Involvement of PKR in the Regulation of Myogenesis

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Abstract
The involvement of the double-stranded RNA-activated protein kinase PKR in the regulation of the myogenic process was investigated. For this purpose, the murine myogenic cell line C2C12 was used. The cells were first cultivated in either growth medium or differentiation medium (DM), and the activation of PKR during differentiation was determined by monitoring its enzymatic activity and by immunoblot analysis. A significant increase in both parameters was detected already at 24 h in DM, whereas in cells grown in growth medium, the increase was evident only after 96 h, when spontaneous differentiation was observed in highly crowded cultures. Consequently, we established the direct effect of PKR activation on the myogenic process. C2C12 cells were transfected with an expression vector harboring a CDNA molecule encoding human PKR fused to the inducible metallothionein promoter. One of the clones (clone 8) expressing high levels of PKR was selected and further analyzed. In the presence of ZnCl₂, which activates the promoter, the rate of cell growth of the transfected cells was clearly reduced compared to that of wild-type C2C12 cells transfected with only the neomycin-resistant gene (C2-NEO). In addition, altered morphology with partial fusion was observed. Biochemically, an increase in creatine kinase activity accompanied by an increased rate of expression of the myogenic transcription factors myoD and myogenin detected in clone 8 cells exposed to ZnCl₂. Most importantly, an induction in the level of cyclin-dependent kinase inhibitor p21WAF1 and an increase in the level of the underphosphorylated active form of the tumor suppressor protein pRb concomitant with the down-regulation of cyclin D1 and c-myc were also evident in the transfected clones. These changes were similar to those observed in normal C2C12 cells cultivated in DM. We conclude that PKR is an important regulatory protein participating in the myogenic process.

Introduction
PKR, a double-stranded RNA-activated serine-threonine protein kinase, has been originally described as an IFN-inducible enzyme implicated in antiviral activity (1, 2). Upon activation, the enzyme molecule is first autophosphorylated at several sites, followed by the phosphorylation of the target molecules (3). Several such targets have been reported. The best characterized of these is the α subunit of the translation initiation factor eIF-2. The factor is phosphorylated on serine residue 51; consequently, the exchange of the eIF-2-bound GDP with GTP by exchange factor eIF-2B is blocked (4), resulting in the inhibition of protein synthesis. In addition, the transcription factor inhibitor IκB (5) and the HIV-specific Tat-binding protein tat (6) were also described as possible (although not necessarily direct) targets phosphorylated by PKR. The genes encoding PKR both from human (7) and mouse (8) origin were isolated and characterized, and the structural domains present in the protein molecule were elucidated. The NH₂-terminal portion of the enzyme appears to contain the double-stranded RNA binding motif and the ability to interact with other protein molecules, including itself (9–11). However, it is still not clear whether dimerization is indeed required for the PKR-mediated biological effects (12). The catalytic domains of the enzyme, on the other hand, are all located in the COOH-terminal region (13). As can be judged from its mode of action, PKR is not involved only in antiviral activity but has a much broader biological significance. It has been clearly demonstrated that ectopic expression of negative dominant mutants of PKR in NIH/3T3 mouse fibroblasts resulted in their malignant transformation (14–16), indicating that the enzyme has a tumor-suppressive effect and is most likely associated with the regulation of cell growth. In addition, overexpression of wild-type PKR was reported to induce apoptosis in susceptible cells, whereas expression of bcl-2 or mutated PKR was able to protect the cells from this event (17, 18). Interestingly, PKR can modulate the function of the signal transducer and activator of transcription STAT1 by association or dissociation between these two proteins (19). Finally, PKR was reported to down-regulate the expression of c-myc in growth-retarded M1 cells transfected with wild-type PKR (20). Taken together, these results suggest that the enzyme fulfills a pivotal regulatory role within the cell. However, it is still not clear whether it is involved in differentiation processes. To address this question, we studied the effect of the ectopic expression of PKR on myogenesis.

Skeletal muscle cell cultures are an excellent experimental tool for the study of differentiation in vitro. Upon withdrawal
from the cell cycle, myogenic committed cells known as myoblasts fuse to form myotubes. This process is well controlled by a series of myogenic specific transcription factors, the myoD family. The family consists of myoD, myogenin, Myf-5, and MRF4, all of which contain a DNA-binding basic region and a helix-loop-helix motif responsible for interaction with other proteins (21), mostly with products of the E2A gene, such as E12 or E47 (22). This complex binds to an element on the DNA termed the E box with the consensus sequence CANNTG. Although myoD homodimers are as stable as the myoD-E12 heterodimers, only the latter bind to the E box (23). It should be noted, however, that additional proteins are involved in modulating the activity of the myoD family (24).

IFN\(^3\) has been previously shown to induce morphological and biochemical changes in several cell systems, including skeletal myogenic cell cultures (25–27). In addition, the expression of both PKR and 2-5A synthetase, another IFN-induced protein, was reported to increase during myogenic differentiation \textit{in vitro} (28). Furthermore, various agents that inhibit myogenesis were also effective in interfering with the expression of these proteins (29, 30). Most recently, it has been shown that an additional IFN-inducible protein, p202, increased during skeletal muscle

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\(^3\) The abbreviations used are: IFN, interferon; GM, growth medium; DM, differentiation medium; CDK, cyclin-dependent kinase.
differentiation (31). However, these elevated levels may be fortuitous, and more direct evidence is needed to clarify whether PKR plays a role in the myogenic process. In this report, we show that ectopic expression of PKR in myogenic cells results in morphological, molecular, and biochemical alterations associated with skeletal muscle differentiation.

Results

Activation of PKR during Myogenic Differentiation of C2C12 Cells. We had to establish first whether PKR is specifically activated during induction of differentiation of the myogenic cell line C2C12. The cells were cultivated in either GM or DM. Cell extracts were prepared at different times, and the presence of PKR was identified by immunoblot analysis using polyclonal antibodies directed against human PKR. The PKR protein appeared as a broad band of 66–68 kDa (Fig. 1A). In parallel, the level of the biologically active protein was determined by monitoring its enzymatic activity under the same conditions. The 32P-labeled autophosphorylated form of PKR, which was 67 kDa in size, was an indication of enzymatic activity (Fig. 1B). The basal level of the PKR protein at the initiation of the experiment (zero time) was low (data not shown) and did not change within the first 24 h in GM (Fig. 1A, DIVISION, lane 1; Fig. 1C). However, a gradual increase was observed with time in culture up to 144 h (Fig. 1A, C2-NEO, clone 8). It should be noted that a few myotubes were always visible in crowded C2C12 cells even when cultivated in GM, an indication of spontaneous differentiation. The kinetics of PKR enzymatic activity obtained with C2C12 dividing cells (Figs. 1, A and B, DIVISION) was similar to that observed with the level of the PKR protein, demonstrating that the gradual increase in this activity reflects an increase in the total amount of PKR. In contrast, in the case of C2C12 cells grown in DM, a significant increase in both the level of PKR and its enzymatic activity was already evident at 24 h after the medium change (Fig. 1, A and B, DIFFERENTIATION). The level of both parameters remained constant up to 96 h and was followed by a decrease. At this time, the cultures were fully differentiated.

Transfection of PKR into C2C12 Cells. The fact that PKR is induced during differentiation of C2C12 cells does not necessarily imply that the enzyme plays a role in myogenesis. To show its direct involvement in the process, we constructed plasmid pMPKR, which harbors a cDNA encoding human PKR fused to the metallothionion promoter (see “Materials and Methods”). This plasmid was cotransfected into C2C12 cells with pSVneo, and 25 neomycin-resistant clones were isolated and expanded. Eight clones expressed high levels of PKR in response to the presence of ZnCl2 (which activates the promoter). The results obtained with a representative clone, clone 8, are presented in Fig. 2; however, two additional clones that were analyzed similarly yielded comparable results that were not included for the sake of simplicity. For control cells, we used a clone transfected with pSVneo only. This clone was designated C2-NEO. Immunoblot analysis as well as a determination of PKR enzymatic activity indicated that the basal level of PKR in either C2-NEO or clone 8 cells was rather low; however, only clone 8 cells exposed to ZnCl2 for 24 h responded with an elevated level of PKR protein (Fig. 2A, clone 8), and an increase in its enzymatic activity (Fig. 2B, clone 8). In contrast, both C2-NEO and clone 8 cells responded equally well to a 24-h treatment with IFN (Fig. 2, A and B, lanes 1 and 2), an indication that the endogenous PKR-encoding gene is functional in these types of cells.

Morphological Alterations and Growth Characteristics of PKR-expressing C2C12 Cells. To establish whether PKR is indeed involved in the initiation of the myogenic process, it was important to study the effect of its ectopic expression on a variety of biological parameters. First, C2-NEO and clone 8 cells were grown in GM in the presence or

![Fig. 2. Expression of PKR in transfected clone 8 cells. C2-NEO and clone 8 cells were treated with either IFN or ZnCl2 (ZnCl2) for 24 h. One group of cultures remained untreated (C). Cell extracts were prepared, and PKR was identified either by immunoblot analysis (A) or by enzymatic activity (B). Densityometry of A and B is presented in C and D, respectively.](image-url)
absence of ZnCl₂ for 96 h, and changes in cell morphology were examined microscopically. Exposure of control cells to ZnCl₂ did not result in any alterations in the morphology or density of treated cells compared to those of untreated cells (compare Fig. 3, A and C). On the other hand, a striking difference was observed when clone 8 cells were similarly analyzed. Cell density was greatly reduced in ZnCl₂-treated cultures, most of the cells developed extended processes, and about 30% formed short myotubes comprised of two to three cells (compare Fig. 3, B and D). The retarded growth rate of clone 8 cells exposed to ZnCl₂ was confirmed in the experiments described in Fig. 5.

Vital cell counts were performed daily after the medium change. Cells were labeled in parallel for 1.5 h with [³H]thymidine, and the rate of DNA synthesis was determined. As expected, both parameters were significantly reduced in differentiating versus dividing C2C12 cells (Fig. 4, A and B). We then compared the rates of growth and DNA synthesis of C2-NEO and clone 8 cells cultivated in GM in the presence or absence of ZnCl₂. It is clearly shown that whereas C2-NEO cells were hardly affected by exposure to ZnCl₂, the growth rate (Fig. 4C) and kinetics of DNA synthesis (Fig. 4D) were decreased in clone 8 cells treated with ZnCl₂. We conclude that cultivation in DM and ectopic expression of PKR induce similar effects on the growth characteristics of C2C12 cells.

Appearance of Muscle-specific Proteins and Transcription Factors in C2C12 Transfected Cells. The reduced rate of proliferation of muscle cultures is accompanied by the expression of muscle-specific proteins. This was confirmed in our study by the following observation. The levels of creatine kinase activity and troponin T gradually increased with time up to 120 h when C2C12 cells were grown in DM. In contrast, when cells were cultivated in GM, the level of these muscle-specific proteins remained low, and an increase became evident only after 96–144 h (Fig. 5, A, I and B, I). At this late time, spontaneous differentiation was observed in highly crowded cultures. We next determined whether the growth inhibition detected in clone 8 cells expressing PKR is also accompanied by an elevated level of muscle-specific proteins. C2-NEO and clone 8 cells were grown in the presence and absence of ZnCl₂, and the levels

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**Fig. 3.** Effect of ZnCl₂ on the morphology of C2C12 cell variants. C2-NEO (A and C) and clone 8 (B and D) cells were seeded in GM (+Zn²⁺) (A and B). Some cultures were exposed to ZnCl₂ 3 h later (+Zn²⁺) (C and D). After an additional 96 h, the cell morphology was examined with a phase-contrast microscope (×125).
of both creatine kinase activity and troponin T were determined at daily intervals. The results demonstrate that the presence of ZnCl$_2$ did not affect the level of either protein in C2-NEO cells (Fig. 5, A, II and B, II). However, the appearance of both proteins was significantly accelerated in ZnCl$_2$-treated versus untreated clone 8 cells (Fig. 5, A, II and B, III), indicating that the ectopic expression of PKR induces the synthesis of muscle-specific proteins.

Next, we wanted to provide evidence that the myogenic transcription factor myoD (32) is also expressed in clone 8 cells expressing PKR. Total RNA was therefore extracted from ZnCl$_2$-treated and untreated clone 8 cultures, and Northern blot analysis was performed using a myoD-specific probe. For comparison, a similar analysis was performed on dividing and differentiating C2C12 cells. The results demonstrate that whereas a significant increase in the level of myoD-specific RNA transcripts was observed in C2C12 cells grown in GM only after 120 h (Fig. 6, A, I), a major increase was detected in differentiating cells cultivated for 24 h in DM, followed by a decrease thereafter. In dividing cells, on the other hand, the amount remained low, and an increase was observed only at 120–144 h (Fig. 6, B, I). These results were confirmed by immunoblot analysis in which the level of the myoD protein as well as that of myogenin, a second myogenic transcription factor, was established. The results indicate that the amount of both proteins increased in differentiating C2C12 cells, with a peak observed at 48 h in DM, followed by a decrease thereafter. In dividing cells, on the other hand, the amount remained low, and an increase was observed only at 120–144 h (Fig. 7, A, I and B, I). We then performed a similar analysis on clone 8 cells grown in GM in the presence or absence of ZnCl$_2$, using C2-NEO cells grown under similar conditions as an additional control. As expected, no effect of ZnCl$_2$ was observed on the level of myogenin or myoD in C2-NEO cells (Fig. 7, A, II and B, II). However, in the case of clone 8 cells, the level of both proteins increased at least 24 h earlier in ZnCl$_2$-treated
Involvement of PKR in Myogenesis

In agreement with the concept that IFN-induced proteins play a role in the regulation of cell growth and differentiation, we show in the present report that PKR is activated during C2C12 myogenic cell differentiation. These results support earlier findings on the induction of PKR activity in rat primary skeletal muscle cultures (28) or in differentiating rat L8 myogenic cells (30). It is not surprising to see that, even in dividing C2C12 cells, an elevated level of PKR was observed after exposure to ZnCl2, respectively (Fig. 8, A–C, III). This is in contrast to a 1.3- and 1.2-fold increase in the level of p21WAF1 detected at 72 and 96 h, respectively, in ZnCl2-treated C2-NEO cells (Fig. 8, A, II). In addition, the down-regulation of cyclin D1 and the accumulation of pRb (underphosphorylated) were accelerated and occurred 24–48 h earlier in ZnCl2-treated versus untreated (control) C2-NEO cells (Fig. 8, B and C, III). Similarly, the reduction in c-myc expression characteristic for C2C12 differentiating cells (Fig. 9I) was detected in ZnCl2-treated clone 8 cells at least 24 h earlier than in control cells (Fig. 9III). It is thus concluded that PKR is involved in the regulation of cell cycle-associated proteins.

Discussion

The IFN system as a whole has been previously shown to exhibit antiproliferation properties against a variety of cell types (34). Furthermore, it seems that molecular events associated with the cell cycle are some of the major targets affected by IFN. Thus, the reduction in c-myc expression, the accumulation of the underphosphorylated form of pRb (36–39) with the concomitant down-regulation of cyclins and CDKs (40–42), the reduced levels of the active E2F family of transcription factors (38, 40, 43), and the induction of CDK-inhibitory proteins (38, 41, 42) were reported to be the result of IFN activity. However, most of the IFN-induced biological activities are mediated by a variety of proteins activated by IFN via a unique signal transduction pathway (44). Accordingly, PKR has been shown to mediate IFN-induced c-myc suppression in M1 myeloid leukemia cells (20). In addition, the ratio of the transcriptional activator of IFN-induced genes (IRF-1) to a suppressor of these genes (IRF-2) is high in growth-arrested cells and low in proliferating cells. Furthermore, deletion of the IRF-1-encoding gene or overexpression of IRF-2 may result in the development of tumors, including those of human origin (45). Finally, it has been demonstrated recently that ectopic expression of 2-5A synthetase in myeloid cells induces cell growth arrest, a reduction in c-myc expression, an accumulation of the underphosphorylated form of pRb, and the appearance of a myeloid differentiation marker (46).

In agreement with the concept that IFN-induced proteins play a role in the regulation of cell growth and differentiation, we show in the present report that PKR is activated during C2C12 myogenic cell differentiation. These results support earlier findings on the induction of PKR activity in rat primary skeletal muscle cultures (28) or in differentiating rat L8 myogenic cells (30). It is not surprising to see that, even in dividing C2C12 cells, an elevated level of PKR was observed at 96 h after the initiation of the experiment (Fig. 1) because spontaneous differentiation is common in crowded cultures.
This was accompanied by cell growth arrest (Fig. 4, A and B) and by elevated levels of muscle-specific proteins (Fig. 5, A, I and B, I; Fig. 7) detected at late times in C2C12 cells cultivated in GM. However, the most striking phenomenon demonstrated in our study was the fact that ectopic expression of PKR in myogenic cells exposed to ZnCl₂ induced a variety of morphological, biochemical, and molecular changes characteristic of myogenic differentiation. Thus, although complete elongated myotubes were not detected in these cultures, a change in cell morphology and the formation of short myotubes consisting of three cells were common (Fig. 3, D). In addition, a retardation of cell growth (Fig. 4, C and D) coupled with the accelerated appearance of muscle-specific proteins creatine kinase and troponin T (Fig. 5, A, II and B, II) and myogenic transcription factors myoD and myogenin (Fig. 6B, II; Fig. 7, A, III and B, III) was evident in transfected cells expressing PKR. Finally, an induction of the expression of p21⁰⁰⁰⁰, accompanied by a reduction in the levels of cyclin D1 and c-myc as well as an accumulation of the underphosphorylated form of pRb, was also observed in these cells (Fig. 8, A–C, III; Fig. 9, III). According to our view, PKR is most likely involved in the down-regulation of gene expression, possibly by the specific inhibition of the translation of certain mRNA molecules. The induction of gene expression in myogenesis, on the other hand, may then follow or be the result of an independent signal transduction pathway and therefore is not directly related to PKR activity.

Recently, Datta et al. (31) reported an additional IFN-induced protein, p202, whose level is increased during the differentiation of C2C12 cells. However, in contrast to the results obtained in our report with PKR, ectopic expression of the gene encoding p202 in C2C12 cells inhibited rather than enhanced muscle differentiation. Thus, overexpression of p202 reduced the level of myoD and inhibited the transcriptional activation of both myoD and myogenin. The discrepancy between the observed elevated level of p202 during differentiation and the inhibition of myoD and myogenin activation by ectopic expression of p202 is explained by Datta et al. (31) to be the result of early expression in the transfected cells. PKR, on the other hand, seems to be
Involvement of PKR in Myogenesis

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NEO (phorylated form during terminal differentiation. Based on the
indicated in the figure, as described in Fig. 5. The levels of myogenin (A)
and myoD (B) were determined by immunoblot analysis.

Fig. 7. Determination of myogenic transcription factors. C2C12 (I), C2-
NEO (II), and clone 8 (III) cells were grown in the appropriate medium
as shown in the figure. As shown, myogenin (A) and myoD (B) were
analyzed by Western blot analysis. The levels of myogenin (A)
and myoD (B) were determined by immunoblot analysis.

sequently, it must be concluded that p202 and PKR operate
on two different levels, although both are activated during
muscle differentiation.

An important finding in our study is the enhanced accumu-
lation of pRb and PKR in PKR-expressing C2C12 cells (Fig. 8C, III). As mentioned above,
this is accompanied by a reduction in the level of both cyclin
D1 and CDK4 and an increase in the synthesis of p21WAF1.

To distinguish between the two constructs,
several plasmid preparations were digested with
HindIII. This plasmid
was digested with HindIII and XbaI. Ligation
in the sense orientation was obtained when the resulting fragments were
0.4- and 5-kb long. In the final stage, one of these plasmids (pBS-SK-PKR)
was digested with Sall and XbaI, and the PKR-containing fragment was
ligated into the polylinker of Bluescript SK, resulting
in two possible orientations. To distinguish between the two constructs,
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was digested with Sall and XbaI, and the PKR-containing fragment was
ligated into the polylinker Sall-XbaI site of plasmid pCMV (50). This plasmid
contains the metallothionein promoter. Before the final step, an extra
Sall site in pCMV was removed by SphI, followed by self-ligation. The final
construct, pMPKR, was used in this study.

Transfection

pMPKR was cotransfected with pSVneo (this plasmid contains the active
neomycin resistance gene fused to the early SV40 promoter; Ref. 51)
into C2C12 cells by electroporation. Approximately 2 × 105 cells/ml were
suspended in 250 μl of GM to which 200 μl of sucrose buffer [272 mM
sucrose and 7 mM Na2PO4 (pH 7.4)] and 50 μl of DNA containing 15 μg
of pMPKR and 1 μg of pSVneo were added. Electroporation was performed
at 400 V and a capacitance of 500 μF using the Bio-Rad gene pulsar II
apparatus (Bio-Rad Laboratories, Hercules, CA). The cells were then
transferred into 10-cm dishes containing DMEM supplemented with 20% FCS.
After 48 h of incubation in GM, the cultures were subdivided at a ratio

Materials and Methods

Cell Cultures and Treatments

Murine myogenic C2C12 cells were maintained in DMEM supplemented
with 15% FCS (Biological Industries, Beth Haemek, Israel; GM) under
10% CO2 at 37°C. The cultures were split every 3–4 days by removing
the cells with a mixture of 0.25% trypsin and 0.05 M EDTA in PBS. Fresh
cultures were prepared from frozen cell pellets every 2 months. Unless
otherwise mentioned, the cells were seeded for experimental purposes at
2 × 105 cells/10-cm tissue culture dish in GM. After 1 day, some of the
cultures were shifted to DMEM supplemented with 10% horse serum and
1 μg/ml insulin (DM).

Mouse α/β IFN (Access BioMedical, San Diego, CA; specific activity,
9.8 × 106 IU/mg) was added to untreated cultures at a concentration of
240 IU/ml.

A stock solution of 100 mM ZnCl2 (Sigma Chemical Co., St. Louis, MO)
in 50 mM HEPES (pH 6.0) was prepared and kept at −20°C. Wherever
applicable, cells were seeded at 2 × 105 cells/10-cm dish in GM, and
ZnCl2 (final concentration, 100 μM) was added 3 h later.

Construction of Plasmids

The Bluescript KS plasmid harboring cDNA encoding human PKR (2.6 kb)
was kindly supplied by B. R. G. Williams (Cleveland Clinic Foundation,
Cleveland, OH).

The cDNA fragment was excised from the vector by HindIII. This
fragment was then subcloned in the HindIII site of Bluescript SK, resulting
in two possible orientations. To distinguish between the two constructs,
several plasmid preparations were digested with Sall and XbaI. Ligation
in the sense orientation was obtained when the resulting fragments were
0.4- and 5-kb long. In the final stage, one of these plasmids (pBS-SK-PKR)
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4 Unpublished results.

5 S. Salzberg et al., manuscript in preparation.
of 1:10, and G418 (Calbiochem-Novabiochem Corp., La Jolla, CA) (800 μg/ml) was added 24 h later. After an additional 14 days, most of the cells died, and single colonies were visible. About 30 clones were removed by trypsin-EDTA solution, resuspended in GM with G418, and expanded. For a negative control, C2C12 cells were transfected with 1 μg of pSVneo only. Eight clones were similarly isolated. A representative clone, C2-NEO, was used throughout this study.

Cell Extracts

Cytoplastmic (S10) Extracts. Cell extracts were prepared after the appropriate treatment by removing the cultured cells with a rubber policeman in PBS. The cells were then centrifuged at 800 × g and resuspended in an ice-cold lysis buffer containing 20 mM HEPES (pH 7.5), 5 mM magnesium acetate, 2.5% NP40, and 1 mM DTT. The extracts were centrifuged at 10,000 × g for 10 min, and the soluble fractions (S10) were stored at −70°C until use. These extracts were used for determination of PKR enzymatic activity.

Total Extracts. Cells were washed twice in cold PBS and centrifuged at 800 × g for 10 min, and the pellets were thawed in 4 volumes of buffer W containing 10 mM HEPES (pH 7.9), 0.4 M NaCl, 0.1 mM EDTA, 1 mM DTT, 5% (v/v) glycerol, 0.5 μM phenylmethylsulfonyl fluoride, 50 mM NaF, 0.1 mM sodium vanadate, 10 mM sodium molybdate, 100 μg/ml leupeptin, 4 μg/ml aprotinin, 2 μg/ml chymostatin, 1.5 μg/ml peptatin, and 2 μg/ml antipain. After repeated pipetting, the lysates were centrifuged at 10,000 × g for 20 min, and the supernatant was frozen in liquid N2. These extracts were used for the identification of the following proteins by immunoblot analysis: PKR; troponin T; myogenin; myoD; c-myc; and pRb.

Nuclear Extract. Frozen pellets were prepared as described for total extracts, thawed in buffer W with 10 mM NaCl only, and centrifuged at 10,000 × g for 20 min. The nuclear pellet was resuspended in original buffer W and centrifuged again. The supernatant was collected and kept in liquid nitrogen. These extracts were used for the determination of cyclin D1 by immunoblot analysis.

Determination of PKR Activity

Cell extracts (S10) were prepared as described above. Heparin (50–100 units/ml) was added to samples containing 500 μg of protein each. The mixtures were incubated at 4°C for 10 min. An equal volume of poly(I):poly(C)-Sepharose beads was added at room temperature for 30 min, with occasional gentle mixing. The beads were washed several times with buffer B [50 mM KCl, 2 mM magnesium acetate, 7 mM 2-mercaptoethanol,
Involvement of PKR in Myogenesis

Protein Analysis by Immunoblotting

Total or nuclear cell extracts were prepared as described above. Samples (20 μg) were loaded on polyacrylamide-SDS gel and analyzed by immunoblotting. We used the rainbow-colored proteins as a molecular weight marker (Amersham International).

Electrophoresis was carried out at 200 V for 1 h at 4°C. Transfer to nitrocellulose sheets was performed in the mini-trans-blot cell (Bio-Rad Laboratories) at 4°C in a buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol for 1 h at 200 mA. The nitrocellulose sheet was immersed in blocking solution containing 10 μg/ml Tris (pH 7.5), 100 mM NaCl, 0.1% Tween 20, 5% FCS, and 3% nonfat milk in PBS for 1 h at room temperature. It was then transferred to a blocking solution supplemented with the following preparation of antibodies: polyclonal antibodies directed against human PKR (dilution, 1:2000, supplied by Dr. A. Vojdani, Immunosciences Laboratory, Inc., Beverly Hills, CA); anti-c-myc monoclonal antibodies (AB-3; dilution, 1:50, Calbiochem-Novabiochem Corp.); anti-Rb monoclonal antibodies (33-245; dilution, 1:250; PharMingen, San Diego, CA); anti-myod (SC-760; dilution, 1:400; Santa Cruz Biotechnology, Santa Cruz, CA); anti-myogenin (SC-576; dilution, 1:400; Santa Cruz Biotechnology); anti-erbB2 (G-9; dilution, 1:2000; Sigma); anti-cyclin D1 (SC-6281; dilution, 1:400; Santa Cruz Biotechnology); and anti-p21WAF1 (SC-6246; dilution, 1:250; Santa Cruz Biotechnology). The mixture was incubated overnight at 4°C and then washed three times with a solution containing 10 μg/ml Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 in PBS. As a secondary detection antibody, we used peroxidase-labeled antitmouse or antirabbit antibodies (Jackson ImmunoResearch; West Grove, PA), and detection was performed by the enhanced chemiluminescence Western blotting procedure as described by the supplier (Amersham International). Light emission was detected by a 2-min exposure to Fuji RX film. Densitometry was determined by the Scion-Image program.

Determination of Specific RNA Transcripts

For each treatment, three 10-cm tissue culture dishes were used. Total RNA was extracted with Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the protocol supplied by the manufacturer. Samples containing 30 μg of RNA were analyzed on 1% agarose gels in running buffer containing formaldehyde, followed by blotting onto nitrocellulose membrane filters (NitroPlus; MSL, Westboro, MA), as described previously (52). The ethidium bromide-stained 18S and 28S bands of cellulose membrane filters (NitroPlus; MSI, Westboro, MA), as described previously (52). The ethidium bromide-stained 18S and 28S bands of cellulose membrane filters (NitroPlus; MSI, Westboro, MA), as described previously (52). The ethidium bromide-stained 18S and 28S bands of

Probes

For the detection of myoD-specific transcripts, a 1.8-kb fragment excised with EcoRI from plasmid pEMC115 was used. This plasmid, generously provided by J. Pierce (Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD) harbors the murine myoD-encoding sequences. The probes were labeled with [α-32P]CTP (specific activity, 3000 Ci/mmol; Amersham) using the rapid multiprime DNA labeling kit as recommended by the supplier (RAN. 1601, Amersham). The specific activity was 2–8 × 10^6 cpm/μg.

Determination of Growth Characterization

In the case of wild-type C2C12 cells, 1 × 10^5 cells/5-cm tissue culture plate were seeded in GM. One day later, the medium was replaced with either GM (for dividing cells) or DM (for differentiating cells). This was considered zero time. With the transfected clones, the cells were seeded at 1 × 10^5 cells/5-cm tissue culture plate in GM, and some of the cultures were treated 3 h later with ZnCl2 (zero time). In all cases, the determination of the growth rate or thymidine incorporation was performed as described below.

At the appropriate times, groups of three plates/point were collected, the medium was removed, the plates were washed twice with PBS, and the cells were collected with trypsin-EDTA, resuspended in PBS, centrifuged, and resuspended again in 0.4% trypan blue in PBS (Sigma). Vital cells were counted in a hemocytometer, using five different fields/count. The SD of the all the counts per time (total, 15 counts) was determined.

Thymidine Incorporation

Cultures were prepared as described above. At the indicated times, the medium was removed from the plate and replaced with fresh medium containing 1 μCi/ml [3H]thymidine (Amersham International) for 1.5 h. The cultures were then washed three times with cold PBS. The cells were lysed with 1% SDS for 10 min at 37°C, and the lysates were moved to test tubes. An equal volume of 20% trichloroacetic acid was added, and the tubes were kept on ice for 20 min. The samples were then filtered through Whatman 25 mm GF/c filters (supplied by Tamar, Ltd., Jerusalem, Israel). The filters were dried, placed in tolue-ene-based scintillation fluid, and counted in Packard 1600 TR liquid analyzer. Each point represents the average of three different measurements.

Determination of Creatine Kinase Activity

Cultures were washed with CaCl2− and MgCl2−free PBS and homogenized in 0.1utm sulfosodium phosphate buffer (pH 7.0) supplemented with 0.1% Triton X-100. The enzymatic activity was determined as described by Shainberg et al. (53). The ATP formed by the interaction of ADP with creatine phosphate phosphorylates glucose in the presence of hexokinase, yielding glucose-6 phosphate. The latter reduces NADP to NADPH, which is determined by recording the absorption at 340 nm.

References


