Regulation of Myogenic Differentiation by Type β Transforming Growth Factor

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Abstract. Type β transforming growth factor (TGFβ) has been shown to be both a positive and negative regulator of cellular proliferation and differentiation. The effects of TGFβ also are cell-type specific and appear to be modulated by other growth factors. In the present study, we examined the potential of TGFβ for control of myogenic differentiation. In mouse C-2 myoblasts, TGFβ inhibited fusion and prevented expression of the muscle-specific gene products, creatine kinase and acetylcholine receptor. Differentiation of the nonfusing muscle cell line, BC_3H1, was also inhibited by TGFβ in a dose-dependent manner (ID_{50} ~0.5 ng/ml). TGFβ was not mitogenic for either muscle cell line, indicating that its inhibitory effects do not require cell proliferation. Inhibition of differentiation required the continual presence of TGFβ in the culture media. Removal of TGFβ led to rapid appearance of muscle proteins, which indicates that intracellular signals generated by TGFβ are highly transient and require continuous occupancy of the TGFβ receptor. Northern blot hybridization analysis using a muscle creatine kinase cDNA probe indicated that TGFβ inhibited differentiation at the level of muscle-specific mRNA accumulation. These results provide the first demonstration that TGFβ is a potent regulator of myogenic differentiation and suggest that TGFβ may play an important role in the control of tissue-specific gene expression during development.

TYPE β transforming growth factor (TGFβ)[1] is a polypeptide homodimer of total M_r 25,000 that is abundant in platelets and is also present in a wide variety of tissues as well as in transformed cells (1, 2, 19, 20, 31, 37). TGFβ was first discovered by its ability to induce transformation in normal cells (31). The cellular actions of TGFβ are highly cell type specific and have been reported to be modulated by other growth factors present in serum (20, 21, 31, 32, 37). The pleiotropic effects of TGFβ include induction of anchorage-independent growth in mesenchymal cells (32), inhibition of adipocyte differentiation (11), induction of collagen and fibronectin synthesis (12), inhibition of epithelial cell proliferation, and stimulation of epithelial cell differentiation (22). In aortic smooth muscle cells, TGFβ also acts by inhibiting proliferation of subconfluent monolayer cultures, whereas it synergizes with mitogens to stimulate growth in soft agar (2). The physiological role of this growth factor in the control of cellular proliferation and differentiation as well as the mechanisms whereby TGFβ elicits its cellular responses remain to be determined.

The process of myogenesis involves withdrawal of proliferating myoblasts out of the cell cycle, their subsequent fusion to form multi-nucleated myotubes, and the coordinate induction of a battery of muscle-specific gene products (23–25). Because activation of muscle-specific gene expression is coupled to cell cycle withdrawal rather than to fusion, a great deal of interest has focused on the role of growth factors in the control of muscle differentiation (4, 6, 7, 9, 10, 13, 14, 16–18, 24, 25, 27–30, 35). The majority of studies on regulation of myogenesis by mitogens have used fetal calf serum (FCS) as a crude source of mitogenic activity. With the exception of fibroblast growth factor, which has been shown to inhibit muscle differentiation in a manner similar to serum (9, 10, 13, 14, 17, 18, 35), little is known of the identities or mechanisms of action of growth factors that regulate myogenesis.

Because of the diverse actions of TGFβ, we examined the possible involvement of this growth factor in the control of myoblast proliferation and differentiation, using the C-2 and BC_3H1 cell lines. These cell lines have been shown to exhibit properties of skeletal muscle and smooth muscle, respectively, after differentiation in media without mitogens (27–29, 34, 36, 38). In fusion-promoting media containing TGFβ, C-2 cells withdrew from the cell cycle but failed to fuse or differentiate as defined by expression of muscle creatine kinase and nicotinic acetylcholine (ACh) receptor. Similarly, in BC_3H1 cells, TGFβ delayed the onset of differentiation and inhibited muscle-specific mRNA accumulation. TGFβ was not mitogenic for either cell line, which indicates

1. *Abbreviations used in this paper*: ACh, acetylcholine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MCK, muscle isoenzyme of creatine kinase; TGFβ, type β transforming growth factor.

that its inhibitory effects on differentiation were not a secondary consequence of cell proliferation. These data demonstrate that TGFβ is a unique regulator of myogenic differentiation and suggest that TGFβ may play an important role in the control of tissue-specific gene expression during early development.

Materials and Methods

Cell Culture

The BC_3Hl mouse muscle cell line (34) was grown in DME containing 20% FCS (Hazelton Research Products, Denver, PA) as described previously (27). To initiate differentiation, media containing 20% serum was removed and replaced with media containing 0.5% serum.

The C-2 mouse muscle cell line (38) was grown in DME containing 20% FCS, 100 μg/ml L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). To initiate fusion, cultures were transferred to DME containing 10 μg/ml insulin, 5 μg/ml transferrin.

TGFβ (R & D Products, Minneapolis, MN) was resuspended in sterile 4 mM HCl at 10 μg/ml and stored at −20°C. Cell numbers were determined using a Coulter Counter (Coulter Electronics Inc., Hialeah, FL) and were confirmed with a hemocytometer. To determine the extent of fusion, nuclei were counted visually in five randomly selected fields at a magnification of 100. Cells containing >2 nuclei were considered to be fused. For metabolic labeling of proteins, cultures were incubated for 2 h with L-[^{35}S]methionine (New England Nuclear, Boston, MA; ~1,000 Ci/mmol) at 100 μCi/ml in DME without methionine and containing 0.5% FCS dialyzed against PBS (140 mM NaCl, 10 mM Na_2HPO_4, pH 7.2). At the end of the labeling period, extracts were prepared as described previously (27).

Creatine Kinase and ACh Receptor Assays

Creatine kinase was assayed as described previously (27). ACh receptors were assayed by specific binding of [^{125}I]α-bungarotoxin to cell monolayers as described (28, 29).

Preparation of Total Cellular RNA

Total cellular RNA was prepared from cultures according to a modification of the 8 M guanidine HCl procedure as described previously (27).

Labeling cDNA Probes

To quantitate steady-state levels of muscle isoenzyme of creatine kinase (MCK) mRNA, a SmaI-EcoRI restriction fragment was isolated from plasmid R21 containing an insert homologous to canine MCK mRNA (33). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was measured using a 1,650-base pair HhaI restriction fragment isolated from a chicken GAPDH cDNA clone, pGAD-28 (5). Inserts were labeled with ^{32}P to a specific activity of 1–5 × 10^9 cpm/μg as described by Feinberg and Vogelstein (8).

Northern Blot Hybridization

Formaldehyde-agarose gel electrophoresis of RNA and Northern blot hybridization were performed as described previously (35). Blots were exposed to Kodak XAR film at −70°C with DuPont Lightning-Plus intensifying screens (DuPont Co., Wilmington, DE). For quantitation of mRNAs, films were exposed for periods during which band intensity was linear with respect to time. Films were then scanned with a densitometer and mRNA abundance was determined from the area under the peak corresponding to individual mRNAs. Hybridization of cDNA probes to total cellular RNA was linear with respect to RNA concentration.

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Two-dimensional gel electrophoresis of [^{35}S]methionine-labeled proteins was performed as described by O'Farrell (26). After staining and destaining, gels were treated with EN^{3}HANCE (New England Nuclear) according to manufacturer's instructions.

Results

TGFβ Inhibits Myoblast Fusion and Appearance of Muscle-specific Proteins

C-2 cells proliferate rapidly in media with 20% FCS and do not express the muscle phenotype. Transfer to media containing insulin and transferrin (fusion-promoting media) results in withdrawal of cells from the cell cycle within ~24 h followed by fusion to form multinucleated myotubes over a period of ~3 d (Fig. 1). The process of fusion is accompanied by induction of creatine kinase and the ACh receptor (Fig. 2).

To examine the possibility that TGFβ might influence myogenic differentiation, proliferating, undifferentiated C-2 cells were exposed to fusion-promoting media with TGFβ (5 ng/ml). In the presence of TGFβ, C-2 cells withdrew from the cell cycle and achieved the same cell density as untreated cells (Fig. 2). However, cells treated with TGFβ retained the bipolar morphology, characteristic of proliferating cells, and failed to fuse at the normal rate (Figs. 1 and 2). TGFβ also delayed induction of ACh receptor and creatine kinase for ~48 h, after which, cultures began to fuse and differentiate

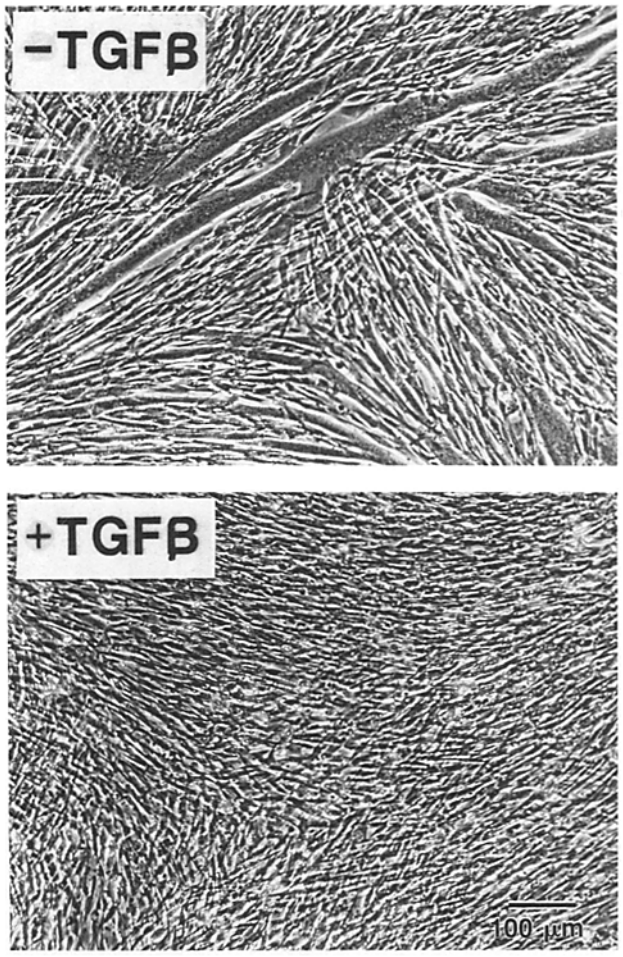

Figure 1. Morphology of C-2 cells in the presence and absence of TGFβ. C-2 cells were cultured in DME with 20% FCS as described in Materials and Methods. To examine the effects of TGFβ, cultures were photographed after exposure to fusion-promoting media with or without TGFβ (5 ng/ml) for 3 d. Conditions are identical to those on day 6 in Fig. 2.

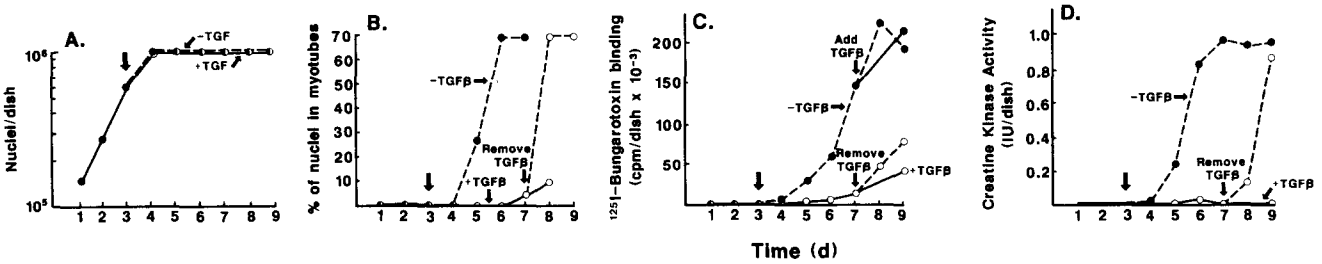

Figure 2. Effects on TGFβ on differentiation of C-2 cells. C-2 cells were cultured in DME with 20% serum as described in Fig. 1. On each day, nuclei per dish (*A*) were counted, the extent of fusion (*B*) was determined, and ACh receptors (*C*) and creatine kinase activity (*D*) were measured. On day 3, cultures were transferred to fusion-promoting media with or without TGFβ (5 ng/ml). On day 7, TGFβ was added to myotubes and was removed from TGFβ-treated cultures. Fresh TGFβ was added every 48 h to TGFβ-treated cultures. Note that TGFβ suppresses differentiation of mononucleated myoblasts and that differentiation proceeds at a normal rate after its removal. However, exposure of myotubes to TGFβ has little or no effect on differentiation. (● - - - ●) Control cultures without TGFβ; (O———O) cultures with TGFβ; (●———●) myotubes exposed to TGFβ; (O- - - -O) TGFβ-treated cultures exposed to fresh media without TGFβ.

very slowly. The low level of induction of muscle-specific proteins in the presence of TGFβ was not due to toxicity or to inhibition of protein synthesis since rates of [^{35}S]methionine incorporation into protein were actually increased slightly in the presence of TGFβ (data not shown). Furthermore, transfer of TGFβ-treated cultures to fresh media without TGFβ resulted in fusion and induction of ACh receptors and creatine kinase with kinetics similar to those observed in untreated cultures (Fig. 2).

We also examined the potential ability of TGFβ to repress expression of muscle-specific gene products in myotubes. As shown in Fig. 2, exposure of myotubes to TGFβ had little or no effect on expression of ACh receptor. In separate experiments, it was found that creatine kinase also continued to be induced after the addition of TGFβ to myotubes (Table I). Together, these results demonstrate that TGFβ inhibits differentiation of C-2 cells through a reversible mechanism that requires its continual presence in culture media. After fusion, C-2 cells become committed to terminal differentiation and lose their sensitivity to the inhibitory effects of TGFβ. Inhibition of differentiation by TGFβ was not a secondary consequence of cell proliferation because TGFβ exhibited no mitogenic activity for C-2 cells (Fig. 2 *A*).

Because of the pleiotropic actions of TGFβ on different cell types, we were interested in whether the inhibitory effects of this growth factor on myogenic differentiation were common to other muscle cell lines. BC_3Hl cells are a nonfusing cell line that has been shown to exhibit smooth muscle characteristics after differentiation in media containing 0.5% serum (34, 36). The effects of TGFβ on proliferation and differentiation of BC_3Hl cells are shown in Fig. 3. Exposure of proliferating undifferentiated BC_3Hl cells to 0.5% serum containing TGFβ (5 ng/ml) resulted in withdrawal of cells out of the cell cycle within 24 h. However, in contrast to control cultures that acquire an elongated refractile appearance after growth arrest (27, 34), TGFβ-treated cultures maintained a flattened stellate morphology characteristic of undifferentiated cells (data not shown). Cultures treated with TGFβ also failed to differentiate at a normal rate as defined by expression of ACh receptor and creatine kinase (Fig. 3). While TGFβ clearly inhibited differentiation of BC_3Hl cells, its effects were more complete in C-2 cells. The incomplete inhibition of differentiation of BC_3Hl cells was probably due, at least in part, to depletion of TGFβ from the media, since daily additions of TGFβ caused greater (although not total) repression (data not shown). As we observed for C-2 cells, inhibition of differentiation by TGFβ was rapidly reversible after its removal from the culture media.

Table I. TGFβ Fails to Block Induction of Creatine Kinase Activity in Myotubes

Culture condition		Creatine kinase activity
		mIU/dish
Day 6	−TGFβ	789
Day 7	−TGFβ	1,690
Day 7	+TGFβ	1,430

Conditions are identical to those on days 6 and 7 in Fig. 2. Undifferentiated C-2 cells were transferred to fusion promoting media for 3 d and creatine kinase activity was determined. Half of the remaining cultures were then exposed to TGFβ (5 ng/ml) for 1 d and creatine kinase activity was determined in the treated and untreated cultures. Note that TGFβ fails to block induction of creatine kinase in myotubes.

To determine whether TGFβ could inhibit expression of muscle-specific gene products in differentiated cells, quiescent differentiated cultures were exposed to TGFβ. As shown in Fig. 3, TGFβ caused a block in creatine kinase induction and a loss of ACh receptors from the cell surface at a rate equivalent to that reported previously for cultures exposed to media with 20% serum (28, 29). Thus, unlike C-2 myotubes which were refractory to TGFβ, BC_3Hl cells remained sensitive to the inhibitory effects of TGFβ after differentiation.

The dose dependence for repression of differentiation by TGFβ was examined by transferring proliferating undifferentiated BC_3Hl cells to media containing 0.5% serum with or without TGFβ. After 48 h, ACh receptors and creatine kinase activity were measured. As shown in Fig. 4, inhibition of differentiation could be observed at a TGFβ concentration as low as 0.20 ng/ml. Half-maximal repression occurred at ~0.5 ng/ml and maximal repression at ~1 ng/ml.

Together, the data in Figs. 1–4 demonstrate that TGFβ inhibits expression of muscle-specific proteins in both C-2 and BC_3Hl cells through a highly transient mechanism that is independent of cell proliferation. Inhibition of differentiation is not a general response to all growth factors because neither epidermal or platelet-derived growth factor had an apparent effect on expression of creatine kinase or ACh receptor (our unpublished results).

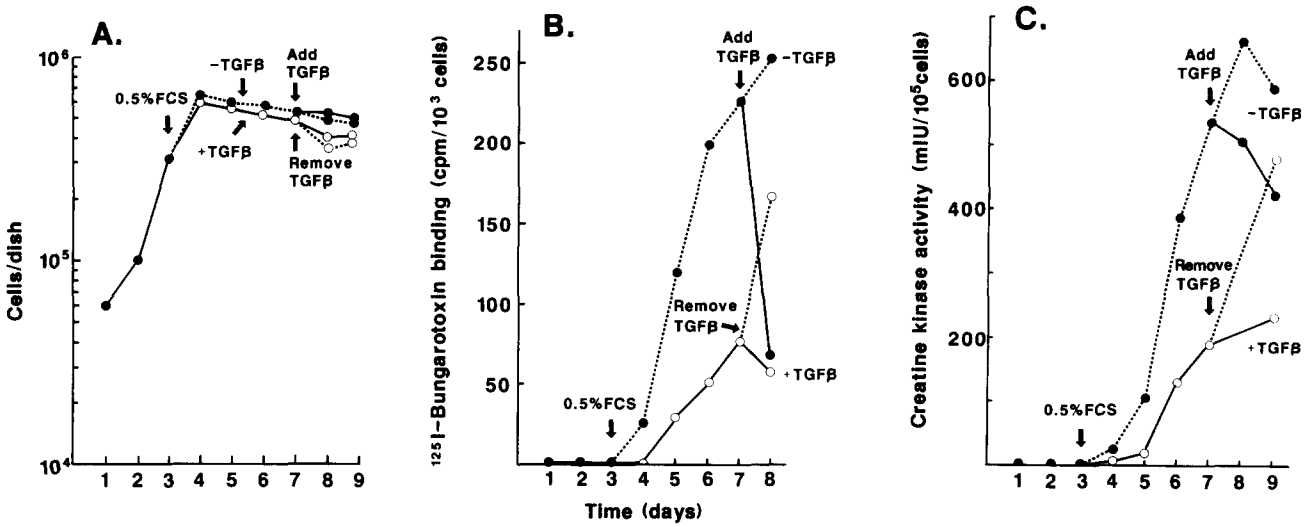

Figure 3. Effects of TGFβ on differentiation of BC_3H1 cells. BC_3H1 cells were cultured in DME with 20% serum as described in Materials and Methods. On each day, cells per dish (*A*) were counted and ACh receptors (*B*) and creatine kinase activity (*C*) were measured. On day 3, cultures were transferred to DME with 0.5% serum with or without TGFβ (5 ng/ml). On day 7, TGFβ-treated cultures were transferred to fresh media without TGFβ and quiescent differentiated cultures were exposed to TGFβ (5 ng/ml). Fresh TGFβ was added every 48 h to TGFβ-treated cultures. Note that TGFβ inhibits appearance of muscle-specific proteins and that after its removal, differentiation proceeds normally. Addition of TGFβ to quiescent differentiated cells also blocks accumulation of muscle proteins. (● - - - ●) Control cultures without TGFβ; (O——O) cultures with TGFβ; (●——●) quiescent, differentiated cultures exposed to TGFβ; (O- - - -O) TGFβ-treated cultures exposed to fresh media without TGFβ.

TGFβ Inhibits Accumulation of MCK mRNA

Previously we reported that differentiation of BC_3H1 cells was accompanied by accumulation of MCK mRNA. Exposure of quiescent differentiated cells to media with 20% serum or fibroblast growth factor resulted in a decline in abundance of MCK mRNA (35). To determine whether TGFβ inhibited differentiation through a mechanism similar to serum and fibroblast growth factor, steady-state levels of MCK mRNA were quantitated by Northern blot hybridization analysis. As shown in Fig. 5, exposure of quiescent differentiated cells to TGFβ or to media with 20% serum resulted in a dramatic decrease in the steady-state level of MCK mRNA. This effect of TGFβ was specific for MCK mRNA as demonstrated by the lack of an effect on the steady-state level of GAPDH mRNA. Steady-state levels of GAPDH mRNA do not change in response to proliferation or differen-

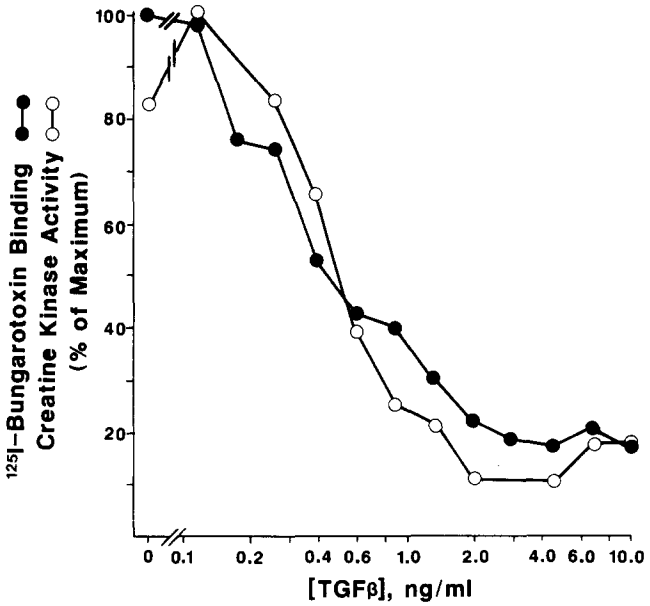

Figure 4. Dose-dependent inhibition of differentiation of BC_3H1 cells by TGFβ. BC_3H1 cells were exposed to media containing 0.5% serum for 2 d with or without TGFβ at the concentrations indicated and ACh receptors and creatine kinase activity were measured. Values are expressed relative to control cultures without TGFβ treatment, which were assigned a value of 100%.

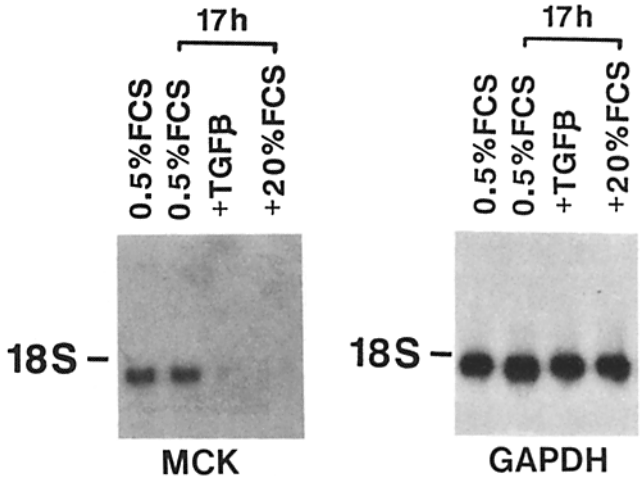

Figure 5. TGFβ specifically inhibits expression of MCK mRNA in BC_3H1 cells. BC_3H1 cells were allowed to differentiate for 4 d in media with 0.5% serum. Cultures were then exposed to media containing 0.5% serum with or without TGFβ (5 ng/ml) or 20% serum, as specified. After 17 h, RNA was isolated and the relative abundance of MCK mRNA and GAPDH mRNA was determined by Northern blot hybridization. Note that TGFβ and 20% serum both cause a loss of MCK mRNA but have no effect on GAPDH mRNA. The position of 18 S ribosomal RNA is indicated.

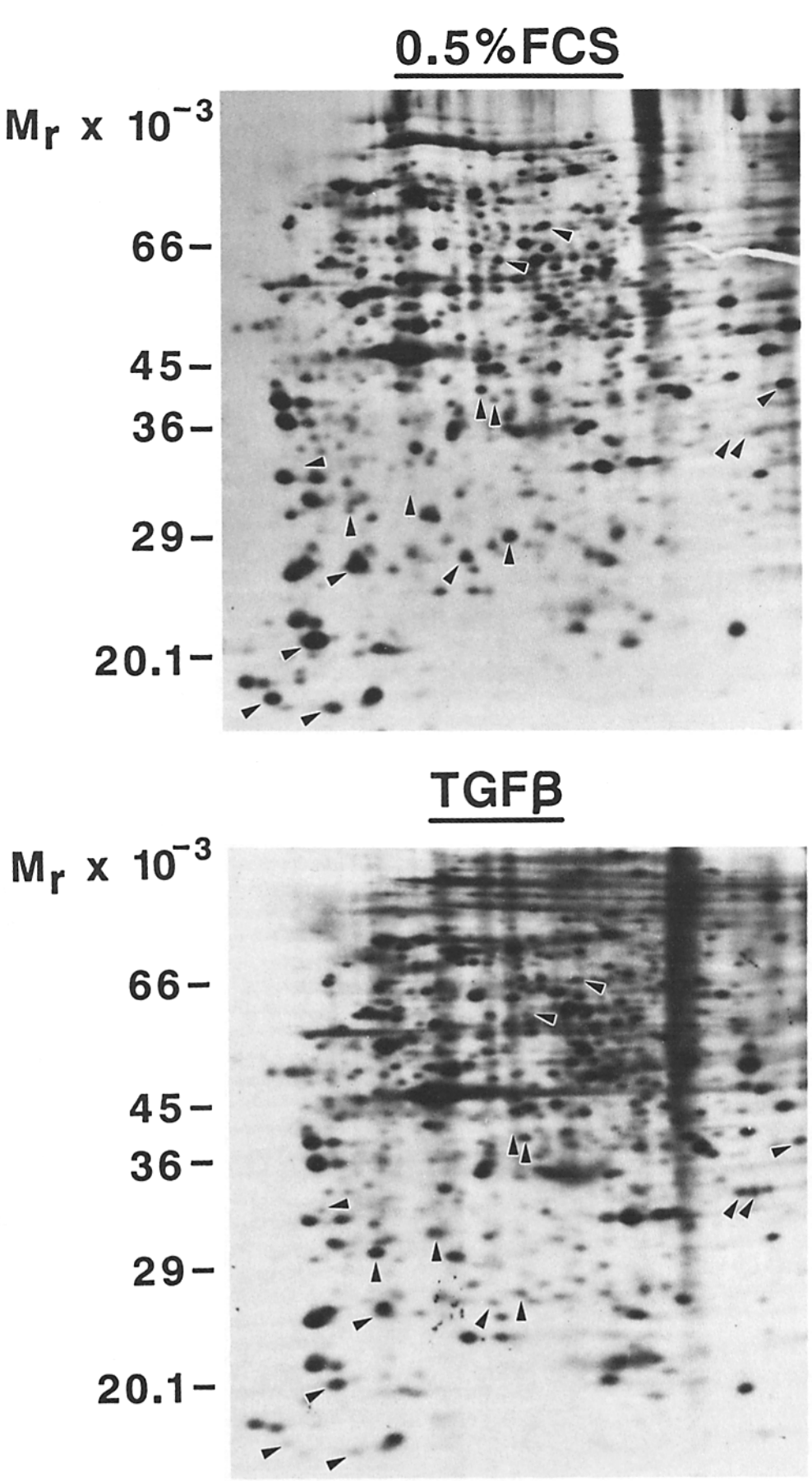

Figure 6. Two-dimensional gel electrophoresis of [^{35}S]methionine-labeled proteins from BC_3Hl cells treated with TGFβ. BC_3Hl cells were allowed to differentiate for 4 d in media with 0.5% serum. Cultures were then exposed to TGFβ (5 ng/ml) for 28 h. Cultures under each condition were labeled with [^{35}S]methionine for 2 h and labeled polypeptides were analyzed by IEF/SDS gel electrophoresis as described in Materials and Methods. Major polypeptides that change in abundance after exposure of differentiated cells to TGFβ are indicated with arrows.

tiation of BC_3H1 cells (35). These data demonstrate that TGFβ can regulate myogenesis by specifically repressing muscle-specific mRNA accumulation.

TGFβ Regulates Expression of a Variety of Proteins in BC_3H1 Cells

Because of the dramatic effects of TGFβ on creatine kinase and ACh receptor, we were interested in determining whether expression of other gene products also might be inhibited by TGFβ. To analyze the effects of TGFβ on the pattern of protein synthesis, quiescent differentiated BC_3H1 cells were exposed to TGFβ for 28 h. Cultures were then labeled for 2 h with [^{35}S]methionine and labeled polypeptides were separated by two-dimensional gel electrophoresis. As shown in Fig. 6, 10 proteins can be observed to decrease in abundance and ~6 proteins to increase in abundance after treatment of quiescent differentiated cells with TGFβ. Because only relatively abundant polypeptides are visualized on these gels, these represent minimum estimates of the total number of gene products whose expression is regulated by TGFβ in these cells. The identities of the proteins that are down regulated by TGFβ are currently unknown, but it seems likely some may represent muscle-specific gene products since they are regulated in parallel with creatine kinase and ACh receptor.

Discussion

During myogenesis, myoblasts cease dividing before activation of muscle-specific genes (4, 6, 7, 9, 10, 13, 14, 16–18, 24, 25, 27–30, 35). While serum components clearly play a key role controlling the onset of myogenic differentiation, the identities and mechanisms of action of the specific growth factors that regulate this process are only beginning to be understood. The characteristics of the quiescent state that allow muscle-specific gene expression also remain unclear.

The results of the present study demonstrate that TGFβ is a potent regulator of myogenesis that specifically inhibits one or more steps leading to activation of muscle-specific gene expression in both smooth muscle–like and skeletal muscle cells. Because TGFβ was not mitogenic for either muscle cell type, this growth factor appears to interfere directly with the differentiation program and does not seem to influence differentiation as a secondary consequence of cell proliferation. In this respect, TGFβ resembles the pituitary-derived and brain-derived fibroblast growth factors and a protein isolated from Buffalo rat liver, referred to as "differentiation inhibitor," or DI, each of which have been shown to inhibit myogenesis through a mechanism independent of cell proliferation (7, 13, 14, 35). TGFβ and the fibroblast growth factors are clearly distinct molecules; however, the relationship, if any, between TGFβ and DI, remains to be determined.

TGFβ did not affect the rate at which C-2 cells stopped dividing when switched into fusion-promoting medium. However, TGFβ inhibited fusion and appearance of muscle-specific gene products almost completely for at least 4 d, despite the fact that cells were in a quiescent state (Fig. 2). Thus, simply prolonging G_1 is not sufficient to induce differentiation. Previous studies have shown that myogenic differentiation occurs early in G_1 and that cells arrested in late G_1 fail to differentiate (4, 14). Moreover, fibroblast growth factor causes transit of quiescent BC_3H1 cells to a restriction point 4–6 h into G_1, that is nonpermissive for differentiation (14). We do not know yet whether TGFβ inhibits myogenesis by causing quiescent myoblasts to progress to this later region of G_1. We also do not presently know whether TGFβ directly represses transcription of muscle-specific genes or alternatively, leads to a generalized metabolic state that is incompatible with expression of this set of genes.

Removal of TGFβ from quiescent undifferentiated C-2 or BC_3H1 cells, allowed the appearance of muscle-specific proteins at a normal rate with no apparent lag, which suggests that the inhibitory signals generated by TGFβ are transient and require continuous occupancy of the TGFβ receptor. After fusion, C-2 cells became refractory to the effects of TGFβ while BC_3H1 cells remained sensitive to TGFβ independent of their state of differentiation. Whether the inability of TGFβ to reverse the differentiation process in myotubes is due to a loss of TGFβ receptors, as has been reported for EGF receptors during differentiation of mouse MM14 myoblasts (16), or is due to some other alteration has not been determined.

In addition to inhibiting expression of creatine kinase and ACh receptor, TGFβ also decreased the synthesis of at least 10 relatively abundant polypeptides in BC_3H1 cells (Fig. 6). The identities of these proteins are currently unknown. However, they may represent other muscle-specific gene products that are subject to coordinate regulation by TGFβ. A set of polypeptides also was induced in BC_3H1 cells by TGFβ. Recent studies by Leof et al. (15) suggest that at least some of these polypeptides may belong to the "competence" gene family, which is induced by platelet-derived growth factor during the transition of from quiescence to proliferation (3). Using AKR-2B cells, these investigators reported that TGFβ caused induction of c-sis and the subsequent induction of c-fos, c-myc, and other PDGF-inducible gene products through an autocrine mechanism (15). Identification of the polypeptides that are up and down regulated by TGFβ in BC_3H1 cells may help reveal the mechanisms through which this growth factor represses myogenic differentiation. Since many polypeptides are regulated by TGFβ in BC_3H1 cells, this will be an interesting system for studies on regulation of gene expression by this growth factor.

Little is known of the mechanisms involved in signal transduction by TGFβ. Tyrosine protein kinase activity, commonly associated with growth factor receptors, has not yet been demonstrated for the TGFβ receptor, nor have other potential signaling mechanisms been identified. The similarities between the effects of TGFβ, fibroblast growth factor, and DI on myogenesis suggest that these growth factors may share a common postreceptor pathway. Currently, we are attempting to identify possible intracellular second messengers that may mediate the effects of TGFβ and fibroblast growth factor on differentiation.

The physiological significance of TGFβ as an inhibitor of myogenesis remains to be determined. TGFβ might be involved in regulating the timing of myoblast fusion during early embryonic development. TGFβ might also be responsible for maintenance of satellite cells, the mononucleated myoblasts that surround mature muscle fibers. Combined with previous studies, the results presented here suggest that TGFβ plays an important role in regulation of tissue-specific gene expression during early development. It will be espe-

cially interesting to determine whether TGFβ interacts with common regulatory pathways in diverse cell types to control proliferation and differentiation.

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