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Metalloendoprotease inhibitors that block fusion also prevent biochemical differentiation in L₆ myoblasts

(creatine kinase/commitment)

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ABSTRACT The effect of metalloendoprotease inhibitors on the biochemical differentiation of the rat skeletal muscle line, L₆, was investigated. Confluent unfused L₆ cells exposed briefly to 1,10-phenanthroline, a chelator of divalent metal cations, or continuously to dipeptide amide metalloendoprotease substrates that are blocked at the NH₂-terminals, *N*-carbobenzyloxyserylleucyl amide and *N*-carbobenzyloxyglycylleucyl amide, did not fuse or express creatine kinase, myosin heavy chain, or α -actin. These effects were reversible and dose-dependent. Exposure to *N*-carbobenzyloxyglycylglycyl amide, which is not a metalloendoprotease inhibitor, had no effect. As the differentiation in a culture progressed, 1,10-phenanthroline became less effective in blocking the accumulation of creatine kinase and myosin heavy chain. Exposure of partially fused cultures to *N*-carbobenzyloxyserylleucyl amide prevented any further accumulation of muscle-specific proteins. In confluent cultures where cell division was blocked before the onset of differentiation, *N*-carbobenzyloxyserylleucyl amide still prevented fusion and the induction of creatine kinase. This indicates that these inhibitors do not act by interfering with the cell cycle. Experiments that measured DNA synthesis rates, plating efficiencies, and the effects of sequential dipeptide and dimethyl sulfoxide treatments indicate that L₆ myoblasts do not irreversibly withdraw from the cell cycle when exposed to *N*-carbobenzyloxyserylleucyl amide. These results are consistent with the role of a metalloendoprotease in initiating the terminal differentiation of cultured muscle cells.

The rat skeletal muscle cell line, L₆ (1), undergoes a developmental program in culture similar to muscle cells *in vivo*. Sparse myoblasts proliferate until confluency, after which they begin withdrawing from the cell cycle (G₀ state) and subsequently lose their proliferative capacity (commitment) (2, 3). These committed myoblasts then fuse their plasma membranes to form myotubes and biochemically differentiate by synthesizing large amounts of muscle-specific proteins, such as proteins for the contractile apparatus, creatine kinase, and the acetylcholine receptor. These processes result in a terminally differentiated muscle fiber and are collectively referred to as myogenesis.

Couch and Strittmatter (4) showed that the metalloendoprotease (MEPr) inhibitors, 1,10-phenanthroline and certain *N*-carbobenzyloxy dipeptide amides, prevent the Ca²⁺-dependent fusion of primary rat myoblasts. They identified a MEPr activity in the cytosol of L₆ myoblasts that is inhibited by the same compounds that block myoblast fusion (5). These inhibitors also prevent Ca²⁺-dependent fusion in vesicle secretory systems (6), suggesting that MEPrs may have a general role in catalyzing biological membrane fusion.

Our laboratory has recently identified dramatic changes in major plasma membrane glycoproteins during myogenesis of L₆ myoblasts. These changes were blocked by the MEPr inhibitors that block fusion (7). We now report that these same MEPr inhibitors also prevent the increased synthesis of muscle-specific proteins during myogenesis. We hypothesize that a MEPr is involved in initiating the terminal differentiation of L₆ myoblasts.

METHODS

Cell Culture. Two subclones of L₆, originally isolated by Yaffe (8), were used: clone A begins fusing 4 to 5 days after plating and is fully fused by day 12, and clone B begins fusing 3 to 4 days after plating becoming fully fused by day 7. Cells were grown on 100-mm, 60-mm, or 35-mm plastic tissue culture dishes (Falcon, Corning) in an 8% CO₂ atmosphere at 37°C using either Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (growth medium) or Ham's F12 medium supplemented with 20% fetal calf serum (nondifferentiating medium). Both contained 1% penicillin/streptomycin (GIBCO).

1,10-Phenanthroline Treatment. Cells grown on 100-mm dishes were washed twice with DMEM and exposed to 10 ml of 80 μ g of 1,10-phenanthroline monohydrate (Sigma) per ml for 2 hr at 37°C. Controls received an equivalent amount of solvent (0.2% methanol). The dishes were then washed three times with 5 ml of growth medium and fed 16 ml of that medium.

Dipeptide Treatment. Stock solutions of 0.75 M carbobenzyloxyserylleucyl amide (Cbz-Ser-Leu-NH₂), 1 M carbobenzyloxyglycylleucyl amide (Cbz-Gly-Leu-NH₂), and 1 M carbobenzyloxyglycylglycyl amide (Cbz-Gly-Gly-NH₂) (Vega Biochemicals) were made in dimethyl sulfoxide (Me₂SO). Cells grown on 35-mm dishes received 1.5 ml of growth medium containing the specified amount of dipeptide or the equivalent amount of Me₂SO (0.1% or 0.2%) daily.

Creatine Kinase Activity. Plates were washed twice with phosphate-buffered saline (0.02 M KPO₄, 0.14 M NaCl) and immediately frozen at -70°C and stored until assayed. Cytosolic extracts were prepared by freeze-thawing twice in 0.4-1.2 ml of 10 mM Tris-HCl/10 mM KCl/0.06% mercaptoethanol and removing the particulate fraction by centrifugation in an Eppendorf centrifuge (15,000 \times *g* \times 10 min). Creatine kinase activity was measured using a coupled enzyme assay (9) with hexokinase and glucose-6-phosphate dehydrogenase (Sigma). The production of NADPH was measured fluorimetrically (excitation wavelength, 349 nm/

Abbreviations: Cbz-Ser-Leu-NH₂, carbobenzyloxyserylleucyl amide; Cbz-Gly-Leu-NH₂, carbobenzyloxyglycylleucyl amide; Cbz-Gly-Gly-NH₂, carbobenzyloxyglycylglycyl amide; FdUrd, 5-fluorodeoxyuridine; DMEM, Dulbecco's modified Eagle's medium; Me₂SO, dimethylsulfoxide; MEPr, metalloendoprotease; mU, millunit(s).

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emission wavelength, 465 nm). Rates are expressed as milliunits per milligram of extract protein as determined by the method of Lowry *et al.* (10).

Labeling of Cellular Proteins with [³⁵S]Methionine. Clone B was grown to confluency on 100-mm culture dishes and then fed daily 10 ml of growth medium containing either 750 μ M Cbz-Ser-Leu-NH₂ or 0.1% Me₂SO for 3 days. Cultures were then labeled in 8 ml of DMEM (3 μ g of methionine per ml) containing 100 μ Ci (1 Ci = 37 GBq) of [³⁵S]methionine (Amersham) (800 Ci/mmol) for 17 hr in the absence or presence of Cbz-Ser-Leu-NH₂. After the labeling period, dishes were rinsed twice with cold phosphate-buffered saline, scraped, and the cells lysed by two freeze-thaw cycles in phosphate-buffered saline. The particulate fraction was collected by centrifugation and frozen at -70°C.

Extraction of Actomyosin. This particulate fraction was extracted with 0.25 or 1 ml of 110 mM Na₂SO₄/40 mM glycine, pH 9.5/2 mM EGTA/1 mM MgSO₄ at 4°C for 2 hr (11). After centrifugation the supernatant was frozen at -20°C.

Electrophoresis. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (NaDodSO₄/PAGE) of high-salt extracts containing actomyosin was done as described by Laemmli (12) and in the figure legends.

Two-dimensional electrophoresis was done on the total particulate fraction as described by O'Farrel (13) using pH 5-7 ampholytes (Bio-Rad) for isoelectric focusing and 10% (vol/vol) acrylamide in the second dimension.

RESULTS

Effect of 1,10-Phenanthroline on Creatine Kinase Expression. 1,10-Phenanthroline forms stable complexes with some divalent metal cations such as Zn²⁺, Fe²⁺, and Cu²⁺; it is thought to inhibit MEPrs by chelating active-site metal ions (14).

Treatment of confluent unfused L₆ cultures with 1,10-phenanthroline monohydrate (80 μ g/ml) for 2 hr delayed the onset of fusion for 2 days. Creatine kinase activity (24 mU/mg; 1 mU/mg = 1 nmol ATP produced per minute per mg of protein) was detected in the cytosolic extracts of treated cells on the third day after treatment, whereas the controls at this time were fused and expressing high levels of creatine kinase activity (121 mU/mg). The treated cells became fully fused by the fifth day after treatment and then expressed high levels of creatine kinase (100 mU/mg) (data not shown).

Treating partially fused cultures with 1,10-phenanthroline (Fig. 1A, curves b and c) prevented the increase in the rate of creatine kinase accumulation for 1 or 2 days after which creatine kinase accumulated at a rate near that of the controls (Fig. 1A, curve d). Both the decrease in the rate of creatine kinase accumulation and the length of the lag time before recovery were less in those cultures that were more differentiated at treatment (compare curves b and c, Fig. 1A). Exposure of the cells to 0.2% methanol for 2 hr (controls) on 3 successive days delayed the increase in creatine kinase activity only slightly. Confluent unfused cultures that were treated every 2 days with 1,10-phenanthroline remained in the undifferentiated state (Fig. 1A, curve a). Cultures treated three times with 1,10-phenanthroline (80 μ g/ml) remained viable and accumulated creatine kinase activity that was less than 9% of the controls (Fig. 1B).

The effect of 1,10-phenanthroline on creatine kinase expression was dose-dependent with the half-maximal effect occurring between 10 and 20 μ g/ml (Fig. 1B).

Effect of Cbz-Ser-Leu-NH₂ on Creatine Kinase Expression. Dipeptides which are blocked at the amino and carboxyl termini and contain hydrophobic carboxyl-terminal residues

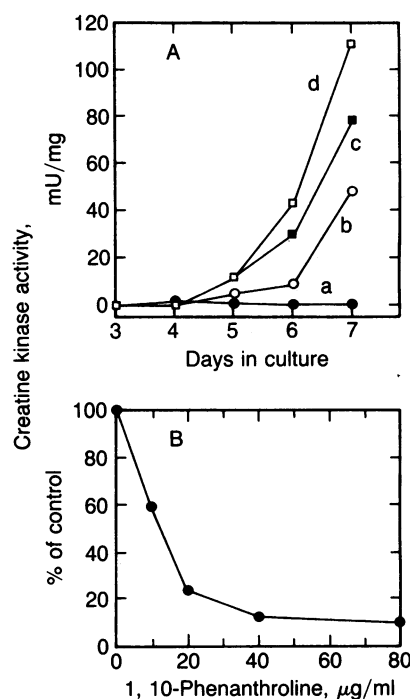


FIG. 1. Effect of 1,10-phenanthroline on creatine kinase induction. (A) One-hundred millimeter culture dishes were inoculated with 3×10^5 cells per dish of clone B. Cultures were treated as follows: curve a, 1,10-phenanthroline monohydrate (80 μ g/ml) on day 3 (confluent) and day 5; curve b, 1,10-phenanthroline monohydrate (80 μ g/ml) on day 4 (began fusing); curve c, 1,10-phenanthroline monohydrate on day 5 (partially fused); and curve d, 0.2% methanol (controls) on days 3, 4, and 5; after plating. Plates were harvested when indicated and assayed for creatine kinase activity as described. Each point represents a single plate. Similar results were obtained with clone A (data not shown). (B) One-hundred millimeter culture dishes were inoculated with clone A as in A. Cultures were treated with 0, 10, 20, 40, or 80 μ g of 1,10-phenanthroline monohydrate per ml of 0.2% methanol on days 4 (confluency), 6, and 8 after plating. The plates were harvested on day 10 and assayed for creatine kinase activity. Each point represents the average of duplicate experiments (100% activity = 195 mU/mg protein).

are substrates and therefore competitive inhibitors of certain MEPrs (15).

Cbz-Ser-Leu-NH₂, a MEPr substrate, has been shown to block myoblast fusion (5). Continuous exposure of confluent L₆ cultures to 750 μ M Cbz-Ser-Leu-NH₂ also prevented creatine kinase accumulation (Fig. 2A, curve a). These cultures did not fuse but remained viable for at least 8 days. When Cbz-Ser-Leu-NH₂ was removed from the medium, subsequent fusion and increased creatine kinase activity occurred within 1 day (Fig. 2A, b), although the cultures took 3 days to become fully fused. Exposing partially fused cultures to Cbz-Ser-Leu-NH₂ resulted in no further increase in creatine kinase activity and a loss of 50% of the specific activity over 3 days (Fig. 2A, c). Although creatine kinase did not continue to accumulate in partially fused cultures exposed to Cbz-Ser-Leu-NH₂, these cultures continued to fuse for approximately another day. Myosin heavy chain content was probed by dot blot immunoassay with a monospecific antibody, and similar results were obtained for both Cbz-Ser-Leu-NH₂ and 1,10-phenanthroline (data not shown).

When a sparse population of myoblasts was exposed to 750 μ M Cbz-Ser-Leu-NH₂, their morphology became dendritic, and some cells became "arborized" (16). The doubling time of the myoblasts increased to \approx 44 hr compared with 20 hr for control cultures (data not shown).

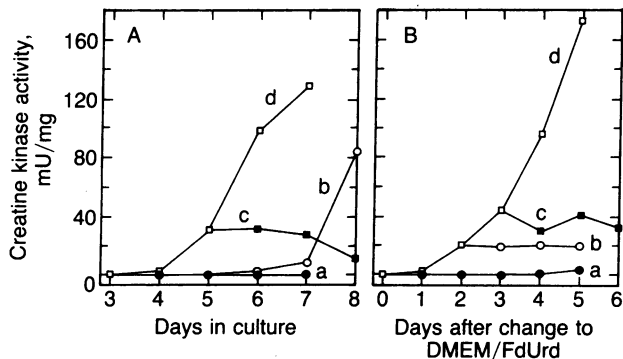


FIG. 2. Effect of Cbz-Ser-Leu-NH₂ on creatine kinase induction in growth medium (A) and growth medium containing 5-fluorodeoxyuridine (FdUrd) at 100 ng/ml (B). (A) Thirty-five millimeter culture dishes were inoculated with 4.5×10^4 cells per dish of clone B in 3 ml of growth medium. The cultures were fed daily 1.5 ml of medium containing the following: curve a, 750 μM Cbz-Ser-Leu-NH₂ from days 3 (confluent) to 7; curve b, 750 μM Cbz-Ser-Leu-NH₂ from days 3 to 5; curve c, 750 μM Cbz-Ser-Leu-NH₂ from days 5 (partially fused) to 8; and curve d, 0.1% Me₂SO (not containing Cbz-Ser-Leu-NH₂)—these control cultures were fed 1.5 ml of medium. Each point represents the average of duplicate experiments. (B) Thirty-five millimeter dishes were inoculated with 2×10^4 cells of clone B in 3 ml of Ham's F12 containing 20% fetal calf serum. After 6 days the medium was changed to DME medium containing 10% fetal calf serum and 100 ng of FdUrd per ml. Cultures received 1.5 ml of this medium daily containing either 750 μM Cbz-Ser-Leu-NH₂ or 0.1% Me₂SO. Curve a, 750 μM Cbz-Ser-Leu-NH₂ from days 0 (confluent) to 5; curve b, 750 μM from days 2 (partially fused) to 5; curve c, 750 μM Cbz-Ser-Leu-NH₂ from days 3 (partially fused) to 6; and curve d, 0.1% Me₂SO (control). Each point represents the average of duplicate experiments.

Effect of Cbz-Ser-Leu-NH₂ in Cultures of Constant Density. The biochemical differentiation and fusion of L₆ myoblasts has been shown to be density dependent (17). Furthermore, the density of L₆ cultures continues to increase after confluency and the initiation of fusion and differentiation. It was therefore possible that L₆ cultures that had been treated with Cbz-Ser-Leu-NH₂ did not reach sufficient density to differentiate because of a lengthened generation time.

To answer this question, we required a culture system in which the cell number would remain constant throughout the experiment. Pinset and Whalen (18, 19) showed that L₆ myoblasts could be grown to high densities without differentiating or fusing in Ham's F12 medium. Changing the medium to DMEM induced the cultures to differentiate and fuse. When cell division was prevented by inhibitors of DNA synthesis, these cultures still fused and differentiated normally.

We grew L₆ myoblasts for 6 days on Ham's F12 medium to a high density. The medium was then changed to DMEM, and the effect of 750 μM Cbz-Ser-Leu-NH₂ on creatine kinase expression was determined. FdUrd (100 ng/ml) was added to the medium to block cell division (20). Control cultures (Fig. 2B, curve d) began fusing within 24 hr and induced high levels of creatine kinase, whereas those exposed to 750 μM Cbz-Ser-Leu-NH₂ continuously (Fig. 2B, curve a) induced only very low levels of the enzyme (<2% of the controls after 5 days). While the majority of dipeptide-treated myoblasts did not fuse, some (<1%) di-nucleated and tri-nucleated myotubes were observed.

When partially fused cultures were exposed to Cbz-Ser-Leu-NH₂ 2 or 3 days after switching to DMEM, no further creatine kinase accumulated for up to 3 days, and the specific activity remained essentially constant (Fig. 2B, curves b and c). These cultures continued to fuse for another day after this exposure to the dipeptide. However, the newly formed

myotubes were elongated and spindly, unlike the amorphous patches observed in untreated cultures. Similar results were obtained when cytosine arabinoside (2 μg/ml) was used in place of FdUrd (data not shown).

Dose Response of Cbz-Ser-Leu-NH₂ and Other Dipeptides. The effect of Cbz-Ser-Leu-NH₂ on confluent and unfused cultures was dose dependent. The maximal effect on fusion and creatine kinase expression was observed between concentrations of 0.5 mM and 0.75 mM, and the half-maximal effect on creatine kinase expression occurred at about 0.18 mM (Fig. 3, curve a). With 0.5 mM dipeptide, no creatine kinase activity (<0.5 mU/mg) was detected, although some myotubes were still present. Cbz-Gly-Leu-NH₂ is another MEPr substrate that has been shown to block myoblast fusion (4, 5). It, too, was effective in preventing creatine kinase accumulation in confluent L₆ cultures (Fig. 3, curve b; maximal effect at 1.5 mM and half-maximal effect at about 0.6 mM). Cbz-Gly-Leu-NH₂ was also less effective in blocking myoblast fusion compared with Cbz-Ser-Leu-NH₂ (4).

Although Cbz-Gly-Gly-NH₂ is chemically similar to the other two dipeptides, it is not an inhibitor of MEPrs or myoblast fusion (4, 5); it was also ineffective in preventing fusion and creatine kinase accumulation in L₆ cultures. At 2 mM Cbz-Gly-Gly-NH₂, 90% of the control creatine kinase activity was still present (Fig. 3, c).

Effect of Cbz-Ser-Leu-NH₂ on Other Markers of Muscle Differentiation. Myosin heavy chain and α-actin (muscle actin) are expressed only in differentiated muscle. Non-muscle-specific β and γ actins are synthesized in both myoblasts and myotubes (21, 22). It was possible that Cbz-Ser-Leu-NH₂ exerted a specific effect on creatine kinase expression but not on biochemical differentiation in general. Therefore, we investigated whether myosin heavy chain and α-actin were expressed in Cbz-Ser-Leu-NH₂-treated cells.

L₆ cultures were exposed to 750 μM Cbz-Ser-Leu-NH₂ for 3 days after confluency and then labeled with [³⁵S]methionine for 17 hr. The cells exposed to Cbz-Ser-Leu-NH₂ remained mononucleated, while control cells fused to form myotubes. Actomyosin was extracted from the particulate fraction and subjected to NaDodSO₄/PAGE. Myosin heavy chain (M_r = 200,000) had been synthesized in the untreated controls but not in the Cbz-Ser-Leu-NH₂-treated cultures. Actins (M_r = 42,000) were synthesized in both cultures (Fig. 4A). The further analysis of [³⁵S]methionine-labeled particulate fractions by two-dimensional electrophoresis showed that α-

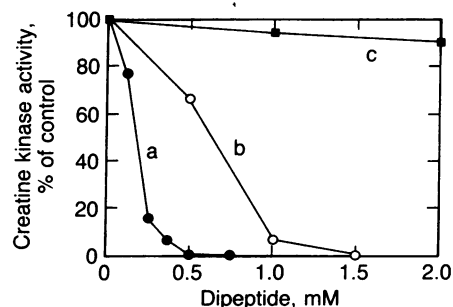


FIG. 3. Effect of concentration of blocked dipeptides on creatine kinase induction. Thirty-five millimeter dishes were inoculated with 3×10^4 cells per dish of clone B in 3 ml of growth medium. After 4 days the confluent and unfused cultures received 1.5 ml of growth media daily containing 0, 0.125, 0.250, 0.375, 0.500, or 0.750 mM Cbz-Ser-Leu-NH₂ in 0.1% Me₂SO (curve a); 0, 0.5, 1.0, or 1.5 mM Cbz-Gly-Leu-NH₂ in 0.2% Me₂SO (curve b); or 1 or 2 mM Cbz-Gly-Gly-NH₂ in 0.2% Me₂SO (curve c). After 3 days the plates were harvested and assayed for creatine kinase activity. Each point is the average of two experiments and is normalized to the appropriate control [100% activity = 64.5 mU/mg (curve a); 34.5 mU/mg (curves b and c)].

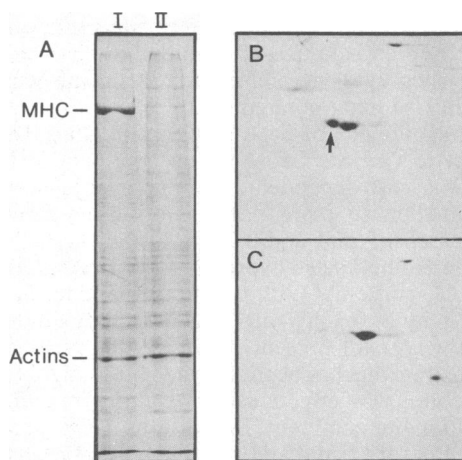


FIG. 4. Effect of Cbz-Ser-Leu-NH₂ on other markers of muscle differentiation. (A) High-salt extracts of the [³⁵S]methionine labeled particulate fraction were prepared as described from L₆ cultures exposed to 0.1% Me₂SO (lane I) or 750 μM Cbz-Ser-Leu-NH₂ (lane II) from confluency. The extracts were electrophoresed using NaDodSO₄/PAGE on 7% acrylamide gels followed by autoradiography. The figure indicates major bands at 200 kDa and 42 kDa corresponding to myosin heavy chain (MHC) and actins, respectively. (B and C) The total particulate fraction from cultures exposed to 0.1% Me₂SO (B) or 750 μM Cbz-Ser-Leu-NH₂ (C) was prepared and analyzed by isoelectric focusing followed by NaDodSO₄/PAGE on 10% gels. The acidic region of the gels is at the left; the figure shows the expanded region of the gel where actins (arrow points to α-actin) appear.

actin was not synthesized in the Cbz-Ser-Leu-NH₂-treated cultures (Fig. 4C) but was present in the controls (Fig. 4B, arrow).

The pattern of protein synthesis in Cbz-Ser-Leu-NH₂-inhibited cultures closely resembles that of fused cultures except for the muscle-specific proteins and several unidentified protein bands (data not shown), indicating that these cells are otherwise normal in protein synthesis.

Effect of Cbz-Ser-Leu-NH₂ on Irreversible Withdrawal from the Cell Cycle. Since biochemical differentiation and fusion of L₆ cells were inhibited by exposure to Cbz-Ser-Leu-NH₂, we investigated whether irreversible withdrawal from the cell cycle (commitment) was also prevented. The results of three types of experiments indicate that Cbz-Ser-Leu-NH₂ does prevent commitment in confluent L₆ myoblasts.

The effects of 750 μM Cbz-Ser-Leu-NH₂ on the rate of DNA synthesis were investigated in confluent cultures by measuring the incorporation of [³H]thymidine into cells over a 2-hr period (data not shown). Cultures exposed to the dipeptide from confluency gradually stopped proliferating; by the fourth day of exposure, these cells incorporated only background levels of [³H]thymidine. When the dipeptide was removed from the medium, the rate of DNA synthesis sharply increased and was followed by the initiation of fusion. In a parallel experiment, confluent cells exposed to Cbz-Ser-Leu-NH₂ for 4 days had the same plating efficiencies as cells at confluency (77 ± 6% and 76 ± 1%, respectively). Although cells exposed to Cbz-Ser-Leu-NH₂ had stopped cycling after 4 days, these cells retained proliferative capacity and were not committed.

Me₂SO (2%) inhibits fusion and differentiation in myoblasts by preventing them from leaving the proliferative state (21). This compound appears to be effective on uncommitted myoblasts only, having little effect on cultures in which a majority of the cells are committed. If Cbz-Ser-Leu-NH₂ allows myoblasts to commit, then cells grown first in the presence of Cbz-Ser-Leu-NH₂ and then given medium containing 2% Me₂SO without the dipeptide would be predicted

to fuse and differentiate to the degree that they are committed. Results of such an experiment indicated that Cbz-Ser-Leu-NH₂ prevents commitment of L₆ myoblasts (see Table 1 and legend).

DISCUSSION

The effect of MEPr inhibitors on myoblast fusion has been previously studied (4). However, the previous work did not address whether these compounds inhibited only fusion or the entire process of terminal differentiation. In this study we focused on the effects of MEPr inhibitors on the expression of muscle-specific proteins and the irreversible withdrawal from the cell cycle (commitment) in differentiating L₆ myoblasts. We have found that the MEPr inhibitors 1,10-phenanthroline and dipeptide amides blocked at the NH₂-terminus prevent the increase in muscle-specific protein expression. Our data also suggest that the dipeptide inhibitor Cbz-Ser-Leu-NH₂ prevents commitment of confluent myoblasts. Experiments that measured DNA synthesis rates, plating efficiencies, and the effects of sequential dipeptide and Me₂SO treatments indicate that myoblasts exposed to Cbz-Ser-Leu-NH₂ stop cycling (enter G₀ state) but do not lose their proliferative capacity (commit).

Certain differences between the effects of 1,10-phenanthroline and Cbz-Ser-Leu-NH₂ on biochemical differentiation are evident. Confluent myoblasts exposed to Cbz-Ser-Leu-NH₂ differentiate more rapidly after removal of the dipeptide than do myoblasts after treatment with phenanthroline. Partially fused cultures exposed to Cbz-Ser-Leu-NH₂ do not continue to accumulate creatine kinase or myosin heavy chain, unlike phenanthroline-treated cultures. These differences may be due to the different mechanisms by which these two compounds inhibit MEPrs or to secondary effects elicited by the dipeptides.

Table 1. Creatine kinase induction after serial treatments with Me₂SO and Cbz-Ser-Leu-NH₂

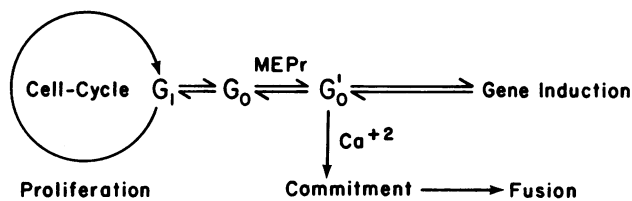
Culture conditions	Fusion	Creatine kinase activity	
		mU/mg	% control
1 Control (0.1% Me ₂ SO)	+	72	100
2 Cbz-Ser-Leu-NH ₂ then 0.1% Me ₂ SO	+	68	94
3 Cbz-Ser-Leu-NH ₂ then 2% Me ₂ SO	-	1.5	2
4 2% Me ₂ SO then 0.1% Me ₂ SO	+	39	54
5 2% Me ₂ SO then Cbz-Ser-Leu-NH ₂	-	2.0	3

Sixty-mm tissue culture dishes were inoculated with 1 × 10⁵ cells per dish of clone B in 10 ml of growth medium. After confluency was reached, the medium was changed to: (1) 5 ml of medium daily containing 0.1% Me₂SO and harvested 3 days later. One day later, the cultures receiving 2% Me₂SO or Cbz-Ser-Leu-NH₂ were still unfused. At this time, the medium was changed so that those cultures previously receiving 2% Me₂SO received either 5 ml of 750 μM Cbz-Ser-Leu-NH₂ (5) or of 0.1% Me₂SO (4) and those previously receiving the dipeptide received either 5 ml of 0.1% Me₂SO (2) or 2% Me₂SO (3) daily for 4 days. After 3 days, cultures that had been switched to 0.1% Me₂SO were fused and expressed creatine kinase activity (2, 4). The culture exposed first to Cbz-Ser-Leu-NH₂ for 4 days, followed by 2% Me₂SO for 4 days (3), was unfused and expressed creatine kinase only 2% of the control. After 4 days in the presence of Cbz-Ser-Leu-NH₂, the majority of the myoblasts must have been uncommitted, because 2% Me₂SO prevented them from fusing and differentiating. As expected, myoblasts exposed to 2% Me₂SO, then Cbz-Ser-Leu-NH₂, also neither fused nor differentiated (5).

Cbz-Ser-Leu-NH₂ does not act by interfering with the cell-cycle. Under those conditions in which undifferentiated cultures of confluent myoblasts were induced to differentiate in the absence of cell division (prevented by FdUrd) (19, 20), Cbz-Ser-Leu-NH₂ still prevented the accumulation of creatine kinase. When these cultures were allowed to partially fuse and then exposed to Cbz-Ser-Leu-NH₂, the specific activity of creatine kinase remained constant for 3 days. When dipeptide treatment of partially fused cultures was performed in normal growth media, creatine kinase specific activity was reduced by 50% over 3 days. We have routinely observed the detachment of myotubes from fusing cultures and the subsequent replacement with dividing myoblasts. Therefore, this reduction in creatine kinase specific activity may be due to a loss of existing myotubes that were not replaced because of dipeptide inhibition of fusion and differentiation. When FdUrd was present in the media, the loss of myotubes in fusing cultures was considerably reduced.

The requirement for an endogenous MEPr in myoblast fusion has been proposed previously (4). In this study we showed that the same MEPr inhibitors that prevent fusion (4) had similar effects on biochemical differentiation. For example, Cbz-Ser-Leu-NH₂ was more potent than Cbz-Gly-Leu-NH₂ in both respects, and Cbz-Gly-Gly-NH₂ was ineffective in blocking both fusion and biochemical differentiation in L₆ myoblasts. Also, the concentrations of dipeptides and phenanthroline that inhibited differentiation were similar to those required to inhibit fusion (4) and the endogenous MEPr activity (5) in L₆ myoblasts. Therefore, evidence for the role of a MEPr in initiating the terminal differentiation of cultured myoblasts is convincing.

Terminal differentiation in muscle is a complex multistep process, the biochemical details of which have yet to be elucidated. In recent years, however, evidence has accumulated that indicates the temporal relationships between the cell cycle, commitment, fusion, and induction of muscle-specific genes. A minimal scheme proposing the relationships of the main events of terminal differentiation, which is consistent with the experimental data (3, 20, 21, 23–27), is shown below:



This scheme may help clarify the stage of terminal differentiation at which MEPr acts as indicated by our data.

In this scheme, proliferating myoblasts withdraw from the cell cycle in response to environmental changes, such as depletion of growth-promoting factors from the media or contact inhibition. Upon replating or replacement of the media, these quiescent cells (G₀ state) may reenter the cell cycle. However, under the appropriate conditions, there is also a probability that these G₀ cells will take the pathway of differentiation (20)—the cells will lose their proliferative capacity irreversibly (commitment), fuse, and induce muscle-specific genes (gene induction). The paths that lead to fusion and gene induction have been shown to be independent (23–27). Recent studies using myoblast cell lines that are temperature sensitive for commitment (25) or cells grown in media with low free Ca²⁺ (24) indicate that gene induction can

occur in the absence of commitment and fusion and that genes are deinduced upon reentry into the cell cycle. Thus, free Ca²⁺ is required for commitment and fusion but not for gene induction.

Our results indicate that in the presence of the MEPr inhibitor Cbz-Ser-Leu-NH₂, confluent L₆ myoblasts enter the G₀ state but do not fuse, commit, or express muscle-specific proteins. Therefore, we propose that a MEPr activity is required for the transition from the G₀ state to a state we designate as G₀'. Cells which have reached the G₀' state are able to reversibly induce muscle-specific genes, but an additional Ca²⁺-dependent step is required for commitment and subsequent fusion. If a MEPr activity is involved in the transition from G₀ to G₀', then inhibiting this activity should prevent myoblasts from undergoing commitment, fusion, and biochemical differentiation. Although the scheme indicates only one site of action for a MEPr, our data do not rule out the possibility that other stages of differentiation might also require MEPr activity.

The effect of MEPr inhibitors on gene induction does not appear to be specific to fusing myoblasts. BC3H1 is a smooth muscle-like cell line derived from mouse (28) that undergoes reversible gene induction upon withdrawal of growth factors or serum from the medium but that does not fuse to form myotubes (29, 30). Our preliminary studies indicate that Cbz-Ser-Leu-NH₂ prevents creatine kinase induction in BC3H1 as well. Therefore, the involvement of MEPrs in cellular differentiation may be a general phenomenon rather than being specific to L₆ myoblasts.

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