Epidermal Growth Factor Controls Smooth Muscle α-Isoactin Expression in BC3H1 Cells

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Abstract. We have examined the effects of epidermal growth factor (EGF), platelet-derived growth factor, and insulin on the differentiation of a mouse vascular smooth muscle–like cell line, the BC3H1 cells. On the basis of cell morphology and smooth muscle α-isoactin synthesis, we demonstrate that EGF at physiological concentrations prevents the differentiation of these cells, whereas platelet-derived growth factor has no apparent effect. The induction of α-isoactin synthesis by serum deprivation is inhibited by EGF in a dose-dependent manner with a half-maximal effect at 3–5 ng/ml and a maximal inhibition at ∼30 ng/ml. Northern analysis also shows that EGF blocks the accumulation of α-isoactin mRNA normally observed during cell differentiation. Addition of EGF to differentiated cells results in a repression of α-isoactin synthesis, a stimulation of β- and γ-isoactin synthesis, and the stabilization of the nonmuscle isoactins. The synthesis of creatine phosphokinase, a muscle-specific noncontractile protein, is also regulated by EGF in a similar fashion. Modulation by EGF of α-isoactin expression is not affected by aphidicolin and is therefore independent of its mitogenic effect on these cells. Insulin is not required for observation of the EGF-dependent effects but instead seems to promote differentiation. Our results show that EGF can replace serum in controlling the differentiation of BC3H1 cells.

THE growth and differentiation of vascular smooth muscle cells can be controlled by factors that are in serum or secreted from neighboring cells (22, 26, 34). The nature of the complex regulation exerted by these factors is an important problem both in cell biology and medicine. A model that has proven useful in addressing this problem is the BC3H1 cell line.

BC3H1 cells, isolated from a mouse brain tumor (27), differentiate in vitro when placed in serum-free medium. Events occurring during the process of differentiation include cessation of cell proliferation, transformation from the morphology of fibroblasts to a long spindly shape typical of smooth muscle cells, and stimulation of the synthesis of several muscle-specific proteins such as myokinase (27), acetylcholine receptor (23), creatine phosphokinase (CPK)[1] (19), and insulin receptor (30). These phenotypic changes induced by serum depletion can be prevented or reversed by addition of serum to the medium.

Previous studies in our laboratory have revealed that expression of actin isoforms in BC3H1 cells also appears to be developmentally regulated. Cell differentiation in serum-free

1. *Abbreviations used in this paper*: CPK, creatine phosphokinase; EGF, epidermal growth factor; ILGF, insulin-like growth factor; N2SF medium, N2 serum-free medium; PDGF, platelet-derived growth factor.

medium results in a selective three- to fourfold increase of smooth muscle α-isoactin synthesis and a five- to sixfold increase in the level of its mRNA, relative to that observed in undifferentiated cells (31, 32).

Serum contains a wide variety of growth factors with various effects on cells (1, 2). To understand the control of BC3H1 cell differentiation exerted by serum, it is necessary to determine the roles played by individual serum growth factors in this model system. Based on changes in the levels of CPK or acetylcholine receptor, two serum growth factors, fibroblast growth factor and β-transforming growth factor, have been shown to modulate the behavior of BC3H1 cells (14, 21, 29). In similar experiments, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) were reported to have no effect on these cells (14, 21). Recently, it has been shown that fibroblast growth factor can exert an effect similar to that of serum on α-isoactin expression in these cells (36).

In the work presented here, we report that results of our studies on the effects of EGF, PDGF, and insulin on the expression of smooth muscle α-isoactin in BC3H1 cells. These factors have all been implicated as possibly being important in regulating smooth muscle cell growth (9, 16, 35). Our results show that although PDGF has no significant effect on actin expression in BC3H1 cells, EGF, like FCS, causes an inhibitory effect on α-isoactin expression. This effect of EGF is independent of the DNA replication.

Materials and Methods

Materials

L-[^{35}S]Methionine (>800 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). [^{32}P]dCTP (3,000 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Receptor-grade EGF and HPLC-purified PDGF were obtained from Collaborative Research Inc. (Lexington, MA). Insulin, aphidicolin, putrescine, and progesterone were purchased from Sigma Chemical Co. (St. Louis, MO). For routine use, EGF was stored as an aqueous solution, insulin was stored in 0.1 N HCl, and aphidicolin was stored in absolute ethanol. Antiserum against human muscle CPK, which cross reacts with the mouse enzyme, was generously provided by Ventrex Co. (Portland, ME). All other chemicals were reagent grade.

Cell Culture

BC3H1 cell monolayers (27) were grown and maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 in T-75 flasks (Corning Glass Works, Corning, NY) with DME supplemented with 10% heat-inactivated FCS (Gibco, Grand Island, NY or HyClone Laboratories, Logan, UT), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were kept subconfluent by subculturing twice a week as described (31). For experiments in this report, cells from passages 10 to 20 were cultured in 35-mm dishes.

To initiate cell differentiation or to test the effects of growth factors on this process, cells were placed in N2 serum-free (N2SF) modified hormone-supplemented medium (3) containing RPMI-1640, 5 µM BSA (Miles Laboratories Inc., Naperville, IL; Pentex, fatty acid-free), 5 µg/ml insulin, 100 µg/ml transferrin, 20 nM progesterone, 100 µM putrescine, 30 nM Na_2SeO_3, and 10 mM Hepes buffer solution, pH 7.2. Confluent cells were rinsed and incubated in N2SF medium, with growth factors added when indicated, for 4 d except where otherwise indicated. Cells were then radiolabeled as described below.

Radioactive Labeling of Cells

Cells were incubated in methionine-deficient DME supplemented with 90 µCi/ml L-[^{35}S]methionine for 4 h at 37°C. The labeled monolayers were washed twice with PBS and scraped off the dishes in 1 ml of PBS. The cells were then collected by centrifugation in a microfuge (Beckman Instruments, Inc., Palo Alto, CA) for 3 min. Cell pellets were dissolved in 70 µl of the 9 M urea-containing lysis buffer described by O'Farrell (18).

Electrophoretic Analysis of Labeled Proteins

Two-dimensional gel electrophoresis was performed essentially as described by O'Farrell (18), except that a modified IEF protocol was used (8). The IEF gels were prefocused for 15 min at 300 V, 30 min at 650 V, and 30 min at 1,000 V. After sonicating for 10–15 s, 20 µl of the [^{35}S]methionine-labeled cell lysate was subjected to IEF in a pH 5–7 gradient at 1,000 V for 16 h. Throughout the prefocusing and focusing, the gel system was maintained at 18°C. Gels were then processed for electrophoresis on 10% polyacrylamide slab gels containing SDS. After autoradiography, spots of interest were excised from the dried gels and quantitated by liquid scintillation counting. Experiments were performed in triplicate, and gels of samples from three independent cultures were analyzed.

Analysis of Total Cell Actin by DNAse I Affinity Chromatography

The experiment was performed essentially as described previously (15, 37). Cells were labeled with [^{35}S]methionine for 4 h as described above. Cells were then harvested, sonicated in a buffer containing 10 mM Tris-HCl, pH 7.4, 0.2 mM $CaCl_2$, 0.2 mM ATP, 10% formamide. A small aliquot was reserved for determination of total cell protein. The remaining solution was applied to a 0.6-ml column of DNAse I-agarose and the actin isolated as described previously (15, 37). For each determination, triplicate plates were analyzed in parallel, and the results are expressed as cpm labeled actin ±SD/µg cell protein.

Analysis of CPK Activity

CPK activity was determined as described previously (14, 20), except that

the concentration of NP-40 was reduced to 0.2% in the solution used to homogenize the cells so that protein concentrations could be analyzed with the protein assay from Bio-Rad Laboratories (Richmond, CA). CPK activity was assayed using a CPK reaction kit (Sigma Chemical Co.), and the results were normalized to total cell protein.

Immunoprecipitation of CPK

Cells from a 35-mm dish were labeled for 4 h in the presence of [^{35}S]methionine, harvested, and sonicated in Tris buffer, pH 7.4, containing 1% NP-40, 2 mM EDTA, 0.15 M NaCl, 1 mM PMSF, and 100 kallikrein inhibitor units/ml aprotinin. Immunoprecipitation of radioactively labeled CPK was performed essentially according to the procedure of Olson et al. (19) using sufficient antiserum to completely remove all CPK from the sample. The immunoprecipitated protein was eluted from fixed killed *Staphylococcus aureus* (Boehringer Mannheim Biochemicals, Indianapolis, IN) containing protein A, and the labeled protein was subjected to SDS gel electrophoresis. For each precipitation equal amounts of total labeled cell protein were used, and following immunoprecipitation, the entire sample was loaded on the gel.

RNA Isolation and Characterization

Preparation of whole cell RNA was performed as previously described (5, 32).

Northern analysis of BC3H1 cell RNA was performed as described previously (32) using human skeletal muscle actin cDNA subjected to nick translation (17) in the presence of [α-^{32}P]dCTP as a probe. The hybridization and washing were performed at 42°C.

Results

Effect of EGF on the Differentiation of BC3H1 Cells

When quiescent cells are cultured in serum-free medium for 4 d, they differentiate into a muscle-like phenotype as previously described (27). This change is reflected by an alteration of cell morphology from a fibroblast-like appearance to a long spindle-shaped appearance typical of muscle cells. At the same time, smooth muscle α-isoactin synthesis is stimulated (31) as is the synthesis of CPK and the acetylcholine receptor (19, 23).
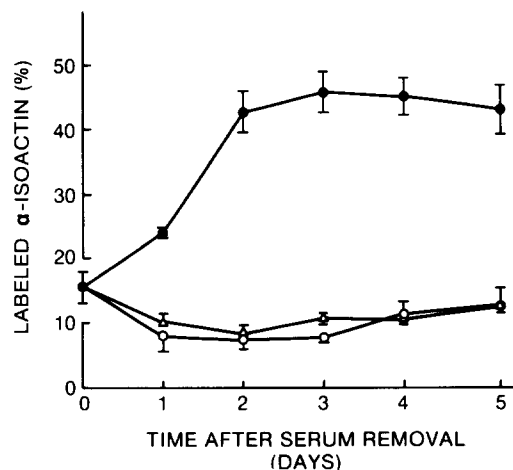

Figure 1. Effects of EGF and aphidicolin on α-isoactin synthesis in differentiating BC3H1 cells. Confluent cells were placed in the medium indicated. At selected times cells were labeled with [^{35}S]methionine and the percent newly labeled α-isoactin was determined as indicated in Materials and Methods. Each point shown represents the mean ±SD of three independent samples. (●) N2SF; (○) N2SF containing 75 ng/ml EGF; (△) N2SF containing 75 ng/ml EGF + 5 µg/ml aphidicolin.
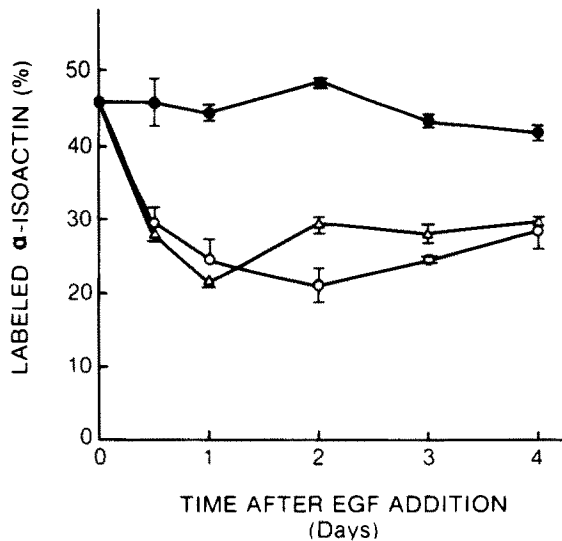
Figure 2. Effect of EGF-induced dedifferentiation on α-isoactin synthesis in BC3H1 cells. Cells differentiated 4 d in N2SF were placed in the medium indicated. At selected times, the cells were labeled with [^{35}S]methionine for 4 h, and the percent α-isoactin of the total labeled actin was determined as described in Materials and Methods. Each point shown represents the mean ±SD of three independent samples. (●) N2SF; (○) N2SF + 75 ng/ml EGF; (△) N2SF + 75 ng/ml EGF + 5 μg/ml aphidicolin.

Inclusion of EGF in the serum-free medium at the start of the differentiation process prevents the morphological change usually observed in these cells (data not shown). In addition, it prevents the induction of smooth muscle α-isoactin synthesis (Fig. 1). Interestingly, in the presence of added EGF, the level of synthesis of this actin isoform relative to the nonmuscle actins is actually lower than it is in cells grown in the presence of 10% FCS (Fig. 1).

Effect of EGF Addition to Differentiated Cultures of BC3H1 Cells

Addition of EGF to 4-d differentiated cultures caused a decrease in the amount of α-isoactin synthesis relative to the synthesis of the nonmuscle β- and γ-actin isoforms (Fig. 2). To determine whether this relative change represented a decrease in the actual amount of α-isoactin synthesized, we measured the total amount of newly synthesized actin by isolating labeled whole cell actin by DNAse I chromatography as described in Materials and Methods and normalizing the amount of radioactive actin to total cell protein. In one experiment, we obtained 1,130 ± 110 cpm actin/μg cell protein in the control cells and 1,240 ± 220 cpm actin/μg cell protein in cells dedifferentiated with EGF for 12 h. In an identical experiment after 4 d of treatment with EGF, the values obtained were 990 ± 130 cpm actin/μg cell protein in the control and 920 ± 130 cpm actin/μg cell protein in the treated cells. These results showed that there was no significant change in the amount of newly synthesized actin per microgram of cell protein after treatment of the differentiated cells with EGF.

The results just described, combined with the data shown in Fig. 2 demonstrate that in cells dedifferentiated with EGF for 12 h, there is ~46% less newly synthesized α-isoactin and 39% more of the newly synthesized β- and γ-actin iso-

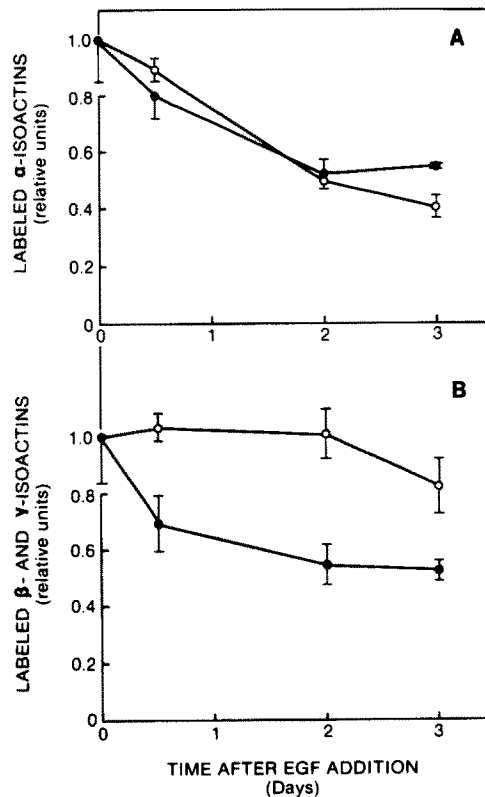*Figure 3.* Effect of EGF-induced dedifferentiation on the turnover of (*A*) α-isoactin and (*B*) β- and γ-isoactins. Labeling of cells with [^{35}S]methionine and the analysis and determination of labeled isoactins are described in Materials and Methods. Cells differentiated 4 d in N2SF were pulse labeled for 4.5 h, washed with unlabeled N2SF, and then transferred to a chase medium consisting of N2SF, 10 mM unlabeled methionine alone (●) or in the presence of 75 ng/ml EGF (○) for the indicated times. The radioactivity in each of the isoactin spots was normalized to the amount in each of the species on day 0. Each point represents the mean ±SD of three independent samples.

forms in comparison with cells continuously grown in the absence of EGF over the same time period. For cells dedifferentiated for 4 d in EGF the absolute amount of newly synthesized α-isoactin decreases by 32% and the absolute amount of β- and γ-isoactins increases 22% compared with the control cells.

We also determined whether dedifferentiation induced by EGF was accompanied by a selective change in the turnover of actin isoforms by studying the disappearance of pre-labeled isoactins after the start of dedifferentiation. As shown in Fig. 3, there is a slower decrease in the relative level of labeled β- and γ-isoactins in dedifferentiating cells over a 3-d period when compared with that in differentiated cells in the absence of EGF over the same period of time. On the other hand, the turnover of α-isoactin is about the same under both growth conditions. These results inducate that not only is the synthesis of α-isoactin decreased relative to the β- and γ-isoforms, but that the existing β- and γ-isoactins are selectively stabilized during dedifferentiation.

Effects of EGF on CPK Levels in BC3H1 Cells

We next investigated whether the effects we observed with

Table I. Effects of EGF and Aphidicolin on CPK Activity
in BC3H1 Cells

Growth condition	CPK activity
	IU/mg protein
Differentiation	
N2SF	0.70 ± 0.05
N2SF + EGF	0.06 ± 0.01
N2SF + EGF + aphidicolin	0.07 ± 0.01
Dedifferentiation	
N2SF	1.22 ± 0.12
N2SF + EGF	0.61 ± 0.03
N2SF + EGF + aphidicolin	0.60 ± 0.03

In the differentiation experiment, confluent cells were transferred into the indicated medium for 4d, and the CPK activity was then determined as described in Materials and Methods. For the dedifferentiation experiment, differentiated cells that had been growing 4 d in N2SF medium were transferred to the medium indicated for an additional 4 d, and the CPK activity in the cells was then determined as indicated in the text. Each value shown is the mean ±SD of three independent samples. For these experiments, EGF was used at 75 ng/ml and aphidicolin at 5 µg/ml.

Figure 5. Concentration dependence of the inhibition of α-isoactin synthesis on EGF. Confluent cultures were transferred to N2SF medium containing the level of EGF indicated. After a 4-d incubation, the cells were labeled with [35S]methionine and the percent labeled α-isoactin was calculated as described in Materials and Methods. Each point represents the mean ±SD of three independent samples.

EGF were specific for cytoskeletal and contractile proteins or whether they occurred as well on muscle-specific metabolic enzymes such as CPK whose synthesis is stimulated during the BC3H1 cell differentiation process (19). Our results, shown in Table I, demonstrate that in cells treated with EGF to prevent differentiation, CPK activity is inhibited 91% relative to the activity observed in differentiated cells. Immunoprecipitation of metabolically labeled CPK from differentiated cells and cells treated with EGF at the beginning of growth in serum-free medium showed that this inhibition of CPK activity was actually due to an inhibition of enzyme synthesis (Fig. 4).

Treatment of differentiated cells with EGF to induce dedifferentiation also resulted in a decrease in CPK activity (Table I). Again, immunoprecipitation experiments indicated that this decrease could be largely accounted for by a decrease in the synthesis of CPK. Equal amounts of radioactively labeled total and cells dedifferentiated 4 d with EGF
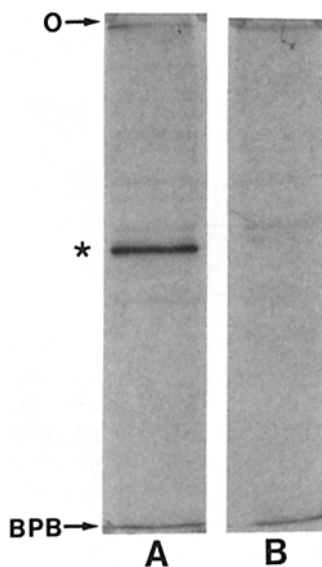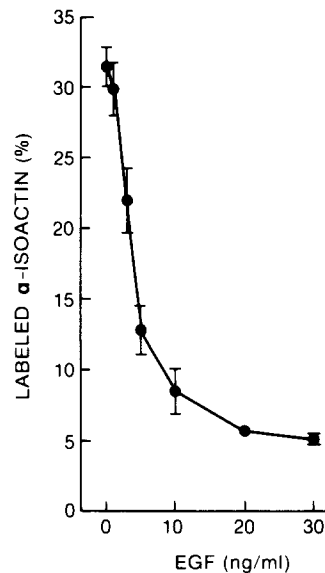

Figure 4. Immunoprecipitation of [35S]methionine-labeled CPK from undifferentiated and differentiated cells. The experiment was performed as described in Materials and Methods and an autoradiogram of the gel was generated. (A) CPK from cells differentiated 4 d in N2SF medium. (B) CPK from undifferentiated cells incubated for 4 d in N2SF medium containing 75 ng/ml EGF. (*) Denotes the position of CPK with an apparent molecular mass of 40 kD. O, origin. BPB; Bromphenol blue marker.

were subjected to immunoprecipitation, and the precipitate was further resolved on SDS gels. Scanning of the CPK band from three separate experiments showed that the band intensity from the dedifferentiated cells was 0.22, 0.20, and 0.27 of that in the respective differentiated cells. These results indicate that as well as controlling the differential expression of actin isoforms, EGF can also inhibit the expression of CPK in these cells.

EGF Dose-Response Study

To determine whether the effects observed in response to EGF occurred at physiological levels of the growth factor, we performed a dose-response study with EGF at concentrations varying between 0 and 30 ng/ml. The results, shown in Fig. 5, indicate that the half-maximal inhibition of α-isoactin synthesis occurs at 3-5 ng/ml, whereas the maximum inhibition is observed at ∼30 ng/ml. The half-maximal effect is in the range seen for other EGF responsive processes (4, 9, 25, 33).

Effects of Insulin on the Differentiation of BC3H1 Cells

Insulin has been shown to promote cell differentiation in 3T3 cells (10), and it has also been shown to potentiate or to act synergistically with other growth factors in promoting cell proliferation (12, 28). Since insulin at 5 µg/ml is a component of N2SF medium, it is important to know whether it is required along with EGF to produce the effects we have observed. We thus tested media with different combinations of these two factors for their effects on BC3H1 cell differentiation. The results presented in Table II show that whether insulin is totally excluded or present at a reduced concentration in the medium, EGF inhibits α-isoactin synthesis to the same extent it does in regular N2SF, demonstrating that EGF exerts its effect in an insulin-independent fashion.

Even though insulin does not influence the effects of EGF, it appears to be required for maximal differentiation of these cells. When insulin was omitted from the N2SF medium during cell differentiation, the observed levels of α-isoactin syn-

Table II. Effects of Insulin and EGF on α-Isoactin and Total Protein Synthesis in BC3H1 Cells

Additions	α-Isoactin	Total protein
	% total labeled actin	*cpm/dish (×10⁻⁷)*
None	39.4 ± 2.2	1.07 ± 0.05
EGF	8.0 ± 1.5	4.64 ± 0.34
5 ng/ml insulin	38.1 ± 1.8	1.14 ± 0.16
5 ng/ml insulin + EGF	8.9 ± 1.0	4.71 ± 0.19
50 ng /ml insulin	50.2 ± 2.6	1.55 ± 0.09
50 ng/ml insulin + EGF	9.8 ± 1.3	4.76 ± 0.20
5,000 ng/ml insulin	52.1 ± 0.3	3.88 ± 0.14

The basic medium used in this experiment is insulin-deficient N2SF. EGF was used at 75 ng/ml. Confluent cells were placed in the medium indicated for 4 d and labeled with [^{35}S]methionine, and the labeled isoactins were analyzed as described in Materials and Methods. Each value shown is the mean ±SD of three independent samples.

thesis were only ∼80% as high as the insulin-containing control (Table II).

Relationship between the Mitogenic Effect of EGF and Its Effect on α-Isoactin Synthesis

We next studied whether EGF could stimulate cell proliferation in BC3H1 cells and, if so, whether its ability to inhibit the expression of α-isoactin was dependent on its mitogenic properties. Differentiated cells were treated with EGF, and the cell number per plate was determined. The results, shown in Table III, indicate that addition of EGF for 48 h to a differentiated culture (4 d incubation in serum-free medium) resulted in a 75–80% increase in cell number. When 20% serum was used as a mitogenic stimulus for comparison, a doubling of the cells occurred over the same time. Thus EGF itself was able to produce almost the same degree of cell proliferation as did 20% serum. Insulin was not required for EGF to exhibit its mitogenic effect on these cells. However, the absence of insulin resulted in an overall lowering of the cell number either because of increased cell death or cell shedding (Table III).

We then studied whether the effect of EGF on α-isoactin synthesis in differentiating cells was independent of the mitogenic effects of EGF. To do this, we added 5 μg/ml aphidicolin (11), a specific inhibitor of DNA polymerase-α, with EGF

Table III. The Mitogenic Effect of EGF in Differentiated BC3H1 Cells

Additions	Cell number/dish
	×10⁻⁴
None	61 ± 8
EGF	107 ± 8
20% FCS	134 ± 4
Insulin	100 ± 9
Insulin + EGF	178 ± 2
Insulin + 20% FCS	219 ± 8

Confluent cells in 35-mm dishes were differentiated in N2SF medium for 4 d and were then transferred to insulin-deficient N2SF medium supplemented with growth factors or serum as indicated. Insulin was used at 5 μg/ml, and EGF was used at 75 ng/ml. After incubation for 48 h, triplicate cultures from each growth condition were trypsinized with 0.05% trypsin, and the cells were pelleted in DME plus 10% FCS and resuspended in 1 ml PBS. Cell numbers of the suspensions were counted in quadruplicate with a hemocytometer and expressed as the mean ±SD.
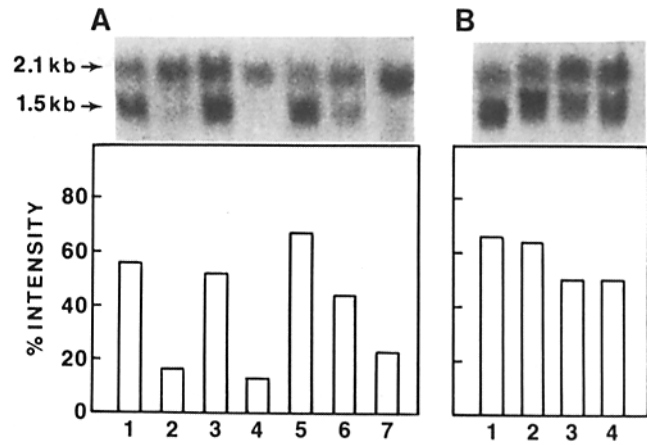

Figure 6. Northern analysis of the muscle and nonmuscle isoactin mRNAs in BC3H1 cells grown under different conditions. The Northern analysis was performed as described in the Materials and Methods. 40 μg of RNA was loaded per lane. Thus, the band intensity observed reflects the actual amount of each actin mRNA present in the cells in the various stages tested allowing for lane to lane comparison. The radioactive band at 2.1 kb represents the β and γ nonmuscle isoactin mRNAs while the band at 1.5 kb represents the α-isoactin mRNA as described previously (32). The autoradiogram of the Northern blot was analyzed by scanning densitometry using an Eikonix digital camera system in the University of Iowa Image Analysis Facility. The percent intensity of the 1.5-kb band was calculated by dividing the intensity of the 1.5-kb band by the sum of the intensities of the two bands, and the results are displayed in the histogram. (*A*) For lanes *1–4*, RNA was collected 4 d after transfer of confluent cells to the medium indicated. (*Lane 1*) N2SF; (*lane 2*) N2SF + 75 ng/ml EGF; (*lane 3*) insulin-deficient N2SF; (*lane 4*) insulin-deficient N2SF containing 75 ng/ml EGF; (*lane 5*) cells differentiated for 4 d in N2SF (differentiated cells) were placed for 4 d in fresh N2SF; (*lane 6*) differentiated cells were placed for 4 d in N2SF containing 75 ng/ml EGF; (*lane 7*) confluent cells before the start of differentiation in N2SF. (*B*) Effect of short-term dedifferentiation on α-isoactin mRNA. For all lanes, 4-d differentiated cultures were used. (*Lane 1*) Starting cells; (*lane 2*) 12 h in fresh N2SF; (*lane 3*) 12 h in N2SF + EGF; (*lane 4*) 12 h in N2SF + 20% FCS.

in serum-free medium. This concentration of aphidicolin is sufficient to inhibit DNA synthesis >97% (data not shown). Under these conditions, the inhibitory effect of EGF on α-isoactin synthesis remained unchanged both in the differentiation (Fig. 1) and dedifferentiation (Fig. 2) processes. Identical results were also seen on the repression of CPK activity (Table I). These results show that the effect of EGF on the inhibition of the synthesis of these muscle-specific proteins occurs independent of its effect on DNA synthesis.

Analysis of α-Isoactin mRNA Levels in Differentiated and Dedifferentiated Cells

Previous work from our laboratory showed that the induction of smooth muscle α-isoactin synthesis was accompanied by a significant increase in the level of α-isoactin mRNA (32). We wished to determine whether the effect caused by EGF on α-isoactin synthesis in these cells occurred in conjunction with a change in the levels of the mRNAs for the muscle and nonmuscle isoactins.

We carried out a Northern analysis on RNA samples ob-

tained from cells in various states of differentiation. As a probe, we used a human skeletal muscle α-isoactin cDNA previously shown to hybridize to the mRNAs for both muscle and nonmuscle actin isoforms (32). The findings shown in Fig. 6 are representative of three different experiments carried out, and equal amounts of total cell RNA were loaded in each lane of the gel. The results in Fig. 6 A indicate first that α-isoactin mRNA accumulates during the differentiation phase relative to the mRNAs for the nonmuscle actin isoforms (lanes 1 and 7), and addition of EGF to N2SF prevents this accumulation (lane 2). Omission of insulin during the differentiation phase results in a smaller increase in the relative level of α-isoactin (lane 3) mRNA compared to the case when insulin is included. Lane 4 demonstrates that insulin is not required for the suppression of α-isoactin mRNA level resulting from the addition of EGF to N2SF. Finally, when dedifferentiation is caused by the addition of EGF for 4 d, a significant decrease in the relative level of α-isoactin mRNA occurs compared to the control in fully differentiated cells (lanes 5 and 6). Fig. 6 B shows that in as short a time as 12 h after addition of either EGF or 20% FCS, there is a 22–25% decrease in the amount of α-isoactin mRNA. Thus, for both differentiating and dedifferentiating cells, changes in α-isoactin levels are accompanied by similar changes in the levels of its mRNA. Early in dedifferentiation, the decrease in α-isoactin mRNA does not equal the decrease in the synthesis of α-isoactin, possibly suggesting the operation of translational control in this process as well.

Discussion

The differential change observed in isoactin synthesis can be accounted for by changes in the levels of the muscle and nonmuscle isoactin mRNAs. During dedifferentiation, there is also a selective stabilization of the nonmuscle isoactins, possibly due to the preferential use and subsequent protection of the nonmuscle actin isoforms at this time.

In our hands, EGF controls the synthesis of CPK during differentiation and dedifferentiation in a manner parallel with smooth muscle α-isoactin as determined both by the measurement of enzyme activities and by immunoprecipitation of newly synthesized CPK. This observation indicates that EGF may coordinately control the expression of a number of muscle-specific proteins. Furthermore, although EGF acts as a mitogen for these cells, this activity is not required for EGF to inhibit the synthesis of these muscle-specific proteins. It has been shown in other cells as well that EGF can cause major effects in specific gene expression independent of its ability to act as a mitogen (13). Furthermore, in BC3H1 cells, Spizz et al. (29) reported a similar finding for FGF.

When assessing the effects of EGF during the dedifferentiation process, we noticed that in the control cells maintained in serum-free medium, there is a continuous increase in the level of muscle actin mRNA relative to that of nonmuscle actin mRNA (compare lanes 1 and 5 in Fig. 6 A). There was not, however, a concomitant increase in the percent of newly synthesized α-isoactin relative to the nonmuscle isoforms during this time (control; Fig. 2). This discrepancy may be due either to an isoform-specific translational control or it may result from the synthesis of nontranslatable 1.5-kb mRNA that will still hybridize with the actin clone. We cannot at this time distinguish between these possibilities.

Inclusion of insulin in the serum-free medium potentiates the degree of differentiation achieved by these cells. However, the concentrations of insulin required for this effect are much higher than those needed to exert an effect through the high affinity insulin receptor (30). Although high affinity insulin receptors have been detected in BC3H1 cells (30), our particular subclone does not express these (Pessin, J., personal communication). Since insulin at high concentrations also binds to the insulin-like growth factor-I (ILGF-I) receptor (7) which is present in our cells (Ginsberg, B., personal communication), the insulin effect we observed was probably mediated via the ILGF-I receptor. Interestingly, in primary smooth muscle cell cultures, ILGF-I, working presumably through the same receptor, exhibits the opposite effect: promotion of cell proliferation (6, 9, 24).

Our results are in disagreement with those of Lathrop et al. (14) and Olson et al. (21) who reported that EGF had no effect in BC3H1 cells. Their protocols differed from ours in that their differentiated cells were grown on collagen layers in 0.5% serum while our cells were differentiated in serum-free medium and grown on plastic. When we substituted their culture conditions individually for ours, however, the EGF effects were still observed. Thus, the most likely explanation for our differing results is that we have a different subclone of the cell line which responds to EGF whereas theirs does not. Growth of our cells on a collagen layer did seem to produce a more pronounced differentiated phenotype in terms of the amount of α-isoactin expression (data not shown). We agree with Lathrop et al. (14) and Olson et al. (21) that PDGF has no observable effect on BC3H1 cell differentiation. Thus, these cells do not require exogenous PDGF to make them competent before becoming sensitive to the influence of EGF.

The effects we observed with EGF during the dedifferentiation process are similar to those reported by Wice et al. (36) for fibroblast growth factor. This observation suggests that these two factors at some point may work through similar pathways after binding of the growth factors to their receptors. Finding this connection will be important in understanding the nature of the control of differentiation in these cells.

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