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Differentiation of BC_3H1 and Primary Skeletal Muscle Cells and the Activity of Their Endogenous Insulin-Degrading Enzyme Are Inhibited by the Same Metalloendoprotease Inhibitors

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Upon reduction of serum in their media, mouse BC_3H1 muscle cells withdraw from the cell cycle and begin to differentiate. In differentiating cells, the induction of muscle-specific genes is accompanied by a distinct morphological chance. However, differentiated BC_3H1 cells do not fuse with each other; they remain mononucleated. Metalloendoprotease inhibitors selectively block the differentiation of BC_3H1 cells while inhibitors of other protease types are ineffective. In these cells, the degradation of the internalized insulin is initiated by a 110 kDa, non-lysosomal protease known as the insulin-degrading enzyme. The same metalloendoprotease inhibitors that block BC_3H1 differentiation also inhibit, with a similar specificity and potency, the *in vitro* and the *in vivo* degradation of insulin by the insulin-degrading enzyme. When the serum in the medium is reduced, the activity of the insulin-degrading enzyme in the cell cytoplasm increases rapidly. This increase precedes any detectable change in the differentiation state of these cells by about 12 hours. These results, together with very similar ones obtained with primary rat skeletal muscle cells, support our earlier proposal that the insulin-degrading enzyme is the metalloendoprotease involved in the initiation of the morphological and biochemical differentiation of muscle cells in culture.

Key words: serum, mouse BC_3H1 muscle cells, cell cycle, muscle-specific genes, internalized insulin

Our laboratory has been interested in the identification and characterization of key enzymes that are involved in the regulation of muscle cell differentiation. L_6 myoblasts, a skeletal muscle cell line of rat origin, proliferate until confluency, irreversibly withdraw from the cell cycle (commitment), and fuse to form multinucle-

Abbreviations used: Cbz, benzyloxycarbonyl; HEPES, 4-(2 hydroxyethyl)-1-piperazineethanesulfonic acid; IDE, insulin-degrading enzyme; MEPr, metalloendoprotease(s); NEM, N-ethylmaleimide; PCMPS, p-chloromercuriphenylsulfonate.

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ated myotubes. Concomitant with fusion, a number of muscle-specific proteins such as creatine kinase, myosin, and α-actin are induced [1]. Previously, we have reported that the activity of an endogenous metalloendoprotease (MEPr) was required for the differentiation of L_6 myoblasts [2,3]. More recently, we proposed that this MEPr was the 110 kDa insulin-degrading enzyme (IDE) which we showed to initiate the degradation of the internalized insulin in these cells [4,5].

In order to find out how general IDE involvement in muscle differentiation might be, we undertook a series of similar studies with the nonfusing, mouse muscle cell line, BC_3H1 [6], and with primary rat skeletal muscle cells grown in culture. BC_3H1 cells undergo reversible induction of muscle-specific genes upon reduction of serum in their media but they do not fuse to form myotubes. This makes BC_3H1 a particularly attractive system to study muscle-specific gene induction in the absence of potentially complicating events associated with cell-cell adhesion and membrane fusion. Original studies with BC_3H1 had suggested that these cells were smooth muscle-like [6]. However, a more recent report emphasizes their similarity to skeletal muscle cells which somehow have lost their ability for commitment and fusion [17].

In this study, we first demonstrated the involvement of an endogenous MEPr in BC_3H1 differentiation. We then established the presence of IDE and its role in insulin degradation. Finally, we showed a very close correlation between the inhibition of IDE activity and the inhibition of BC_3H1 differentiation by a variety of MEPr inhibitors. A parallel study with primary rat skeletal muscle cells gave essentially the same results. Thus our original finding of IDE involvement in the differentiation of L_6 myoblasts is now extended to nonfusing BC_3H1 and fusing, primary skeletal muscle cells.

EXPERIMENTAL PROCEDURES

Materials

The irreversible metalloendoprotease inhibitor and phosphoramidon were purchased from Enzyme Systems Products; Cbz-Ser-Leu-NH_2, Cbz-Gly-Leu-NH_2, and Cbz-Gly-Gly-NH_2 were from Vega Biotechnologies, Inc.; and all the other protease inhibitors were from Sigma. ^{125}I-Insulin (monoiodinated, 90 μCi/μg) was from Du Pont-New England Nuclear; ^{35}S-methionine (800 Ci/mmol) was from Amersham Corp.

Cell Culture

Stock cultures of mouse BC_3H1 muscle cells were obtained from Dr. David Schubert of the Salk Institute (La Jolla, CA) and maintained as described [6]. Cells were grown in Dulbecco's modified Eagle's medium containing 20% fetal calf serum and 100 units of penicillin/streptomycin/ml and were incubated at 37°C in 8% CO_2 in air. To induce differentiation, the fetal calf serum was reduced to 1%, usually 3 days after plating cells on 35-, 100-, or 150-mm plastic tissue culture dishes (Falcon, Corning).

Primary cultures of rat myoblasts were prepared from thigh muscles of newborn rat pups, essentially as described [1,18]. Cells were initially plated on Falcon tissue culture dishes for 1 h in order to reduce fibroblast contamination. Nonadherent cells were replated on gelatin-coated Falcon plates in Dulbecco's modified Eagle's medium containing 10% horse serum, 0.5% chicken embryo extract, and 2 mM glutamine.

Preparation of Cell Extracts

Cytosolic and particulate fractions were prepared from 3-day cells, essentially as described [5]. Cells were washed, collected by scraping, and lysed by homogenization in 10 mM Tris-Cl, 10 mM KCl with 0.06% mercaptoethanol, pH 8.1. The homogenate was centrifuged at 100,000g for 2 h. Supernatant was designated as the cytosolic, and the pellet as the particulate fraction.

Creatine Kinase Activity

Creatine kinase activity was measured using a coupled assay with hexokinase and glucose-6-phosphate dehydrogenase, and the formation of NADPH was monitored fluorometrically as described [3].

Northern Analysis

The preparation of total cellular RNA and the ^{32}P-labeling of cDNA probes for creatine kinase and glyceraldehydephosphate dehydrogenase was as described [5]. Ten micrograms of total cellular RNA per lane was electrophoresed in formaldehyde-agarose gels [7]. RNA was then transferred to nitrocellulose in 20 × SSC (1 × SSC, 0.15 M Nacl, 0.015 M sodium citrate, pH 7.0) and prehybridized in 50% formamide, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 5 × SSC, 50 mM Na_2HPO_4, 0.1% $NaDodSO_4$, and 250 μg/ml denatured salmon testes DNA. Blots were hybridized in the same buffer containing 10% (w/v) dextran sulfate with ^{32}P-labeled cDNA probes (approx. 10^6 cpm/ml) for 16 h at 42°C. Blots were washed with 2 × SSC, 0.1% $NaDodSO_4$ for 1 h at room temperature and with 0.1 × SSC, 0.1% $NaDodSO_4$ for 3 h at 68°C. Blots were autoradiographed with Kodak XAR film at −70°C, using intensifying screens.

Labeling of Cellular Proteins With ^{35}S-Methionine

Three-day cells on 100-mm culture dishes were labeled in 8 ml of Dulbecco's modified Eagle's medium (3 μg of methionine/ml) containing 100 μCi of ^{35}S-methionine (800 Ci/mmol) for 20 h. After labeling, cells were rinsed with ice-cold phosphate-buffered saline, scraped, and lysed by two freeze-thaw cycles. Cytosolic fraction was separated by centrifugation and stored frozen at −20°C.

Preparation of Monoclonal Antibodies

Monoclonal antibodies were raised in BALB/c mice against purified human erythrocyte IDE, essentially as described [8]. The splenic lymphocytes of the mouse with the highest titer were fused with SP2/0 myeloma cells [15,16]. Hybridoma supernatants were screened for their ability to precipitate ^{125}I-insulin cross-linked to IDE. Positive hybridomas were further cloned and corresponding ascites tumors were produced. Immunoglobulin from ascites fluid was prepared using protein A-Sepharose. Among a large number of monoclonal antibodies reactive with human IDE, two were found to immunoprecipitate mouse IDE from BC_3H1 cytosolic extracts, without inactivating the enzyme significantly. One of these monoclonal antibodies, 3C9, was used for the immunoblotting and immunoprecipitation experiments in this study. At 10^{-7} M concentration, 3C9 immunoprecipitated over 90% of the IDE activity in the cytoplasmic extracts of both BC_3H1 and primary muscle cells. The immunoprecipitated IDE from metabolically labeled cells appeared as a pure, 110 kDa protein in polyacrylamide gels.

Immunoblotting

Cytosolic fractions were electrophoresed, transferred to nitrocellulose, probed with the monoclonal antibody 3C9 and ^{125}I-labeled rabbit antimouse IgG antibody, and autoradiographed as described [5].

Immunoprecipitations

IDE in cytosolic fractions was immunoprecipitated by incubating first with the monoclonal antibody 3C9, then with *Staphylococcus aureus* coated with anti-mouse IgG and by centrifugation, as described [5,8].

Insulin Degradation Assay (*In Vitro*)

Insulin-degrading activity of cell extracts was assayed as described [5]. Forty micrograms of cellular protein was mixed with ^{125}I-insulin (approx. 2×10^4 cpm; final concentration: 0.163 ng/ml) to initiate the reaction. Assay was terminated by the addition of ice-cold trichloroacetic acid (10% final concentration). After spinning in an Eppendorf centrifuge, the radioactivity in both supernatant and pellet was determined in an LKB Ria Gamma counter. Acid-soluble counts in the supernatant indicated the extent of insulin degradation.

The activity of the IDE immunoprecipitated by the antibody 3C9 was assayed similarly, after the immunoprecipitate was washed and resuspended in the assay buffer as described [5].

Insulin Binding Assay

Three-day cells were washed with the binding buffer (0.1 M HEPES, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose, and 1% bovine serum albumin, pH 8) and incubated with the same buffer containing ^{125}I-insulin (100 pM) for 5 h at 14°C. Cells were then washed with phosphate-buffered saline at 4°C, solubilized with 1 N NaOH, and counted for radioactivity. The binding obtained in the presence of excess unlabeled insulin (10 μM) was considered to be nonspecific binding and was subtracted from that obtained in its absence to yield specific insulin binding.

Insulin Degradation Assay (*In Vivo*)

Three-day cells were washed and incubated with the binding buffer containing ^{125}I-insulin (100 pM) at 37°C for 5 h. At indicated times, aliquots of the medium were removed and intact insulin was precipitated by trichloroacetic acid. Samples were centrifuged and the radioactivity was counted in both the supernatant and the pellet. The acid-soluble counts in the supernatant indicated the extent of insulin degradation.

RESULTS

The growth and differentiation of BC_3H1 cells can be controlled by the amount of serum added to their media. Figure 1A shows proliferating cells, 3 days after plating in medium with 20% fetal calf serum (FCS). Upon reduction of FCS down to 1%, these cells stop dividing and start differentiating as they attain an elongated, spindle-like morphology shown in Figure 1B (6 days in 1% FCS). This morphological differentiation is accompanied, at the biochemical level, by the coordinate expression of a number of muscle-specific proteins such as the muscle isoenzyme of creatine kinase. Creatine kinase activity is undetectable in proliferating cells (in 20% FCS), but

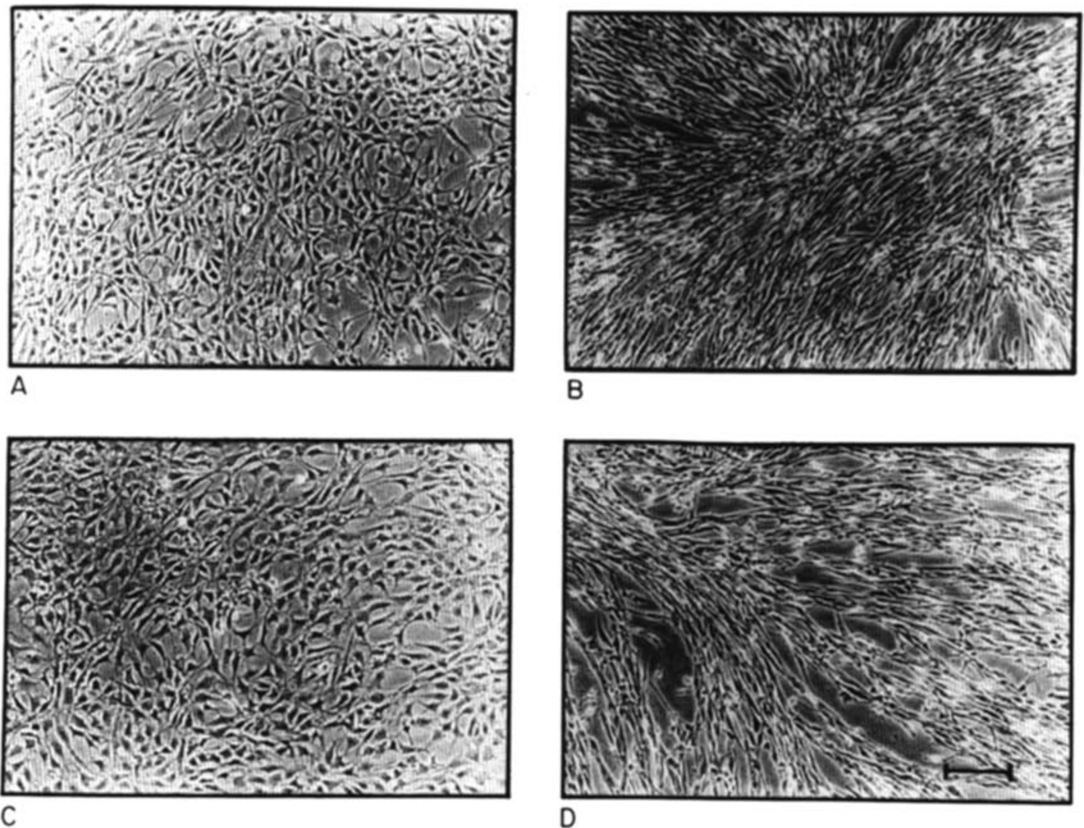

Fig. 1. Morphological differentiation of BC_3H1 cells and the effect of a reversible, dipeptide MEPr inhibitor. **A:** Proliferating 3-day cells in 20% FCS. **B:** Differentiated cells, after 6 days in 1% FCS. **C:** Undifferentiated cells, after 6 days in 1% FCS and 2 mM Cbz-Gly-Leu-NH_2. **D:** Differentiated cells, after 6 days in 1% FCS and 2 mM Cbz-Gly-Gly-NH_2. Bar = 200 μm.

it starts increasing dramatically once FCS in the medium is reduced to 1%. Thus, creatine kinase activity provides a quantitative measure of the state of differentiation of BC_3H1 cells.

The Effect of Protease Inhibitors on Differentiation

Cellular proteases are classified into four different types: metal-, serine-, carboxyl-, and thiol-dependent [9]. In order to investigate the possible involvement of an endogenous protease in BC_3H1 differentiation, we treated these cells with various protease inhibitors of known specificity. We then measured the expression of creatine kinase activity over a period of 7 days while untreated control cells proceeded to differentiate in 1% FCS media (differentiation media).

Table I summarizes the effects of all the nontoxic protease inhibitors on the expression of creatine kinase activity. All MEPr inhibitors except phosphoramidon were effective. None of the inhibitors of the other three protease types had a significant effect, with the exception of the nonspecific sulfhydryl reagent PCMPS. (The metal atom at the active site of a MEPr is often coordinated by cysteine residues,

TABLE I. Effect of Protease Inhibitors on BC_3H1 Differentiation*

Addition	Protease type inhibited	Concentration	Duration of treatment	Creatine kinase, % control
None				100
1,10-phenanthroline	Metal-	100 μg/ml	2 h	0
Irreversible MEPr inhibitor	Metal-	1 mM	1 h	0
Cbz-Ser-Leu-NH_2	Metal-	2 mM	Continuous	0
Cbz-Gly-Leu-NH_2	Metal-	2 mM	Continuous	3
Cbz-Gly-Gly-NH_2	None	2 mM	Continuous	92
Phosphoramidon	Metal-	0.1 mM	Continuous	100
PCMPS	Thiol-(Metal-)	0.1 mM	2 h	5
Leupeptin	Thiol-(Serine-)	20 μg/ml	Continuous	98
PMSF	Serine-	1 mM	2 h	100
Chymostatin	Serine-	20 μg/ml	Continuous	94
Pepstatin	Carboxyl-	20 μg/ml	Continuous	97

*Cells were switched from 20% to 1% FCS, and treated with the protease inhibitors 3 days after plating. Same treatments with the irreversible MEPr inhibitor, 1,10-phenanthroline, phenylmethylsulfonyl fluoride (PMSF), and PCMPS were repeated every other day. Cells were assayed for creatine kinase activity 9 days after plating. This activity for the untreated, control culture was 700 milliunit/mg (1 milliunit/mg = 1 nmol of ATP formed per min/mg protein). The second most common protease type known to be affected by a given inhibitor is shown in parentheses.

rendering the enzyme susceptible to sulfhydryl reagents [10]). Figure 1C shows the effect of the reversible, dipeptide MEPr inhibitor Cbz-Gly-Leu-NH_2 on cell morphology. A close chemical analogue Cbz-Gly-Gly-NH_2 is not a MEPr inhibitor and thus provides a useful control. In its presence, BC_3H1 cells readily attained the distinctive morphology of their differentiated state (Fig. 1D), as well as expressing near-normal levels of creatine kinase activity (Table I). All the other MEPr inhibitors that inhibited creatine kinase expression similarly inhibited the morphological differentiation of BC_3H1 cells. The morphology of cells whose differentiation was blocked by the MEPr inhibitors listed in Table I were visually indistinguishable from the undifferentiated cells shown in Figure 1A,C. (pictures not shown). The MEPr inhibitors affected neither the proliferation of cells nor their withdrawal from the cell-cycle significantly (data not shown).

The Effect of MEPr Inhibitors on Creatine Kinase mRNA Levels

We investigated whether the inhibition of creatine kinase expression by MEPr inhibitors was at the mRNA level. Total cellular RNA was prepared from BC_3H1 cells during various stages of differentiation. Muscle creatine kinase (MCK) was probed in Northern blots by a cDNA specific for this isoenzyme at high stringency. Proliferating cells had undetectable levels of MCK mRNA. After switching to differentiation medium, MCK mRNA gradually increased for the next 6 days (Fig. 2A, lanes 1–4). A parallel culture was treated with the irreversible MEPr inhibitor Cl-CH_2-(C-O-)(OH-N)-Phe-Ala-Ala-NH_2 for 1 h, prior to the media switch and the same treatment was repeated every other day. (This inhibitor blocks MEPr activity irreversibly, by alkylating the active site [11]). Inhibitor-treated cells did not express a significant level of MCK mRNA (Fig. 2A, lanes 5,6). Unless the 1-h inhibitor treatment was repeated, MCK

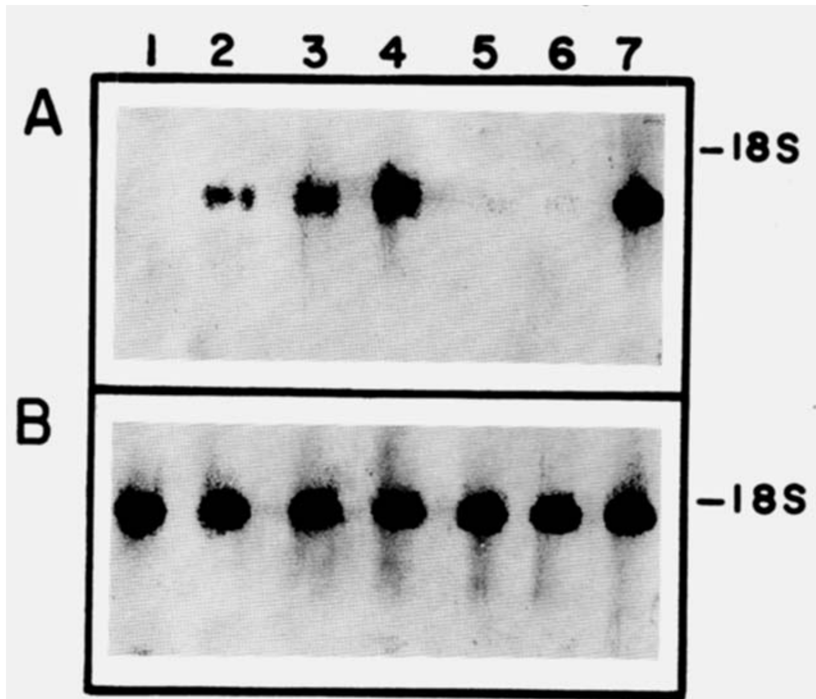

Fig. 2. The effect of the irreversible MEPr inhibitor on the mRNA levels of creatine kinase and glyceraldehydephosphate dehydrogenase. Total cellular RNA (10 μg/lane) was probed in Northern blots by ^{32}P-labeled cDNA specific for **(A)** muscle creatine kinase or **(B)** glyceraldehyde phosphate dehydrogenase. **Lane 1:** Three-day proliferating cells. **Lanes 2–4:** Differentiating cells, after 2, 3, and 4 days in 1% FCS. **Lanes 5, 6:** Cells treated (as in Table I) with the irreversible MEPr inhibitor, after 3 and 4 days in 1% FCS. **Lane 7:** Differentiated cells, 6 days after a single treatment with the inhibitor and in 1% FCS.

mRNA levels began to increase, indicating that the differentiation potential of these cells was not lost (Fig. 2A, lane 7).

A duplicate Northern blot probed with a cDNA specific for the constitutive enzyme glyceraldehydephosphate dehydrogenase showed no effect of inhibitor treatment on the mRNA levels of this enzyme (Fig. 2B). A series of RNA dot blot assays with other inhibitors confirmed the conclusion that the effect of the MEPr inhibitors on creatine kinase expression was at the mRNA level (data not shown).

The Role of the Insulin-Degrading Enzyme in Insulin Degradation

Insulin degradation by BC_3H1 cell extracts was measured by the generation of acid-soluble ^{125}I counts from ^{125}I-insulin. The insulin-degrading activity of the cytosolic fraction prepared from proliferating cells (3 days after plating) was approximately ten times that of the 100,000*g* particulate fraction (Fig. 3).

Degradation of ^{125}I-insulin by metabolizing BC_3H1 cells was measured by the appearance of acid-soluble radioactivity in the medium (Fig. 4a). Pretreatment of cells with the lysomotropic agent chloroquine had little effect on the degradation rate, indicating that lysosomal proteases were not involved (Fig. 4b). Similar results with another lysomotropic agent, NH_4Cl, confirmed this conclusion (data not shown).

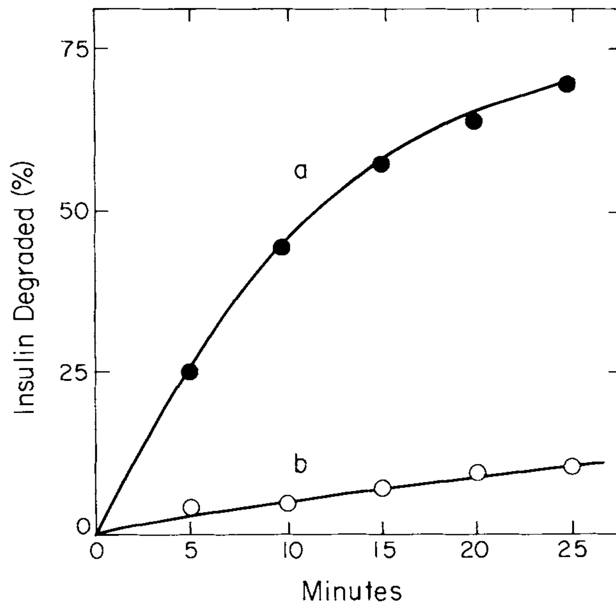

Fig. 3. Insulin degradation by cell extracts. Degradation of ^{125}I-insulin (0.150 ng/ml) by 40 μg of protein from 3-day cells was assayed at 37°C. a: Cytosolic fraction; b: particulate fraction.

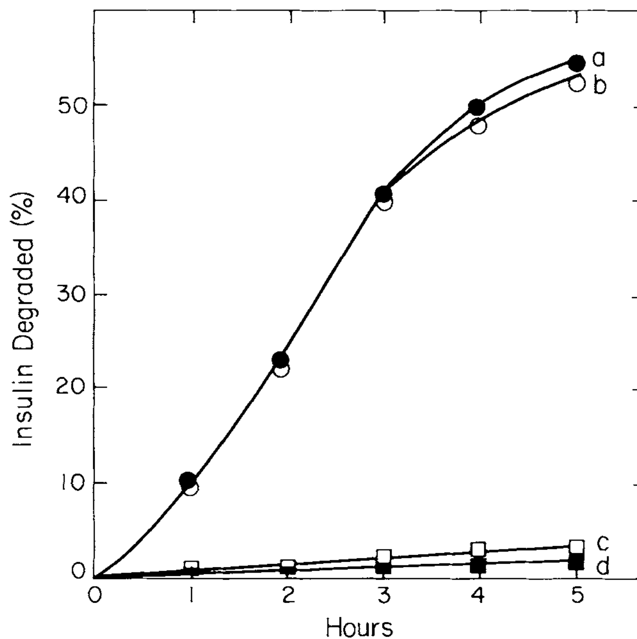

Fig. 4. Insulin degradation by metabolizing cells. a: Three-day cells incubated with ^{125}I-insulin (100 pM) at 37°C; b: same as a, except medium also contained chloroquine (0.1 mM) added 1 h before ^{125}I-insulin; c: same as a, except medium also contained KCN (1 mM) added 1 h before ^{125}I-insulin; d: same as a, except at 14°C. ^{125}I-insulin was added at 0 h.

Endocytosis is a temperature- and energy-dependent process which can be inhibited by lowering the temperature below 16°C or by treating cells with certain energy poisons [12]. *In vivo* insulin degradation was found to be very low when measured at 14°C or after treatment of cells with KCN (1 mM) (Fig. 4c,d). No significant insulin-degrading activity was detected in a cell-free, conditioned media which had been preincubated with cells for 6 h at 37°C (data not shown). These results are therefore in accordance with previous findings that *in vivo* insulin degradation requires the internalization of insulin (receptor-bound) via endocytosis [12,13].

In a number of cell types including L_6 myoblasts, a 110 kDa cytosolic protease referred to as the insulin-degrading enzyme (IDE) initiates the degradation of the internalized insulin [5,8,13]. In order to demonstrate the presence of this enzyme in BC_3H1 cells, we used the monoclonal antibody 3C9 which was raised against IDE purified from human erythrocytes. This antibody immunoprecipitated a peptide of 110 kDa from the cytosolic extracts of BC_3H1 cells (Fig. 5A, lane 1). In Western blots, it recognized a protein of the same apparent molecular weight (Fig. 5B, lane 1). Normal mouse IgG used as control neither immunoprecipitated nor recognized any peptides in these cell extracts (Fig. 5A,B, lanes 2).

Antibody 3C9 immunoprecipitated over 90% of the insulin-degrading activity in the cytosolic extracts whereas the normal mouse IgG was completely ineffective at the highest concentration used (10^{-7} M) (Fig. 6a,b). Binding of the antibody 3C9 to IDE in cylosolic extracts did not inactivate the enzyme (Fig. 6c). This made it possible to subsequently assay the activity of the immunoprecipitated enzyme (please see below).

The Inhibition of Insulin Degradation by MEPr Inhibitors

All the protease inhibitors of interest were tested for their possible inhibition of the degradation of insulin by the cytosolic extracts, the immunoprecipitated IDE, and the metabolizing cells. (Table II). All the MEPr inhibitors except phosphoramidon were effective. The inhibitors of the other three protease types were ineffective with the exceptions of bacitracin and the nonspecific sulfhydryl reagents PCMPS and NEM. (Bacitracin as well as nonspecific sulfhydryl reagents have been shown to be potent inhibitors of IDE and thus of insulin degradation in other cells [5,13]).

The inhibition of the *in vivo* degradation of insulin by the inhibitors listed in Table II does not stem from a reduced binding of insulin to its receptor on the cell surface. In accordance with previous reports [13], these inhibitors in fact enhanced insulin binding up to 30% (Table II).

IDE Involvement in the Differentiation of Primary Skeletal Muscle Cells

It was also desirable to investigate whether IDE might be similarly involved in the differentiation of primary muscle cells. For this purpose, a parallel study with rat primary skeletal muscle cells was carried out. First, the presence of the 110 kDa IDE was demonstrated by immunoprecipitating the active enzyme from the cytoplasmic extracts of cultured primary myoblasts. Then, the effects of all the protease inhibitors of interest on the *in vitro* and *in vivo* degradation of insulin by IDE were determined. Results were compared to the effects of the same inhibitors on the differentiation of primary muscle cells in culture. The summary of these results are presented in Table III.

As has been the case with the cell lines L_6 [5] and BC_3H1 (this report), all the MEPr inhibitors that blocked the differentiation of primary myoblasts also effectively

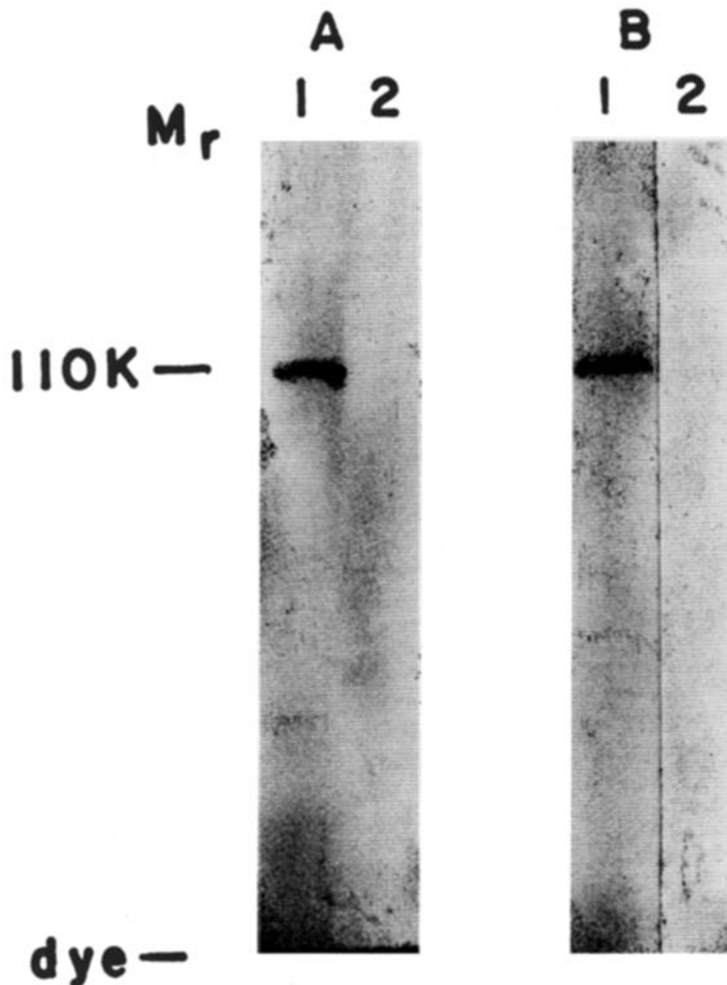

Fig. 5. Detection of IDE by monoclonal antibody 3C9. **A:** Immunoprecipitation from the cytosolic extracts of 3-day cells, metabolically labeled with ^{35}S-methionine. **Lane 1:** Antibody 3C9. **Lane 2:** Normal mouse IgG. **B:** Immunoblotting of the cytosolic extracts. **Lane 1:** Antibody 3C9. **Lane 2:** Normal mouse IgG. Electrophoresis was on 7.5% polyacrylamide-SDS gels. A: Proteins were visualized after autoradiography of the dried gel. B: Proteins were transferred from the gel to nitrocellulose paper, reacted first with antibody 3C9, second with ^{125}I-labeled rabbit anti-mouse IgG, and then autoradiographed.

inhibited the *in vivo* and the *in vitro* degradation of insulin by IDE, with a similar specificity and potency (Table III).

Time-Course of Change in the Insulin-degrading Activity of BC_3H1 Cells During Differentiation

Cytoplasmic extracts of proliferating, undifferentiated BC_3H1 cells (in 20% FCS) have a basal insulin-degrading activity of about 18 fmoles. min^{-1} insulin degraded per mg of cell protein (Fig. 7). Antibody-removal experiments in Figure 6 indicated that over 90%, and possibly all of this activity was dependent on IDE. In

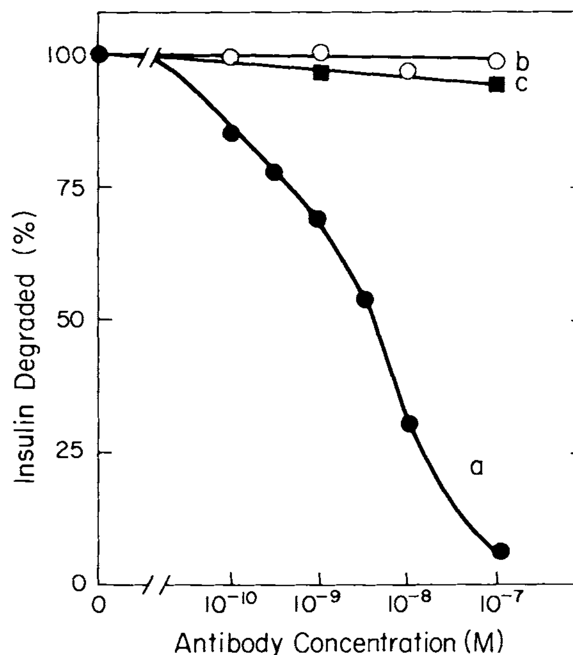

Fig. 6. Immunoprecipitation of the insulin-degrading activity from cytosolic extracts. Cytosolic extracts of 3-day cells were incubated with (a) antibody 3C9 or (b) normal mouse IgG. Antibodies were precipitated by the addition of *S. aureus* coated with anti-mouse IgG and by centrifugation. Supernatants were tested for ^{125}I-insulin degradation. c: Same as a, but without the addition of *S. aureus*.

order to investigate whether there might be a cause-and-effect relationship between insulin-degrading activity and differentiation, we determined the time-course of this activity at 3-h intervals, after differentiation was initiated by switching the cell medium from 20% to 1% FCS. The insulin-degrading activity in the cytoplasmic extracts of control cells rapidly increased upon the media-switch, approximately doubling within 9 h, and continued to increase thereafter. During this time, there was no accompanying change in the activity of creative kinase in the same extracts. (This activity is essentially zero in proliferating, undifferentiated cells.) The creatine kinase activity was first detectable at 15 h, after which it gradually increased over the course of the experiment (measured upto 96 h) (Fig. 7A).

If the cells were treated with the reversible MEPr inhibitor Cbz-Ser-Leu-NH$_2$ (2 mM) 1 h prior to the media-switch, they did not differentiate and express any creatine kinase activity (as long as the reversible MEPr inhibitor was present in their media) (Fig. 7B). Interestingly, the insulin-degrading activity in their cytoplasmic extracts still increased rapidly, albeit with a slightly lower rate, approximately doubling within 12 h. The insulin-degrading activity in the cytoplasmic extracts of these differentiation-inhibited cells was about 80% of that measured for differentiating, control cells, 24 h after the media-switch (Fig. 7A,B). Over 90% of this activity in the cytoplasmic extracts of BC$_3$H1 cells at any stage of their differentiation could be removed by

TABLE II. Effect of Protease Inhibitors on the Degradation and Cell Binding of Insulin*

	Degradation rate, % control			
Addition	Cytosolic extract	IDE	Metabolizing cells	Cell binding
None	100	100	100	100
1,10-Phenanthroline (100 μg/ml)	12	11	15	122
Irreversible MEPr inhibitor (1 mM)	2	3	11	127
Cbz-Ser-Leu-NH_2 (2 mM)	54	49	29	117
Cbz-Gly-Leu-NH_2 (2 mM)	57	53	35	114
Phosphoramidon (0.1 mM)	100	100	100	103
PCMPS (0.1 mM)	6	4	17	128
NEM (1 mM)	7	3	15	130
Bacitracin (1 mM)	1	4	8	129
Leupeptin (20 μg/ml)	98	97	99	106
PMSF (1 mM)	96	100	100	99
Chymostatin (20 μg/ml)	94	99	97	108
Pepstatin (20 μg/ml)	99	100	100	102

*Insulin degradation rates with the cytosolic extracts and IDE were determined as in Figure 3a, and as described in "Experimental Procedures." Inhibitors were added at the indicated concentrations 20 min before ^{125}I-insulin. Assays were started with the addition of ^{125}I-insulin at 0 min. Insulin degradation rates of 3-day cells were determined at 37°C as in Figure 4a, from the linear range of each curve. Cells were treated with 1,10-phenanthroline, NEM, PCMPS, and phenylmethylsulfonyl fluoride (PMSF) for 2 h and with the irreversible MEPr inhibitor for 1 h prior to ^{125}I-insulin addition. All other inhibitors were added 1 h prior to ^{125}I-insulin and were kept in the medium during the assay. ^{125}I-insulin was added at 0 h. Specific cell binding of ^{125}I-insulin was determined at 14°C. Inhibitor treatments in the binding assay was otherwise identical with those described for the degradation assay with 3-day cells.

immunoprecipitation with the anti-IDE monoclonal antibody 3C9, at 10^{-7} M (data not shown).

DISCUSSION

In this study, we demonstrated that a variety of MEPr inhibitors blocked the biochemical and morphological differentiation of BC_3H1 cells (Table I; Fig. 1). The effects of these inhibitors were at the mRNA level and restricted to the induction of muscle-specific genes (Fig. 2). These results indicate that an endogenous MEPr activity is required for the initiation of the differentiation process in BC_3H1 cells.

An important question this study tried to answer is the identity of the endogenous MEPr. Our previous studies have indicated that a similar MEPr involved in the differentiation of the skeletal muscle cell-line L_6 is IDE, a 110 kDa protease responsible for the initiation of the degradation of internalized insulin [4,5]. A series of *in vitro* and *in vivo* insulin degradation and antibody precipitation studies with BC_3H1 cells revealed the presence of IDE and its involvement in the degradation of insulin in these cells as well (Figs. 3–6).

The finding that every MEPr inhibitor that blocked BC_3H1 differentiation also inhibited insulin degradation by the 1) cytosolic extracts, 2) metabolizing cells, and 3) IDE, immunoprecipitated by the monoclonal antibody 3C9, with a similar specificity and potency, suggests that the MEPr in question is IDE (Table II). IDE from other sources was previously shown to be inhibited by a number of nonspecific sulfhydryl reagents [13]. Our results here (Tables II, III) and previously with L_6 myoblasts [5] indicate that IDE is in fact a MEPr which is also inhibited by nonspecific sulfhydryl

TABLE III. Effect of Protease Inhibitors on Differentiation and Insulin Degradation in Primary Skeletal Muscle Cells*

	Differentiation, % control		Insulin degradation, % control	
Addition	Creatine Kinase	Fusion	IDE	*In vivo*
None	100	100	100	100
1,10-Phenanthroline (100 μg/ml)	0	0	8	15
Irreversible MePr inhibitor (1 mM)	0	0	2	12
Cbz-Gly-Leu-NH2 (2 mM)	3	1	51	29
Cbz-Ser-Leu-NH2 (2 mM)	1	0	48	24
Phosphoramidon (0.1 mM)	100	100	100	100
PCMPS (0.1 mM)	5	6	7	11
NEM (1 mM)	ND	ND	3	11
Bacitracin (1 mM)	ND	ND	2	10
Leupeptin (20 μg/ml)	95	97	97	98
PMSF (1 mM)	100	100	95	100
Chymostatin (20 μg/ml)	94	92	91	93
Pepstatin (20 μg/ml)	97	99	98	97

*Cytoplasmic extracts were prepared from cells, 6 days after plating. Creatine kinase activity was assayed; IDE was immuno-precipitated and assayed for insulin degradation as described in "Experimental Procedures." The extent of fusion was determined by microscopy, 6 days after plating as described [2]. The *in vivo* insulin degradation was measured with cells 3 days after plating as described in "Experimental Procedures." Protease inhibitor treatments were started on 3-day cells and performed as in Tables I and II, with minor modifications. The values measured with untreated, control cells were taken as 100%. ND, not determined due to the cell toxicity of the inhibitor.

reagents. This is not surprising in light of the fact that in many well-characterized MEPr, the metal atom at the active site of the enzyme is coordinated by cysteine residues [10]. However, the exact function of the cysteine residue(s) essential for IDE activity remains to be determined.

The correlation between the inhibition of IDE and the inhibition of muscle cell differentiation now seems to be well established. Present results with the nonfusing BC_3H1 cells also argue against any suggestion that in fusing L_6 cells, the observed inhibition of muscle-specific gene induction by the inhibitors of IDE might have been a consequence of the inhibition of myoblast-myoblast fusion. The results with the primary skeletal muscle cells indicate that the involvement of IDE in differentiation is not restricted to cell-lines: IDE may have a similar role during the embryonic development of the muscle tissue.

IDE accounts for over 90% of the insulin-degrading activity in the cytoplasmic extracts of the BC_3H1 cells (Fig. 6). Therefore this activity is essentially equivalent to the activity of IDE present in these extracts. The time-course experiments show that the IDE activity in the cytoplasmic extracts of BC_3H1 cells rapidly increases in response to the differentiation medium (Fig. 7A). In cells whose differentiation is blocked by the presence of the reversible MEPr inhibitor Cbz-Ser-Leu-NH_2, this rapid increase in the IDE activity upon media-switch still takes place as demonstrated by assaying the cytoplasmic extracts of inhibitor-treated cells for insulin degradation *in vitro* (Fig. 7B) [Cytoplasmic extracts are prepared from cells that are extensively washed before being harvested. This and the subsequent dilution of extracts during cell lysis by homogenization, and their further dilution in the *in vitro* insulin degradation assay reduce the concentration of any remaining inhibitor to negligible levels. Therefore, the insulin-degrading activity of the cytoplasmic extracts prepared

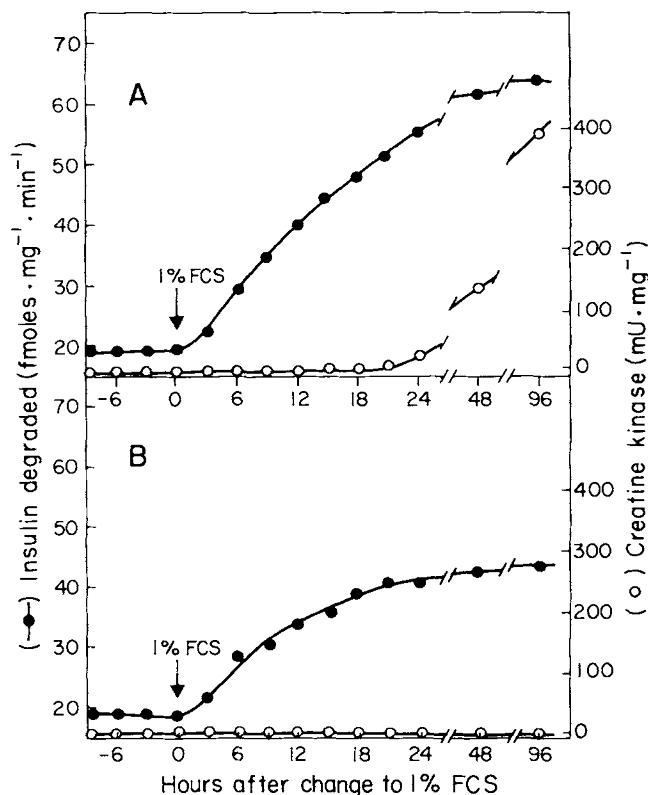

Fig. 7. Time-course of creatine kinase and insulin-degrading activities in differentiating and inhibitor-treated BC_3H1 cells. Cytoplasmic extracts were prepared from cells at indicated times. Creatine kinase and insulin-degrading activities of the extracts were assayed as described in "Experimental Procedures." Medium was switched from 20% to 1% FCS, 3 days after plating. The time of media switch is indicated with an arrow and at 0 h in both graphs. **A:** Control cells. **B:** Cells treated continuously with the reversible MEPr inhibitor Cbz-Ser-Leu-NH_2 (2 mM), starting 1 h before the media switch.

from reversible MEPr inhibitor-treated cells represents a true measure of the active IDE levels in the cytoplasm of these cells. Obviously, in the presence of the inhibitor, the *in vivo* IDE activity is significantly reduced as measured by the *in vivo* insulin degradation assay (Table II)].

The rapid increase in the active IDE levels upon switching to the differentiation media precedes any detectable change in the creatine kinase activity by about 12 h (Fig. 7A), and it takes place even in the complete absence of differentiation (Fig. 7B). Apparently, this rapid increase in the active IDE levels is one of the early cellular responses to the differentiation-signal received by these cells from their media. Therefore, the increase in the active IDE levels may be one of the prerequisites and/or causes for the initiation of differentiation: when the *in vivo* enzymatic action of IDE on its natural substrate(s) is blocked by various inhibitors, differentiation is not initiated.

Upon switching to differentiation media, the IDE activity increases rapidly and without a significant lag (Fig. 7). This suggests an activation of already existing IDE molecules, possibly by chemical modification, rather than *de novo* synthesis of the enzyme. Obviously, there is enough precedence for such enzyme activation by medium

components, usually via a receptor-mediated signaling process across the plasma membrane (e.g., EGF binding stimulates the tyrosine kinase activity of its receptor which in turn activates certain endogenous enzymes via phosphorylation [19].) The elucidation of the molecular nature of the rapid IDE response uncovered in this study will require further investigation.

Another important question that remains to be answered is how the inhibition of IDE would lead to the inhibition of muscle differentiation. In principle, an endogenous protease such as IDE can control the intracellular concentration of a regulatory peptide(s) which somehow functions either as a "repressor" or an "inducer" of differentiation. It is conceivable that this regulatory peptide(s) is either the internalized, intact insulin (repressor), or a fragment of it (inducer) generated via proteolysis, or both. Interestingly, it has been reported that the total absence of insulin or its presence at relatively high concentrations in the medium would inhibit muscle differentiation [20–22, and unpublished observations by C.K.]. These observations are consistent with a repressor/inducer model for insulin as discussed above. However, there is not sufficient data to prove such a model at this time. Our future experiments will include a more extensive study of insulin effects and its metabolism in cultured muscle cells within the context of cell differentiation.

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