; Chambard et al., 1987). Because PT did not completely inhibit thymidine incorporation, it remains a possibility that there are two distinct populations of BC3H1 cells, one sensitive to thrombin and another to FGF, rather than both pathways being present in individual cells. However, the fact that purified α -thrombin or acidic FGF could each completely block CK turn-on would argue strongly against this possibility.

We have also found that a BC3H1 muscle cell line stably transfected with the EJ *ras* oncogene (designated BCT31) does not differentiate, and this result is consistent with recent reports demonstrating that oncogenic H- or N-*ras*-transfected C2 cells (Olson et al., 1987; Gossett et al., 1988) or H-*ras*-transfected BC3H1 cells did not differentiate (Payne et al., 1987). *ras* thus becomes a candidate for acting in a signal-transducing pathway. The goal of the present study was to try and identify where *ras* functioned in the transduction of growth factor signals in BC3H1 cells. The rationale was that if *ras* functioned in the PT-sensitive pathway but before the PT block then PT should rescue differentiation in EJ *ras*-transfected cells. Alternatively, if *ras* functioned in the PT pathway, but after the PT block then PT would not be able to rescue differentiation. Likewise if *ras* functioned outside of the PT toxin pathway then PT would also not be able to rescue differentiation.

We found that PT toxin was not capable of rescuing the differentiation phenotype in EJ *ras*-transfected cells. We also found that EJ *ras*-transfected cells were capable of proliferating in high concentrations of PT. These data indicate that *ras* functions after the PT block or in a PT-insensitive path-

way. It is unlikely these results are due to the inability of PT to ADP ribosylate its "normal" G protein substrate as no differences were seen in substrates ribosylated in BC3H1 or BCT31 cells. Furthermore, this does not reflect a difference between the endogenous p21 *ras* and the transfected human *ras*, as no substrates were seen to be ribosylated in the range of 21 kD (Fig. 7). Magee et al. (1987) have also reported that p21 (N-*ras*) could not be ADP ribosylated by PT.

It has been proposed that p21 *ras* controls the activity of phospholipase C (Wolfman and Macara, 1987; Fleishman et al., 1986; Marshall, 1987). However, Lacal et al. (1987) reported that *ras*-transfected cells had higher constitutive levels of diacylglycerol, but levels of inositol phosphate were not affected; the report concluded that *ras* is working through another "novel" pathway to stimulate diacylglycerol production. Seuwen et al. (1988) found that expression of oncogenic Ha-*ras* or Ki-*ras* did not abrogate the sensitivity of phospholipase C activation to PT, and concluded that the transforming potential of activated forms of *ras* does not result from persistent activation of phospholipase C. Moreover, microinjected *ras* antibodies block mitogenic responses, induced by calcium ionophore and 12-O-tetradecanoyl phorbol-13-acetate (TPA), prostaglandin F_{2a}, or phosphatidic acid (Yu et al., 1988), strongly suggesting that *ras* does not control phospholipase C-induced production of inositol phosphates and Ca²⁺ but rather functions downstream from these mitogenic signals. The data we have presented also suggest that *ras* is downstream of the PT site in growth factor signaling pathways. Our hypothesis is summarized diagrammatically in Fig. 8. Two distinct pathways are capable of regulating proliferation and differentiation in BC3H1 cells. We speculate that *ras* functions in the PT-insensitive pathway; alternatively it may function at a common point or downstream of the PT substrate in the PT-sensitive pathway. We are currently investigating the functional location of *ras* using microinjected *ras* antibodies and growth factors.

ras stimulates the transcription of genes containing a regulatory sequence to which the transcription factors jun/AP-1 can bind (Wasylyk et al., 1987; Imler et al., 1988; Mermoud et al., 1988), and several proliferation active genes contain this regulatory sequence. *ras* also induces *fos* gene expression in NIH 3T3 cells and this *fos* gene expression is critical for the *ras* or TPA-induced activation of the collagenase promoter, a promoter activated by jun/AP-1 (Schonthal et al., 1988). Furthermore, *fos* interacts directly with jun/AP-1 to stimulate responsive genes (Chiu et al., 1988; Sassone-Corsi et al., 1988), and TPA, serum, and EGF can stimulate jun/AP-1 expression (Quantin and Breathnach, 1988; Lamph et al., 1988; Ryseck et al., 1988). In *ras*-transfected BCT31 cells, such transcription factors could bind to regulatory sequences of proliferation-specific genes, and as a result inhibit muscle gene expression. It is interesting to note that the muscle-specific CK enhancer region is not functional in N-*ras* transfected C2 muscle cells (Olson, E., personal communication). Alternatively, these transcription factors may bind to regulatory sequences of a differentiation "master gene," analogous to the recently described myoD gene (Davis et al., 1987), and inhibit its expression. Alternatively, *ras* may activate a protein that inactivates myoD or differentiation-specific enhancer binding proteins. These interesting studies suggest a plausible mode whereby growth factors could modulate differentiation in muscle

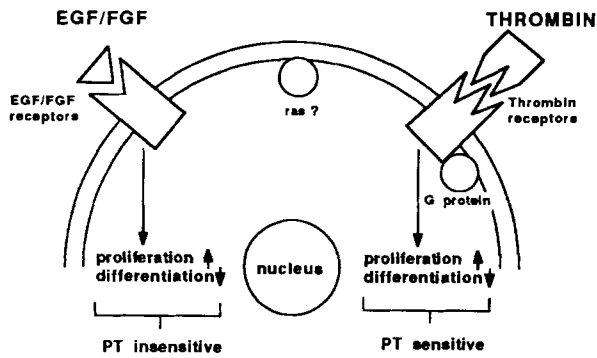


Figure 8. Model for signal transducing pathways that regulate proliferation and differentiation in BC3H1 cells. Two unique pathways, only one of which is sensitive to PT, are indicated. The *ras* oncogene, which can also modulate proliferation and differentiation in these cells, has been hypothesized to be working in one of these pathways.

cells, and we are currently investigating the validity of these possibilities.

In summary, we conclude that two signal transducing pathways identified in this study impinge on a common focal point that promotes proliferation and inhibits differentiation. *ras* functions in a PT-insensitive pathway or downstream from the PT substrate in BC3H1 cells.

We would like to thank D. Spandidos, Beatson Centre for Cancer Research, for providing the pHO constructs, and J. Ofosu, McMaster University Health Sciences Centre, Hamilton, for the α -thrombin. We would also like to thank S. Chance for excellent technical assistance and R. Dunn for technical advice.

This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada to J. A. Connolly. D. J. Kelvin is an Ontario Graduate Scholar.

Received for publication 10 May 1988, and in revised form 14 September 1988.

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