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Peptide Elongation Factor eEF1A-2/S1 Expression in Cultured Differentiated Myotubes and Its Protective Effect against Caspase-3-mediated Apoptosis*

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Abstract

Peptide elongation factor eEF1A-2/S1, which shares 92% homology with eEF1A-1/EF-1 α , is exclusively expressed in brain, heart, and skeletal muscle. In these tissues, eEF1A-2/S1 is the only type 1A elongation factor expressed in adulthood because a transition from eEF1A-1/EF-1 α to eEF1A-2/S1 occurs in early postnatal development. In this article, we report that the expression of eEF1A-2/S1 protein is activated upon myogenic differentiation. Furthermore, we show that upon serum deprivation-induced apoptosis, eEF1A-2/S1 protein disappears and is replaced by its homolog eEF1A-1/EF-1 α in dying myotubes; cell death is characterized by the activation of caspase-3. In addition, we show that the continuous expression of eEF1A-2/S1 resulting from adenoviral gene transfer protects differentiated myotubes from apoptosis by delaying their death, thus suggesting a prosurvival function for eEF1A-2/S1 in skeletal muscle. In contrast, myotube death is accelerated by the introduction of the homologous gene, *eEF1A-1/EF-1 α* , whereas cells transfected with antisense *eEF1A-1/EF-1 α* are protected from apoptosis. These results demonstrate that the two sister genes, *eEF1A-1/EF-1 α* and *eEF1A-2/S1*, regulate myotube survival with the former exerting prodeath activity and the latter a prosurvival effect.

Apoptosis is an active process enabling normal metazoan development and tissue homeostasis by permitting the deletion of unwanted or damaged cells (1,2). In mammalian cells, the control of cell death or survival is governed by the interplay between the Bcl-2 family members and the activation of caspase proteases (for review, see Refs. 3-7). Once activated, caspases can cleave several proteins to inactivate their functions, which are essential to survival. Upon differentiation induction of skeletal muscle cell lines, undifferentiated myoblasts are susceptible to apoptosis but become resistant as they differentiate into myotubes. It is suggested that this resistance may be dependent upon the induction of two repressors of cell cycle traverse: p21Cip1 and Rb (8,9). However, no study has yet discussed the apoptotic induction mechanism in differentiated myotubes.

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During skeletal muscle development, a switch occurs in the expression of the two homologous peptide elongation factor 1As. In mouse and rat, eEF1A-1¹/EF-1 α is expressed during embryonic and early postnatal development and is replaced by eEF1A-2/S1 2 weeks after birth; the latter is the only homolog expressed in adult muscle (10,11). In fact, eEF1A-2/S1 is expressed in brain, heart, and skeletal muscle, all tissues largely composed of long lived terminally differentiated cells (12-14). eEF1A-2/S1 has been shown by *in vitro* translation assay to support elongation activity similarly to eEF1A-1/EF-1 α ; it binds aminoacyl-tRNA and delivers it to the ribosomal aminoacyl site during the elongation phase of protein biosynthesis (15-17). Deletion of the promoter and first noncoding exon of the *eEF1A-2/S1* gene is associated with the *wasted* mouse phenotype, characterized by muscle atrophy, neurological impairment, immunological deficiency, and death after fewer than 30 days postpartum (18). In skeletal muscle, absence of either form of eEF1A homologs is observed 3 weeks after birth in *wasted* mutant mice, thus impairing protein translation (11). No mechanism has yet been identified to explain the switch in peptide eEF1As seen in skeletal muscle, heart, and brain during development. Furthermore, it remains unclear why eEF1A-2/S1 is only expressed in long lasting terminally differentiated cells such as neurons, cardiomyocytes, and myofibers. Because both elongation factors, eEF1A-1/EF-1 α and eEF1A-2/S1, have similar canonical function in protein translation, we hypothesized that the developmental switch could be explained by the noncanonical functions of the proteins.

To unravel this mystery, we analyzed the protein expression and function of eEF1A-1/EF-1 α and eEF1A-2/S1 in culture during both muscle differentiation and apoptotic cell death induced by serum deprivation in cultured differentiated myotubes. In this article, we report that differentiating L6 and C2C12 myoblast cultures express eEF1A-2/S1 protein at a late stage of myotube differentiation. Furthermore, we show that differentiated cultures die after serum withdrawal, an event associated with a decrease in eEF1A-2/S1 protein expression, an increase of eEF1A-1/EF-1 α protein abundance, and the activation of caspase-3. Adenoviral transfer of the *eEF1A-2/S1* gene or *eEF1A-1/EF-1 α* antisense gene transfection rescues cultured myotubes from apoptotic cell death induction. These results suggest that differentiated myotubes can be induced to die via apoptosis, and the suicidal event can be either slowed down or accelerated by homologous peptide elongation factor eEF1A-2/S1 or eEF1A-1/EF-1 α , respectively. This may illustrate one reason behind the peptide eEF1A developmental switch, so that all those long lived, terminally differentiated cells are protected from accidental induction of programmed cell death.

¹The abbreviations used are:

eEF1A	eukaryotic elongation factor 1A
DMEM	Dulbecco's modified Eagle's medium
PBS	phosphate-buffered saline
PARP	poly(ADP-ribose) polymerase
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid
PIPES	1,4-piperazinediethanesulfonic acid
AFC	7-amino-4-trifluoromethylcoumarin
CMV	cytomegalovirus
TUNEL	terminal nucleotidyl transferase-mediated UTP nick end labeling
DEVD	Asp-Glu-Val-Asp peptide
YVAD	Tyr-Val-Ala-Asp peptide

MATERIALS AND METHODS

Cell Culture

The mouse-derived skeletal myoblast C2C12 cell line (American Type Culture Center, ATCC) and rat-derived cultured myo-blast L6A line (a generous gift from Dr. Dan Goldman, University of Michigan, Ann Arbor) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ streptomycin and kept at 37 °C in a 10% CO₂ humidified atmosphere incubator. After confluent cultures were rinsed twice with serum-free DMEM, myoblast differentiation was induced by replacing the 10% fetal bovine serum-containing culture medium by DMEM supplemented with 2% horse serum. The yield of myotubes achieved was visually estimated to be between 60 and 80%. For serum deprivation, myotubes were rinsed twice with serum-free DMEM to remove all trace of serum and then cultured after the third change in the same medium. Cultured C2C12 cells were harvested for analysis at 3, 6, 9, 12, and 15 days of differentiation and at 3, 6, and 9 days of serum deprivation. Cultured L6 cells were collected daily after induction of either differentiation or apoptosis.

DNA Fragmentation Assays

Cultured C2C12 and L6 myotubes were processed in the same manner. The medium of serum-deprived cells was collected and added to the scraped cells to allow centrifugation of both adhering and floating cells. Cell pellets were resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, and 0.5 mg/ml proteinase K) and incubated overnight at 56 °C. Afterward, samples were treated with 50 $\mu\text{g}/\text{ml}$ RNase A for 2 h followed by a phenol/chloroform extraction. The DNA was precipitated using 0.3 M final concentration of sodium acetate, pH 5.2, and isopropyl alcohol. The DNA pellet was then rinsed with 75% cold ethanol and resuspended in water. After being spun for 20 min in a microcentrifuge, samples were loaded onto a 1.8% agarose and Tris-boric acid-EDTA buffer gel containing ethidium bromide to allow electrophoretic separation of the fragmented DNA. The separated fragments were visualized on the agarose gel using a UV transilluminator, and profiles of oligonucleotides were taken with a digital camera.

In Situ DNA Degradation Assay by Terminal Nucleotidyl Transferase-mediated UTP Nick End Labeling (TUNEL) Staining Reaction

DNA fragmentation analysis of individual cell was performed by DNA nicked-end labeling using terminal-*d*-transferase, as described by Gavrieli *et al.* (23). Differentiated L6 myotubes were treated with trypsin and then grown on coverslips; one set was serum deprived 2 days after seeding. Differentiated cells were fixed using cold methanol/acetone (1:1) at -20 °C for 10 min. Fixed cells were rehydrated with PBS for 30 min at room temperature and then rinsed twice with double-distilled water. Afterward, coverslips were covered with terminal transferase reaction buffer (30 mM Tris-HCl, pH 6.8, 100 mM sodium cacodylate, 5 mM cobalt chloride, 0.5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 10 μM biotin-16-dUTP (Roche), and terminal transferase (Promega) at 0.3 unit/ μl); the reaction was performed at 37 °C for 1 h in a humidified chamber and then terminated by transferring the coverslip samples to a TB buffer containing 300 mM sodium chloride and 30 mM sodium citrate for 15 min at room temperature. The specimens were then rinsed in double distilled water and incubated in 2% bovine serum albumin for 10 min. After the addition of serum albumin, the specimens were incubated with 20 $\mu\text{g}/\text{ml}$ streptavidin and fluorescein isothiocyanate (Roche) in PBS for 30 min at room temperature and subsequently washed twice in PBS along with 0.4 $\mu\text{g}/\text{ml}$ propidium iodide in the second washing. The propidium iodide was removed by rinsing the samples twice in PBS. Coverslips were mounted in PBS containing 50% glycerol and examined with a Nikon fluorescence microscope.

Protein Extraction, Gel Electrophoresis, and Western Blotting

Myotubes cultures were rinsed with PBS and then gently scraped into 1 ml of PBS. Collected cells were centrifuged at 4,000 rpm for 3 min. The pellets were resuspended into RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA) containing fresh protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 2 μ g/ml of both leupeptin and pepstatin). Afterward, cell extracts were sonicated for 1 min to break cell membranes; proteins were quantified by a modified Bradford method, using the Bio-Rad protein assay according to the manufacturer's instructions, and compared with a γ -globulin standard curve. Equal amounts of total protein for each sample were separated on a SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane by the standard transfer method. Membranes were blocked in Tris-buffered saline solution containing 0.25% Tween 20 and 5% fat-free powdered milk. Before incubation with primary antibodies, membranes were rinsed in Tris-buffered saline-Tween 20 solution. Primary antibodies used were HT7 for eEF1A-1/EF-1 α , CB5 for eEF1A-2/S1 (22), MF-20 monoclonal antibody for myosin heavy chain (24) (the clone was a generous gift from Dr. L. Chalifour, Lady Davis Institute, Montreal, Canada); Caspase-3 (H-277) and poly(ADP-ribose) polymerase (PARP) (H-250) antibodies were purchased from Santa Cruz, and monoclonal β -actin antibody from Amersham Biosciences, Inc. After incubation overnight at 4 °C or 1 h at room temperature, the membranes were washed four times, 10 min each, in Tris-buffered saline-Tween 20. Blots were subjected to a second hybridization with either horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (Cappel) or goat anti-mouse antibodies (Cappel) and then washed five times with Tris-buffered saline-Tween 20. Bands were detected using a chemiluminescent ECL procedure, according to the manufacturer's protocol (Amersham Biosciences, Inc.). For β -actin detection, previously used membranes were soaked in stripping buffer (70 mM Tris-HCl, pH 6.8, 2% SDS, and 0.1% β -mercaptoethanol) at 60 °C for 30 min and hybridized following the procedures described above. Films were quantified by densitometry (Molecular Dynamics) and expressed as the ratio to the maximal level of detection.

Viability Assay and Caspase-3 Inhibitor

Viability tests were performed using the trypan blue exclusion assay, as described by Duttaroy *et al.* (25). Viability percentages were calculated by dividing the number of dead blue cells by the total cell number. Each experiment was repeated three times. A cell-permeable, irreversible peptide inhibitor of caspase-3, DEVD-FMK (Calbiochem), was dissolved in dimethyl sulfoxide and added to the culture medium at a final concentration of 75 μ M. The culture medium was changed every day, with the addition of fresh inhibitor-containing medium, and control cells were plated with the same concentration of dimethyl sulfoxide without inhibitor. Cell viability was measured as described above.

Caspase Assays

Serum-deprived and control cell cultures were rinsed once in cold PBS and then collected in cold PBS by scraping the cells off the plate. After centrifugation and removal of PBS, cell pellets were kept at -80 °C until caspase assays were performed. The frozen pellets were resuspended in caspase lysis buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, and 0.1% CHAPS) supplemented with protease inhibitors (5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml pepstatin A, 10 μ g/ml aprotinin, and 20 μ g/ml leupeptin). Freeze-thaw cell lysis cycles were performed by alternatively transferring the samples from an ethanol/dry ice bath to a 37 °C water bath five times. The supernatant was collected after 20 min of centrifugation at full speed (\approx 12,000 rpm) in a cold microcentrifuge. Assays were performed in caspase buffer (10 mM PIPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, and 5 mM dithiothreitol, final concentration), to which 50 μ M final concentration of substrate and 5 μ l of protein extract were added to yield a final volume of 100 μ l. Caspase-3 DEVD-AFC and caspase-1 YVAD-

AFC peptide substrates (Biomol) were dissolved in dimethyl sulfoxide. The assays were performed in black wall, clear bottom plates using a Bio-Rad fluorometer and read at 598 nm after excitation at 390 nm wavelengths. The results were compared against an AFC curve generated during the reaction. To measure specific activity, proteins were quantified according to the method described above.

Adenovirus Construction, Cell Infection, and β -Galactosidase Assay

Recombinant adenovirus expressing *eEF1A-2/S1* was prepared with ADENO-QUEST (Quantum Biotechnologies, Montreal). Mouse *eEF1A-2/S1* cDNA (26) was subcloned into the pQBI-AdCMV5 transfer vector. Cotransfection of QBI-293A cells with viral DNA and the transfer vector was performed by the precipitated calcium phosphate procedure, according to the manufacturer's protocol. Plaques were picked up and amplified in QBI-293A cells. Screening for positive recombinants was performed by Western blotting using CB5 antibody, which specifically recognizes eEF1A-2/S1 protein (22). Among the positive clones, one named A5.4 was used to generate the experimental virus. Control virus for mock infection was generated by transfection of QBI-293A cells with nondigested viral DNA. The β -galactosidase gene was already included in the viral DNA by the manufacturer; insertion of a new gene into the vector does not affect its transcription and thus can be used as a marker of infection. After amplification, cells were broken by freeze/thaw cycles; and after centrifugation, the supernatant culture media containing viruses were kept frozen. Virus titration was done according to the manufacturer's instructions. Growing cultures of L6 cells were infected with mock or recombinant adenovirus supernatant diluted to 200 multiplicity of infection, enough to cover the cell monolayer at 37 °C for 1.5 h. After incubation, the medium was changed to fresh DMEM supplemented with 10% fetal bovine serum. Infection efficiency was measured after 2 days and again after 6 days (corresponding to 4 days of differentiation) by β -galactosidase assay, using the following protocol. Cells were fixed for 10 min with 0.2% glutaraldehyde and 2% formaldehyde in PBS and then washed three times for 10 min with PBS. Afterward, an enzymatic reaction was performed in β -galactosidase buffer (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ in PBS) with 1 mM 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (Sigma) as substrate. After 3–5 h at 37 °C, β -galactosidase-positive blue cells were counted, and the results were expressed as percentage of the total number of cells.

eEF1A-1/EF-1 α Transfection and Viability Assay

Cultured L6 myoblasts were grown and differentiated in six-well plates, as described above. After 3 days of differentiation, cells were cotransfected with 5 μ g of constitutively expressing Rous sarcoma virus β -galactosidase vector, along with 10 μ g of pBK CMV-*eEF1A-1/EF-1 α* sense or antisense (full-length), or empty pBK CMV vector used as a control (19,20) using LipofectAMINE 2000 (Invitrogen), with the exception that the cells were not subcultured the previous day. Efficiency of transfection was assessed by β -galactosidase staining, which revealed that myotubes were transfected. Cells were serum deprived 24 h after transfection and collected on a daily basis as described previously into cold PBS. After centrifugation, pellets were kept frozen at –80 °C until protein extraction was performed. For the extraction, the pellets were resuspended in 100 μ l of 0.25 M Tris-HCl, pH 7.8, and then submitted to three freeze-thaw cycles by transferring the samples between an ethanol-dry ice bath and a 37 °C water bath. Afterward, the extracts were centrifuged at 12,000 rpm for 8 min in a refrigerated bench top centrifuge, and the supernatants were retained for the assay. Viability was quantified by measuring β -galactosidase activity, as described by Chen *et al.* (19) and Oral *et al.* (27). The activity was assessed in 300 μ l of reaction solution containing 3.33 μ l of 100 \times magnesium buffer (0.1 M $MgCl_2$, 4.5 M β -mercaptoethanol), 66 μ l of 4 mg/ml *o*-nitrophenyl- β -D-galactopyranoside, 230.67 μ l of 0.1 M sodium phosphate buffer, pH 7.5 (41 ml of 0.2 M Na_2HPO_4 , 9 ml of 0.2 M NaH_2PO_4 , and 50 ml of water), to which 20 μ l of protein extract was added. After a 10-min incubation at 37 °C in a water bath, the reaction was terminated by the

addition of 500 μ l of 1 M Na₂CO₃, and the optical density of the samples was measured by spectrophotometry at 420 nm. Blanks were generated at the same time, and proteins from a positive sample were added after reaction termination with sodium carbonate. Efficiency of transfection was determined by β -galactosidase staining of the cells, as described above. For Western blotting, cells were selected with 250 μ g/ml of Geneticin for 4 days before collection.

RESULTS

Differentiation and Serum Deprivation of Mouse C2C12 and Rat L6A Myoblast Cell Lines

To initiate our study on the functions of eEF1A-1/EF-1 α and eEF1A-2/S1, mouse C2C12 and rat L6 myoblasts were first cultured to confluence and then induced to differentiate into multinucleated myotubes by changing the culture medium from a high (10% fetal bovine serum) to a low (2% horse serum) serum concentration; both cell lines gave rise to large myotubes upon differentiation. After 4 days of differentiation for L6 cells, and 6 days for C2C12, myotube cultures were switched from medium containing 2% horse serum to serum-free medium, to induce apoptotic cell death in differentiated myotubes by serum deprivation.

Western Blot Analysis of eEF1A-1/EF-1 α , eEF1A-2/S1, and Myosin Heavy Chain in L6 and C2C12 Differentiation and Serum Deprivation Assays

Protein samples of L6 and C2C12 cultures were collected at different time points during differentiation and processed for Western blotting with specific antibodies against eEF1A-2/S1, eEF1A-1/EF-1 α , and myosin heavy chain, used as a marker of differentiation. After 3 days of differentiation, L6 cells start expressing both eEF1A-2/S1 protein and the differentiation marker myosin heavy chain (Fig. 1A), whereas eEF1A-1/EF-1 α protein decreases to around 20% of its initial expression after differentiation. In C2C12 cultures, eEF1A-2/S1 and myosin heavy chain also appear after 3 days of differentiation (Fig. 1B), accompanied by a 3-day gradual loss in protein level of eEF1A-1/EF-1 α , which later remains stable at a low level in C2C12 myotube cultures. The kinetic of eEF1A-2/S1 protein appearance correlates with myosin heavy chain, a late marker of differentiation, suggesting that eEF1A-2/S1 is a protein that appears late in the differentiation process.

When C2C12 and L6 myotubes are serum deprived to induce apoptosis, eEF1A-1/EF-1 α and eEF1A-2/S1 expression patterns are reversed from that seen during differentiation. In cultured L6 myotubes, eEF1A-2/S1 protein disappears after 2 days of serum deprivation, whereas eEF1A-1/EF-1 α protein level increases simultaneously, to almost double the level seen in control myotubes (Fig. 2A). A similar pattern is observed in cultured C2C12 myotubes; eEF1A-1/EF-1 α protein expression level remains low for the first 3 days of serum deprivation but then increases and reaches a higher protein expression level than in the differentiated state (Fig. 2B). Unlike eEF1A-1/EF-1 α , eEF1A-2/S1 protein disappears after 9 days of serum deprivation. Myosin heavy chain was used to confirm that cells were differentiated during the serum deprivation experiment; β -actin was used as a loading control.

Cell Viability and DNA Fragmentation Assay

Cell viability of serum-deprived C2C12 and L6 myotubes was measured using the trypan blue exclusion assay, where blue cells are counted as dead. As seen in Fig. 3A, only 15% of C2C12 myotube cultures remain alive after 8 days of serum deprivation, whereas more than 60% of the control cells cultured with 2% horse serum remain viable at the same time point. In L6 myotube cultures, less than 10% of serum-deprived cells survive past 3 days, whereas about 40% of the control cells survive. To characterize myotube death further, DNA fragmentation assays were performed. As shown in Fig. 3B, oligonucleosome fragments appeared in C2C12 myotube cultures after 3 days of serum deprivation and increased steadily up to 9 days of serum deprivation, whereas control myotube cultures do not display any detectable DNA

fragmentation. The earliest dying L6 cells start to show fragmented DNA after 2 days of serum deprivation. Thus, serum deprivation induces apoptotic cell death in differentiated cultures of myotube. TUNEL staining was performed to confirm apoptosis as the mechanism for cell death (Fig. 4). The *left part* of the composite *panel* (Fig. 4, A, C, and E) represents the control differentiated myotube cultures maintained in DMEM supplemented with 2% horse serum; the *right part* (Fig. 4, B, D, and F) represents serum-deprived cultured myotubes. Bright TUNEL-positive stained nuclei, a hallmark of apoptosis, are observed in serum-deprived myotube cultures as indicated by *full arrows* in Fig. 4F, whereas the control counterpart remains negative for TUNEL staining (Fig. 4E, *open arrows*). Propidium iodide staining was used as a counterstain for all nuclei (Fig. 4, C and D), and phase contrast pictures (Fig. 4, A and B) reveal that the cells were indeed differentiated myotube syncytia.

Caspase-3 and PARP Western Blots, Caspase Activity, and Caspase-3 Inhibitor Effects on Cell Viability

We have assessed the apoptotic biochemical program by measuring caspase-3 activity with the cleavage of DEVD-AFC fluorescent peptide substrate. High caspase-3 activity is observed after 2 days of serum deprivation, whereas the activity in control cells remains close to the base-line level for the same period of time (Fig. 5A). As expected for caspase-mediated cell death, caspase-3 activity is seen when the cells are dying as shown by the kinetic of viability measurements (Fig. 5C). Caspase-1 fluorescent YVAD-AFC peptide was used as a negative control; caspase-1 activity remains constant in both serum-deprived and control myotube cultures. To verify that caspase-3 activity indeed occurs in apoptotic myotube cultures, proteins from control and serum-deprived cells were processed for Western blotting with caspase-3 and PARP rabbit polyclonal antibodies. As shown in Fig. 5B, the p17 catalytic fragment of active caspase-3 protein, obtained after activation by cleavage of p32 zymogen form of caspase-3, is seen on the Western blot at both 2 and 3 days of serum deprivation. These time points correspond to the peak of activity level in caspase-3 fluorogenic peptide cleavage assays (Fig. 5A). During the same period of time, a p85-cleaved PARP fragment, a marker of cellular caspase-3 protein activity (28), is detected in the same samples, demonstrating that caspase-3 is indeed activated in differentiated myotube cultures after serum deprivation. To confirm further that a caspase-3-dependent death mechanism indeed occurs, DEVD-FMK caspase-3 peptide inhibitor was used in differentiated cell cultures. As seen in Fig. 5C, after 3 days of serum deprivation in the presence of the inhibitor, around 60% of the differentiated cells are still alive, whereas less than 10% of the serum-deprived control cells remain viable. The inhibitor was potent to protect differentiated cells against serum deprivation-induced myotube cell death. This protection from cell death by the use of caspase-3 inhibitor confirms that upon serum deprivation of differentiated myotubes, the cell death mechanism is caspase-3-dependent, as suggested by Western blotting.

Western Blots and Viability of eEF1A-2/S1-infected Cells

Previous figures have shown that eEF1A-2/S1 was lost in cultures of dying myotubes but present before induction of apoptosis (see Fig. 2). Adenovirus clone A5.4 expressing eEF1A-2/S1 was used to infect undifferentiated L6 cells to see whether eEF1A-2/S1 protects or rescues cells from serum deprivation-induced apoptosis. In general, infection efficiency, determined by β -galactosidase assay, is on average about 85%, whereas infection efficiency for C2C12 cells is too low (around 40%); therefore only L6 cultures were used for this study. Undifferentiated L6 cells were infected and allowed to differentiate into myotubes. After differentiation, cells were serum deprived, and their viability was measured by trypan blue exclusion assay. As shown in Fig. 6A, about 45% of eEF1A-2/S1-infected myotube cultures remain alive after 3 days of serum deprivation, whereas only about 15% of the control (uninfected) and mock-infected (β -galactosidase) cells remain viable. After 2 days of serum deprivation, mock-infected cultures seem protected by the adenoviral infection, but that effect

was not seen after 3 days of serum deprivation, revealing the true protective effect of eEF1A-2/S1. To confirm further the protective effect of eEF1A-2/S1, caspase-3 activity was measured in serum-deprived *eEF1A-2/S1*-infected differentiated L6 cells. After 2 days of serum deprivation, A5.4-infected cultures exhibit between 50 and 60% of the caspase-3 activity of control cultures (Fig. 6B). As mentioned above, the adenoviral infection seems to protect myotubes cultures after 2 days of serum deprivation, but this protection is not seen when caspase-3 activity is measured (no significant difference), thus confirming that the adenovirus does not protect myotubes cultures, as seen after 3 days of serum deprivation viability. The reduced caspase-3 activity and low eEF1A-1/EF-1 α protein expression seen in *eEF1A-2/S1*-infected cells confirm the role of eEF1A-2/S1 as a rescuer from cell death in differentiated skeletal myocyte cultures. As shown in Fig. 6C, eEF1A-1/EF-1 α and eEF1A-2/S1 Western blots were performed to verify the efficiency of eEF1A-2/S1 adenoviral expression as well as the corresponding change in eEF1A-1/EF-1 α expression. As expected, only A5.4-infected cells express eEF1A-2/S1 protein before differentiation, revealing the efficiency of transfection. The absence of myosin heavy chain expression was used as a marker for the undifferentiated state in these samples. After differentiation, all samples express eEF1A-2/S1 protein, but after 3 days of serum deprivation, only the A5.4-infected myotubes still express eEF1A-2/S1 protein as shown by Western blotting. Interestingly, A5.4-infected L6 cultures express less eEF1A-1/EF-1 α protein than their control counterparts, noninfected and mock-infected cultures.

Western Blots and Viability of eEF1A-1/EF-1 α Sense- and Antisense-transfected L6 Cultures

After 3 days of differentiation, L6 cells were transiently transfected with *EF-1 α /EF1A-1* sense or antisense gene cDNA under the control of the CMV promoter (19,20); viability was assessed by measuring β -galactosidase activity, as described previously (19,27), providing the advantage that only transfected cells are counted. However, cells must be dead for a certain period of time to become negative for β -galactosidase activity. Upon serum deprivation, cells transfected with *eEF1A-1/EF-1 α* sense die more rapidly, whereas antisense-transfected cultures are protected from apoptosis and survive better than sense gene-transfected cells (Fig. 7A). After 3 days of serum deprivation, fewer than 25% of the cells transfected with the *eEF1A-1/EF-1 α* sense gene remain viable compared with about 55% of the control (mock transfection) and 70% of the *eEF1A-1/EF-1 α* gene antisense gene, as measured with this technique. The difference between the antisense and the control is small after 3 days of serum deprivation but significant ($p < 0.05$). The efficiency of gene transfer was analyzed by β -galactosidase staining, and Western blotting revealed a 50% increase in the abundance of eEF1A-1/EF-1 α protein presence with sense-transfected cells, whereas antisense-transfected cells show a decrease of 40% in eEF1A-1/EF-1 α protein abundance (Fig. 7, B and C). Both were compared with cells transfected with the empty pBK CMV vector. The experiments show that: 1) the same effect on enhanced apoptosis of sense eEF1A-1/EF-1 α is observed in myotubes, as published previously (19-21) and 2) the effects of eEF1A-1/EF-1 α and eEF1A-2/S1 in myotube apoptosis are opposite.

DISCUSSION

In this report, we demonstrate that eEF1A-2/S1 and eEF1A-1/EF-1 α protein expressions are regulated inversely in differentiated myotubule cultures during differentiation and apoptosis. When eEF1A-2/S1 protein is up-regulated during differentiation, eEF1A-1/EF-1 α protein is down-regulated with the reverse pattern of expression in apoptotic events. Apoptotic cell death is also associated with the activation of caspase-3 protease, which can be inhibited by specific cell-permeable, irreversible inhibitor. The presence of this inhibitor protects differentiated cultures of myotubes from serum deprivation-induced cell death, thus confirming that the death mechanism is caspase-3-dependent in differentiated myotube cultures. DNA fragmentation analysis confirms the apoptotic nature of the death in cultured myotubes and indeed reveals

that differentiated myotubes are capable of apoptotic cell death, as TUNEL-positive multinucleated syncytia are observed.

In an attempt to determine eEF1A-2/S1 function in apoptotic cells, the effect of continuous expression of eEF1A-2/S1 was assessed in dying myotubes. *eEF1A-2/S1*-infected myotubes are more resistant to serum deprivation-induced apoptosis than virus alone infected (β -galactosidase) or uninfected cells, as observed after 3 days. This result is the first to ascribe a protective or rescuing function to eEF1A-2/S1 protein in apoptosis. Cultures of myotubes bearing the transfected sense *eEF1A-1/EF-1 α* gene die more rapidly, whereas cells transfected with the antisense are protected and die more slowly from serum deprivation-induced apoptosis, similar to what was observed previously (19,20). This experiment essentially shows the opposite effects of eEF1A-1/EF-1 α and eEF1A-2/S1 on myotube survival. In terminally differentiated muscle cells, eEF1A-2/S1 may possibly replace eEF1A-1/EF-1 α , functioning not only in protein synthesis but also preventing apoptosis. This is the first noncanonical function associated with eEF1A-2/S1 and may help unravel the mystery behind the developmental switch between peptide elongation factor 1A observed in brain neurons, heart, and skeletal muscles; the switch may be beneficial to preserve and protect these long lasting cells from inopportune apoptotic cell death. Thus, the absence of eEF1A-2/S1 protein, and its replacement by eEF1A-1/EF-1 α in dying muscles, as observed in injured muscles (22) and in cultured myotubes, is a mechanism permitting apoptosis to occur.

Both eEF1A-2/S1 and eEF1A-1/EF-1 α proteins have a similar elongation function *in vitro* (15,16), and probably *in vivo*, because only eEF1A-2/S1 protein is expressed in mature brain neurons, heart, and skeletal muscles, where protein translation is maintained. The similarities between eEF1A-2/S1 and eEF1A-1/EF-1 α may not extend to other noncanonical functions not directly associated with protein biosynthesis. For example, eEF1A-1/EF-1 α is capable of severing microtubules, whereas no similar function has been shown for eEF1A-2/S1 (29). It is known that microtubule severing or anti-microtubule agents induce apoptosis (30,31). The increase in eEF1A-1/EF-1 α protein levels observed during apoptosis may serve to facilitate rapid death of cells, by dismantling their cytoskeleton. In contrast, eEF1A-2/S1, which may not be capable of microtubule severing, may thus indirectly protect cells from apoptosis.

Our study of apoptotic cell death in myotube cultures reveals a caspase-3-dependent mechanism. Caspase-3 activation was confirmed by a specific inhibitor, and by Western blot analysis of caspase-3 and PARP cleavage, a cellular marker of caspase-3 activity (28). In differentiated L6 cells, however, caspase-3 activity is lower when eEF1A-2/S1 is present, as seen in A5.4-infected cells; after 2 days of serum deprivation, half of the control caspase-3 activity is present in A5.4-infected cells. This may explain why, after 3 days of serum deprivation, about twice as many A5.4-infected cells survive serum deprivation-induced apoptosis. These results suggest that the protection exerted by eEF1A-2/S1 may include a mechanism involved in the regulation of caspase-3 activity. Disruption of the muscle architectural organization triggers the activation of caspase-3, as observed previously in the muscle cell death in muscular dystrophy (32). It will be interesting to analyze the possible analogy between caspase-3 activation in muscular dystrophy and how the expression of both elongation factors eEF1A-1/EF-1 α and eEF1A-2/S1 is regulated in this disease state.

The mechanism regulating caspase-3 activation may be related to the efficiency of differential translation of some genes mRNA by eEF1A-1/EF-1 α or eEF1A-2/S1. Accordingly, in such mechanism, eEF1A-2/S1 may favor the translation of prosurvival genes mRNA, or likewise repress the translation of proapoptotic genes, whereas eEF1A-1/EF-1 α may work in the opposite manner. Recently, two different studies reveal that the translation of the mRNA of some genes is modified according to the state of the cell, and a change in abundance of a single protein determines the translation speed of some mRNAs (33,34). This differential translation

mechanism may explain why eEF1A-1/EF-1 α protein is less abundant in *eEF1A-2/S1*-infected cells, which survive serum deprivation-induced apoptosis longer, and also why the eEF1As exert opposite effects on apoptosis.

In conclusion, we demonstrate that a switch between two peptide eEF1As occurs in cultured differentiation, as well as in tissue during development (11). This switch from eEF1A-1/EF-1 α to eEF1A-2/S1 may confer to the long lived, terminally differentiated myotube a translation elongation factor, which also prevents accidental cell death from occurring. The eEF1A-2/S1 protective function is the first noncanonical function associated with the protein. More experiments are needed help our understanding of how the protective function of eEF1A-2/S1 in skeletal muscle operates to regulate apoptotic cell death. Furthermore, it will be interesting to see whether this protective function of eEF1A-2/S1 behaves in heart and neurons in a manner that is similar to its behavior in skeletal muscles. It will also be interesting to study *in vivo* the expression and effect of eEF1A-2/S1 in skeletal muscle during aging and to determine whether the loss of eEF1A-2/S1 may expedite the age-dependent myofiber decline in skeletal muscle.

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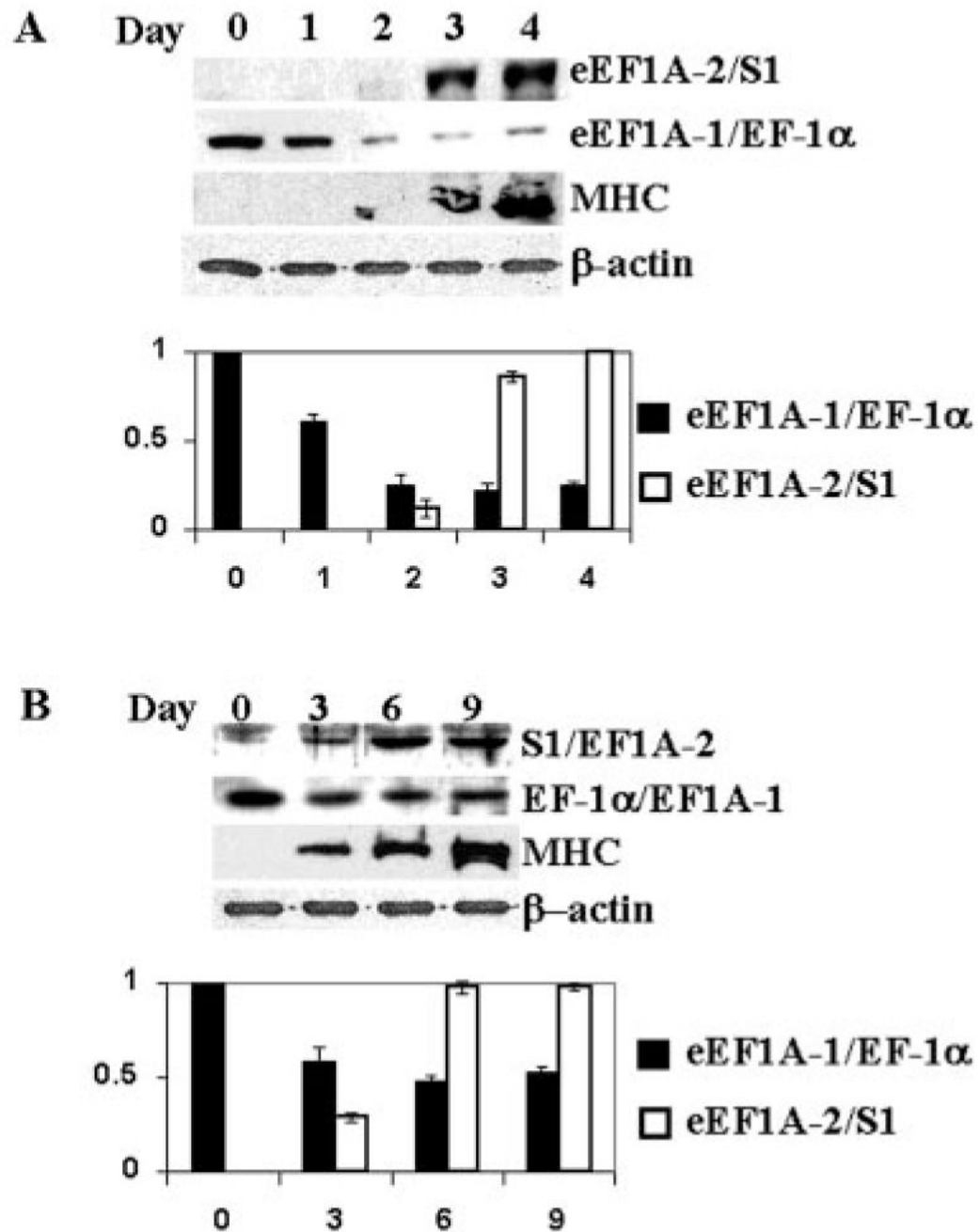


Fig. 1. eEF1A-2/S1 and eEF1A-1/EF-1 α protein expression during muscle differentiation in L6 and C2C12 myoblasts

eEF1A-2/S1 and eEF1A-1/EF-1 α proteins were detected using CB5 and HT7 specific antibodies, respectively. *Panel A*, L6 cells were differentiated under low serum conditions and collected on a daily basis for protein analysis. *Lane 0* corresponds to undifferentiated cells, and *lanes 1–4* represent 1–4 days of differentiation, respectively. MF-20 monoclonal antibody recognizing myosin heavy chain (*MHC*) was used as a marker of differentiation. Relative expressions of eEF1A-2/S1 and eEF1A-1/EF-1 α during differentiation are shown in the graph, and the *error bars* represent that S.D. *Panel B*, as in *panel A*, but for mouse C2C12 myoblasts

collected at 0, 3, 6, and 9 days of differentiation, respectively. In both *panels A and B*, β -actin was used as a loading control.

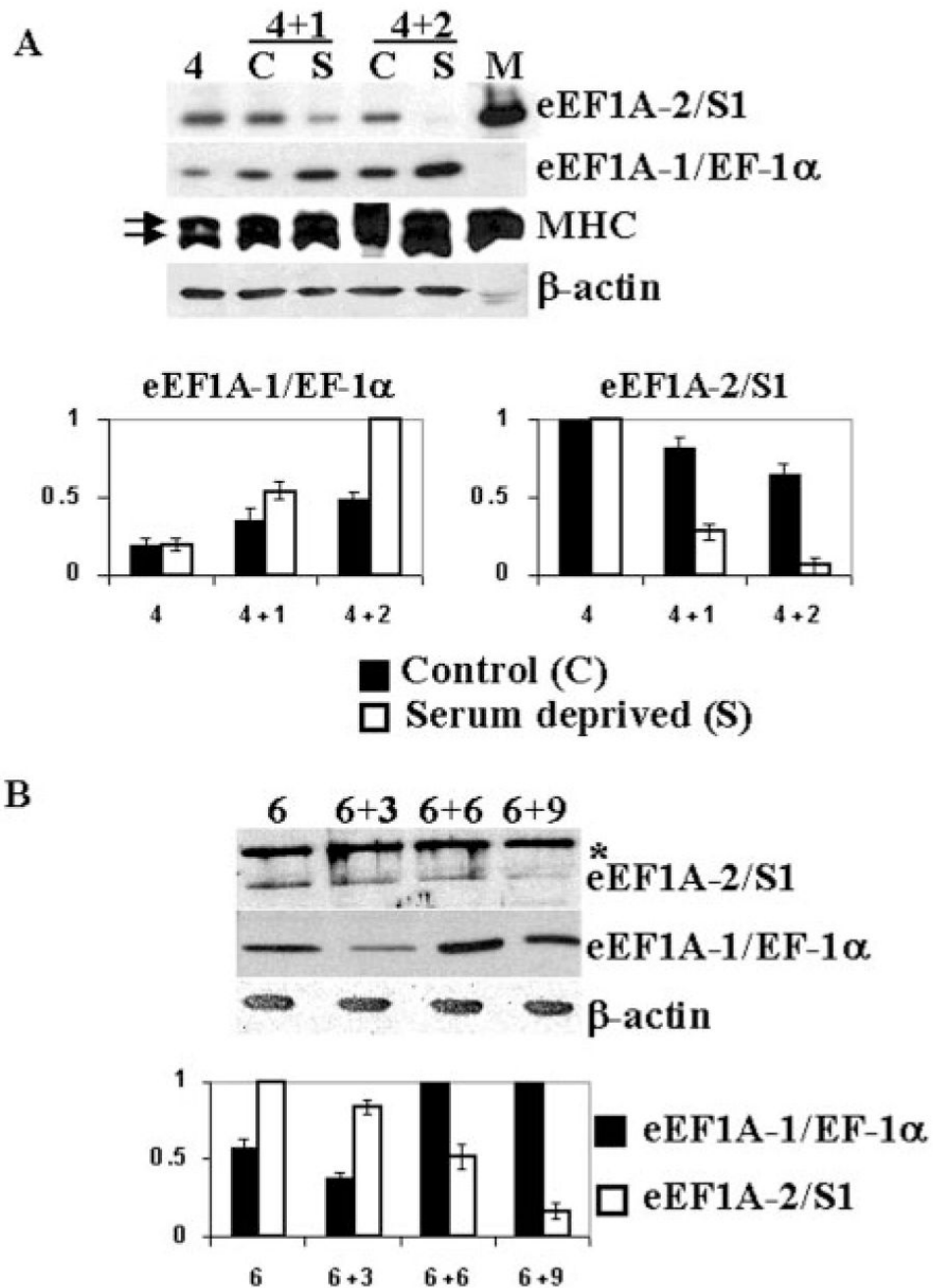


Fig. 2. eEF1A-2/S1 and eEF1A-1/EF-1 α protein expression in serum deprivation-induced apoptosis of L6 and C2C12 myotube cultures

After 4 days of L6 differentiation and 6 days of C2C12 differentiation, myotube cultures were serum deprived to induce apoptosis. +1 and +2 represent the number of days of serum deprivation after differentiation, indicated by the first number (4 or 6). *Panel A*, eEF1A-2/S1 and eEF1A-1/EF-1 α protein expression in L6 myotube cultures during serum deprivation. Control cells (C) were maintained in DMEM supplemented with 2% horse serum, and serum-deprived cells (S) were kept in serum-free medium. The *lane* marked M indicates 3-month-old rat skeletal muscle protein extract used as a control. Myosin heavy chain (MHC) was used as a marker of differentiation and β -actin as a loading control. Graphs represent the relative

expression of both elongation factors in control and serum-deprived samples, and the *error bars* represent S.D. *Panel B*, serum-deprived C2C12 myotube cultures. As above, the graph represents the relative expression of eEF1A-2/S1 and eEF1A-1/EF-1 α in C2C12 cells. The *asterisk* marks a contaminant band.

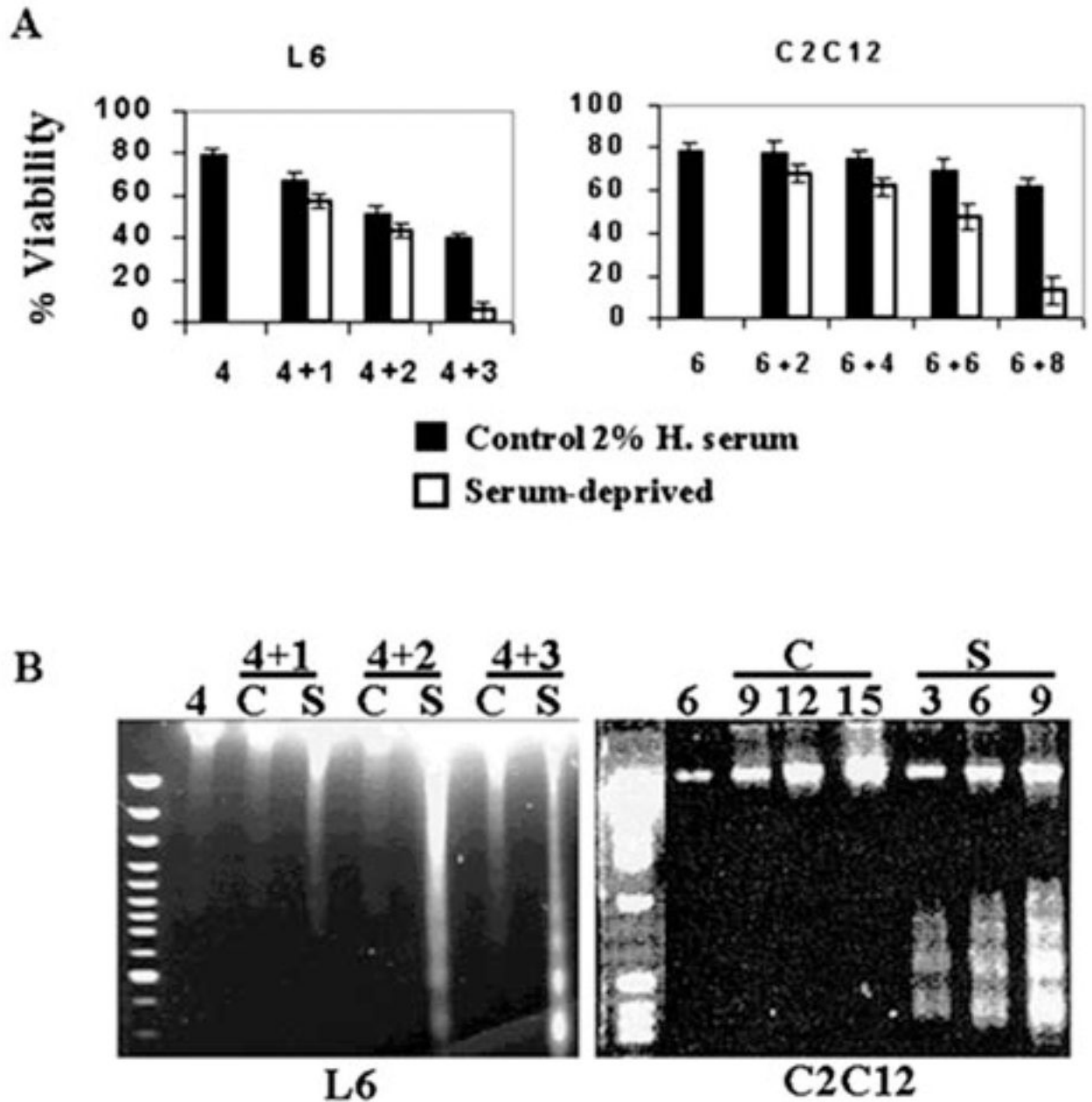


Fig. 3. Viability and apoptosis detection in L6 and C2C12 differentiated myotube cultures
Panel A, serum deprivation viability results of differentiated L6 and C2C12 myoblasts assessed by trypan blue exclusion assay (the S.D. is represented by the *error bar*). *Panel B*, DNA fragmentation was performed to demonstrate the apoptotic nature of cell death. For L6 cells, control samples at 4–7 days of differentiation are marked C. Serum deprivation was initiated on the 4th day of differentiation. Serum-deprived cell samples are labeled S. As in Fig. 2, the *+day* system is used to label the samples from the day of serum deprivation. In C2C12 cells, control cells at 6 (initial day of serum deprivation), 9, 12, and 15 days of differentiation and serum-deprived cells for 3, 6, and 9 days were used.

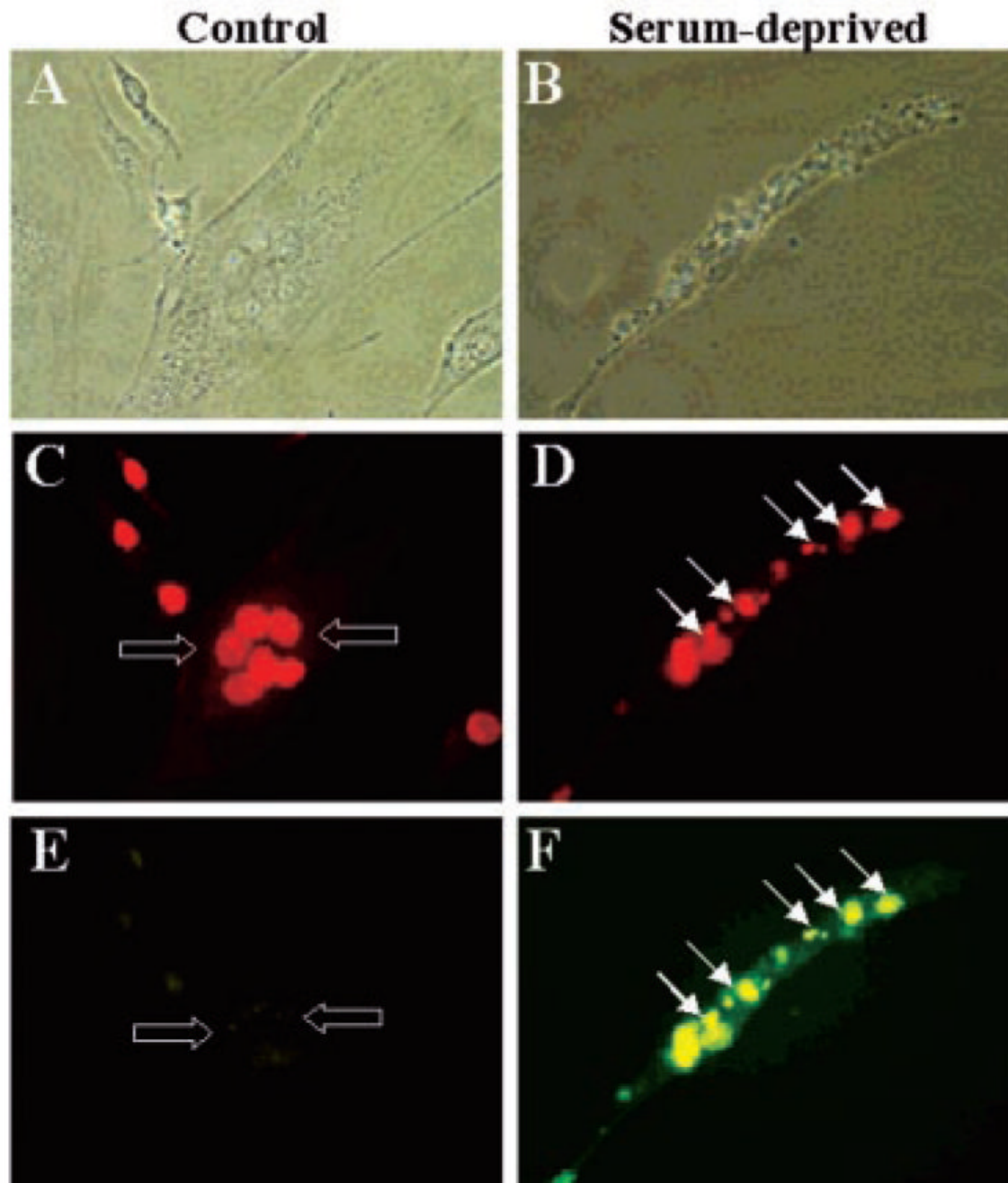


Fig. 4. Detection of apoptotic myotubes by TUNEL staining

Differentiated L6 myotube cultures were grown on coverslips and then serum deprived to induce apoptosis. Control cells were maintained in DMEM supplemented with 2% horse serum during the duration of the experiment (*panels A, C, and E*). After 3 days of serum deprivation, L6 myotube cultures were processed for TUNEL staining (*panels B, D, and F*). Phase contrast pictures show control (*panel A*) and apoptotic (*panel B*) myotubes. Propidium iodide was used as a counterstain to reveal the presence of multinucleated myofibers (*panels C and D*). TUNEL staining revealed the absence of positive apoptotic myonuclei in the control myotube (*panel E*) and bright, condensed positive myonuclei in apoptotic myotube (*panel F*). *Open arrows* indicate the presence of non-apoptotic nuclei (*panels C and E*), and *full arrows* indicate the

presence of apoptotic nuclei (*panels D and F*). All pictures were taken at the same magnification ($\times 400$).

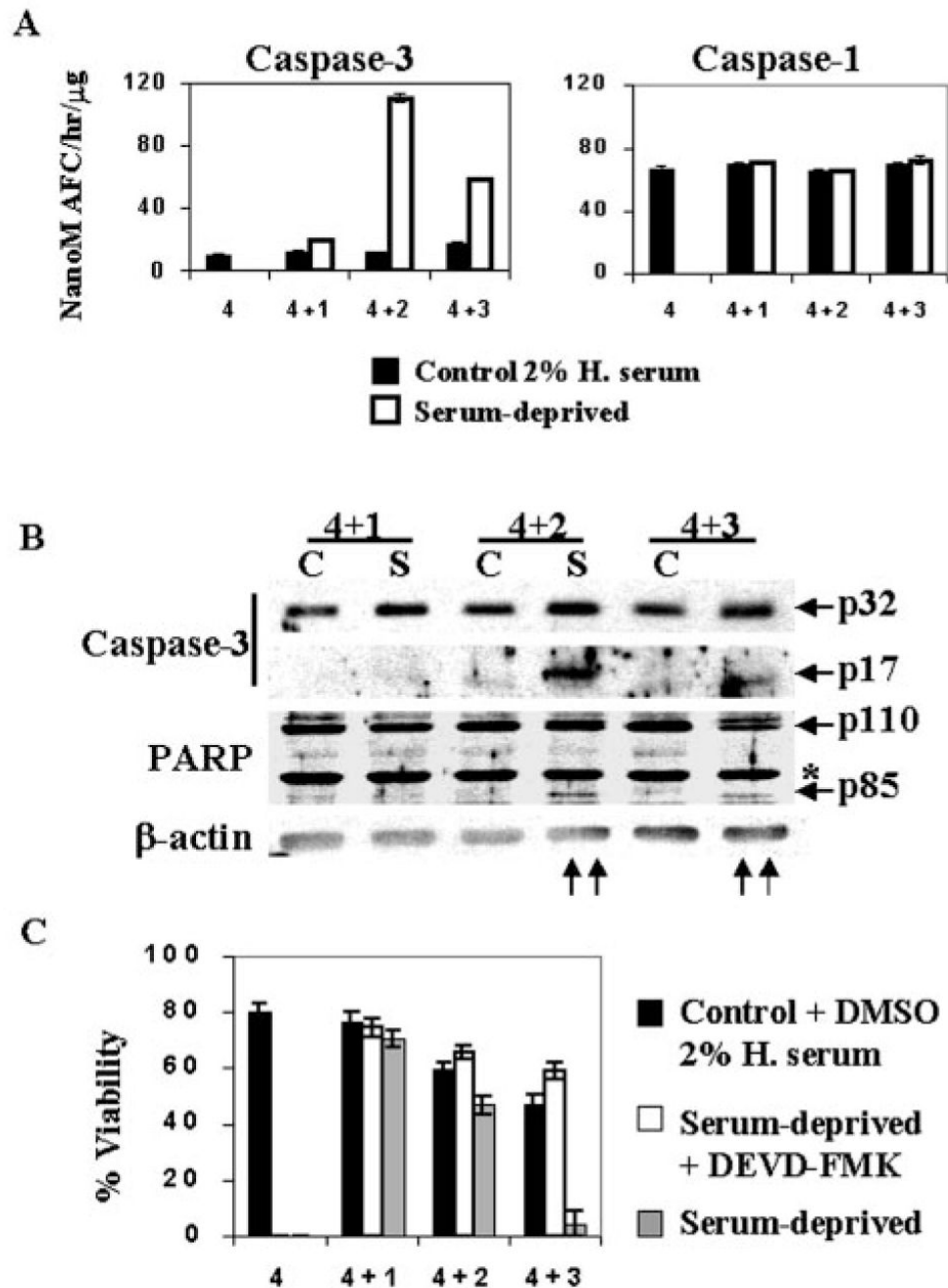


Fig. 5. Caspase activity in serum deprivation-induced apoptosis of differentiated L6 cells
 After 4 days of differentiation (*initial point*), L6 cells were serum deprived for 1, 2, or 3 days to induce apoptosis, and the cells were harvested for caspase activity assays and Western blotting. *Panel A*, caspase-1 and -3 activity was measured by the cleavage of specific fluorescent substrates and calculated as nmol of AFC released/h/ μ g of protein. The *black bars* represent 2% horse serum control samples, and the *white bars* represent serum-deprived samples. *Panel B*, Western blotting reveals the presence of both inactive p32 (zymogen) and active p17 caspase-3 in serum-deprived samples (S); control protein samples are represented by C. Western blotting reveals the presence of cleaved p85 PARP in 2- and 3-day serum-deprived L6 cell samples. The native form of PARP is detected at 110 kDa. Note that in rat

samples, PARP antibody detects a contaminant band marked by an *asterisk*. *Double arrows* indicate the lane with p17 active caspase-3 and PARP cleavage. *Panel C*, viability of serum-deprived L6 cells cultured with caspase-3 DEVD-FMK peptide inhibitor (*gray bars*) was assessed by trypan blue exclusion assay. Cells cultured with dimethyl sulfoxide and 2% horse serum (*black bars*) and serum-deprived cells (*white bars*) were used as controls. The *error bars* represent the S.D.

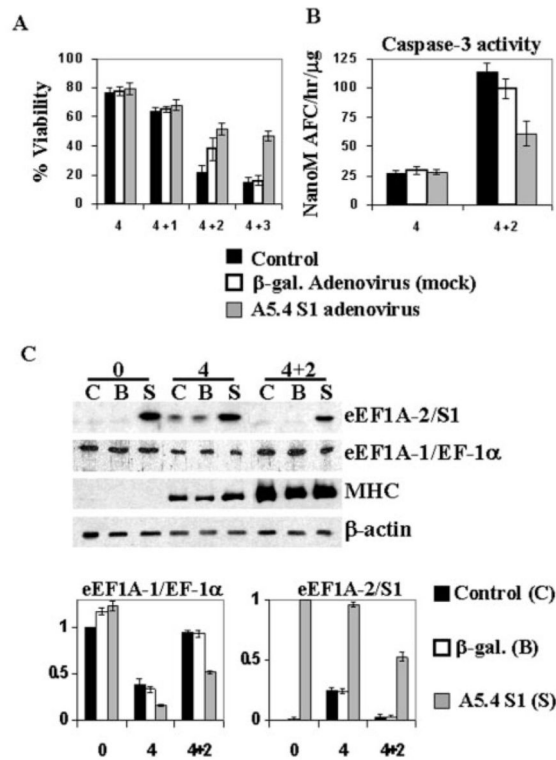


Fig. 6. Effect of continuous expression of eEF1A-2/S1 in serum-deprived L6 myotube cultures
Panel A, a trypan blue exclusion assay was used to measure the viability of differentiated L6 cells after serum deprivation following adenoviral infection. Uninfected cells (*black bars*) and cells infected with the control β -galactosidase adenovirus (*white bars*) were serum deprived and used as controls. The *gray bars* represent cells infected with eEF1A-2/S1 A5.4 adenovirus. *Panel B*, caspase-3 activity was measured in all cells. The same labeling system developed in Fig. 2 was used to label the *lanes*. *Panel C*, Western blotting of adenovirus-infected cells before (0), after 4 days of differentiation (4), and after 2 days of serum deprivation (4+2). The uninfected control cells are indicated by C, the β -galactosidase control by B, and A5.4-infected cells by S. CB5 and HT7 rabbit polyclonal antibodies were used to detect eEF1A-2/S1 and eEF1A-1/EF-1 α , respectively. MF-20 monoclonal antibody was used to detect myosin heavy chain (MHC), a marker of differentiation. Graphs represent the relative expression of eEF1A-2/S1 and eEF1A-1/EF-1 α proteins. All *error bars* represent the S.D.

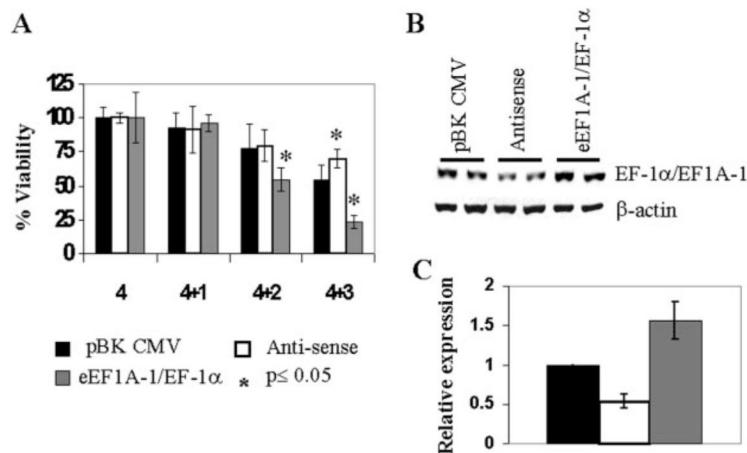


Fig. 7. Effect of transient sense and antisense *eEF1A-1/EF-1α* transfection in serum-deprived L6 myotube cultures

Panel A, viability of differentiated L6 cells after serum deprivation is measured by β -galactosidase activity after transient cotransfection of β -galactosidase reporter vector and the gene of interest. Cells cotransfected with pBK CMV empty vector were used as control (*black bars*). Cells cotransfected with antisense *eEF1A-1/EF-1α* in pBK CMV vector are represented by the *white bars*, and cells cotransfected with the sense *eEF1A-1/EF-1α* gene in pBK CMV vector by the *gray bars*. The *asterisks* represent a significant difference analyzed by Student's *t* test ($p < 0.001$). *Panel B*, Western blotting of cotransfected cells to show the efficiency of the transfection. The experiment was performed in duplicate from two different cell samples. β -Actin was used as a loading control. *Panel C*, relative expression of eEF1A-1/EF-1 α protein in transfected L6 myotube cultures, including the S.D.