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Induction of Terminal Differentiation by the c-Jun Dimerization Protein JDP2 in C2 Myoblasts and Rhabdomyosarcoma Cells*

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Muscle cell differentiation is a result of a complex interplay between transcription factors and cell signaling proteins. Proliferating myoblasts must exit from the cell cycle prior to their differentiation. The muscle regulatory factor and myocyte enhancer factor-2 protein families play a major role in promoting muscle cell differentiation. Conversely, members of the AP-1 family of transcription factors that promote cell proliferation antagonize muscle cell differentiation. Here we tested the role of the c-Jun dimerization protein JDP2 in muscle cell differentiation. Endogenous expression of JDP2 was induced in both C2C12 myoblast and rhabdomyosarcoma (RD) cells programmed to differentiate. Ectopic expression of JDP2 in C2C12 myoblast cells inhibited cell cycle progression and induced spontaneous muscle cell differentiation. Likewise, constitutive expression of JDP2 in RD cells reduced their tumorigenic characteristics and restored their ability to differentiate into myotubes. JDP2 potentiated and synergized with 12-*O*-tetradecanoylphorbol-13-acetate to induce muscle cell differentiation of RD cells. In addition, JDP2 induced p38 activity in both C2 and RD cells programmed to differentiate. This is the first demonstration of a single transcription factor that rescues the myogenic program in an otherwise non-differentiating cancer cell line. Our results indicate that the JDP2 protein plays a major role in promoting skeletal muscle differentiation via its involvement in cell cycle arrest and activation of the myogenic program.

Skeletal muscle differentiation is characterized by terminal withdrawal from the cell cycle, muscle-specific gene activation, and subsequent fusion of myoblasts into multinucleated myotubes. The basic helix-loop-helix proteins MyoD and Myf5 are key factors that, upon activation, induce the expression of myogenin and myocyte enhancer factor-2 proteins, which subsequently play a major role in the terminal differentiation step (1). On the other hand, the disruption of cell growth regulatory pathways in muscle precursor cells results in solid tumors (known as rhabdomyosarcoma) that fail to withdraw from the cell cycle and are unable to differentiate into muscle cells, despite the expression of the muscle regulatory protein MyoD (2, 3). Multiple lesions were reported to be responsible for the inability of these cells to undergo muscle differentiation (3–5). Activation of several signaling pathways such as the p38 MAPK[1] pathway (6), suramin (7), and TPA (8, 9) was observed to partially rescue the differentiation phenotype; yet the precise molecular mechanism that promotes rhabdomyosarcoma differentiation is not fully understood. Transcription factors that promote cell proliferation such as those from the basic leucine zipper (bZIP) family and the AP-1 family (10) are potent inhibitors of muscle cell differentiation. Ectopic expression of c-Jun, v-Jun, and JunB in myogenic cells inhibits muscle differentiation (11, 12). The antagonism between MyoD and Jun proteins may be related to the competition of these proteins for a limited amount of an essential coactivator. Indeed, following the onset of muscle cell differentiation, c-Jun and JunB levels decrease (13), whereas JunD remains constant (1). Recently, we isolated a cDNA encoding an 18-kDa protein, a member of the bZIP family of transcription factors designated JDP2 (14). JDP2 was isolated based on its ability to bind c-Jun and was shown to bind activating transcription factor-2 (15), CAAT/enhancer-binding protein-γ (16), and the progesterone receptor (17). The JDP2 protein is constitutively expressed in multiple cell lines tested and represses AP-1 transcriptional activity (14). Recently, JDP2 was shown to inhibit retinoic acid-induced differentiation of F9 teratocarcinoma cells by recruitment of the histone deacetylase-3 complex to the c-*jun* promoter (18). JDP2 binds both CRE and TRE DNA elements as a homodimer and as a heterodimer with activating transcription factor-2 and Jun family members, respectively (14, 15). JDP2 expression levels remain constant following a wide range of stress stimuli, but JDP2 is phosphorylated at threonine 148 by JNK (19). Here we show that the expression of endogenous JDP2 was induced during the differentiation of C2 myoblasts. Ectopic expression of JDP2 in myoblasts and rhabdomyosarcoma tumor cells strongly promoted muscle cell differentiation. JDP2 efficiently induced the spontaneous differentiation of C2 cells growing in high serum-containing medium and accelerated the differentiation of myoblasts growing in serum-free medium. Moreover, JDP2 expression in rhabdomyosarcoma cells caused a partial cell cycle arrest, induction of myogenic markers, and partial fusion of these tumor-derived cells. We suggest that JDP2 is actively involved in down-regulation of cell cycle progression and induces muscle cell differentiation. This is the first dem-

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; CRE, cAMP response element; TRE, 12-*O*-tetradecanoylphorbol-13-acetate response element; JNK, c-Jun N-terminal kinase; CREB, cAMP response element-binding protein; MHC, myosin heavy chain; BrdUrd, bromodeoxyuridine; GFP, green fluorescent protein; RD, rhabdomyosarcoma; GM, growth medium; DM, differentiation medium; CMV, cytomegalovirus; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinase.

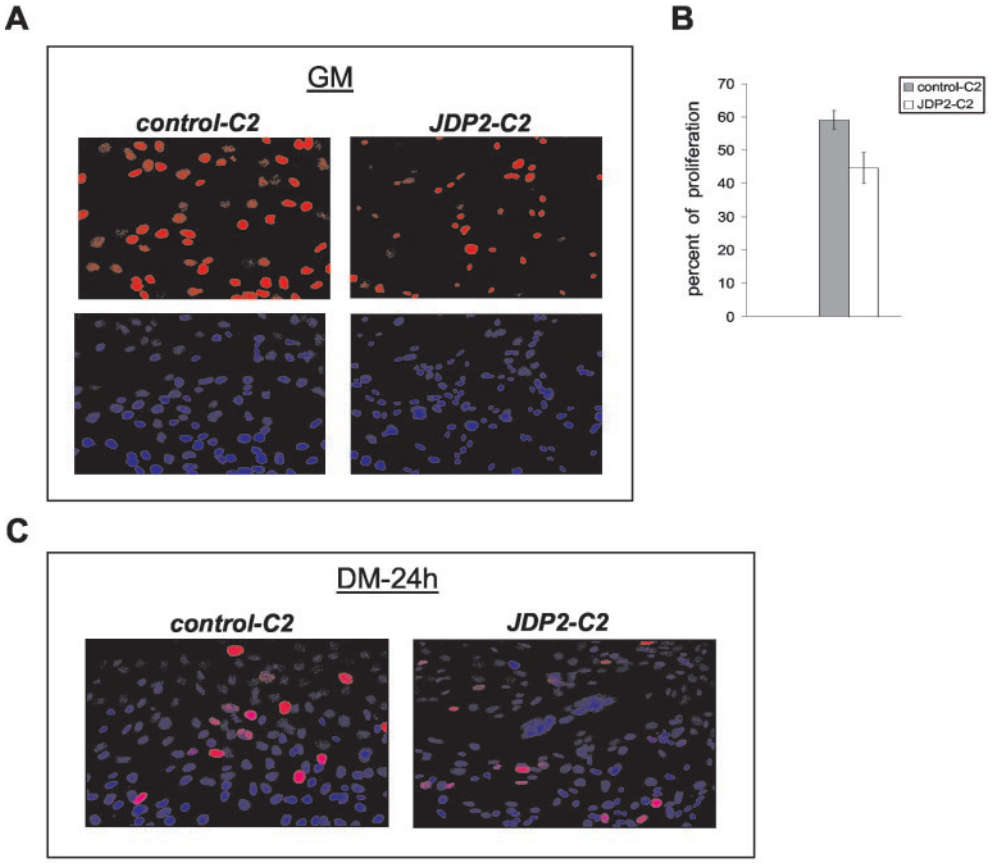

FIG. 1. **JDP2-dependent suppression of DNA synthesis in C2 cells.** *A*, DNA synthesis was determined following incubation of cells with BrdUrd for 3 h. Control-C2 and JDP2-C2 cells were grown in GM, followed by fixation and anti-BrdUrd immunostaining (*upper panels*, *red* staining). Cell nuclei were visualized by DAPI (*lower panels*, *blue* staining). Representative stained fields are shown. *B*, BrdUrd-labeled cells were counted relative to the number of nuclei. The results presented were derived from at least 250 counted cells and represent the mean ± S.E. of three independent experiments. *C*, cells were treated as described for *A*, except that cells were grown in DM for 24 h prior to incubation with BrdUrd and cell staining. Representative co-stained fields are shown.

onstration of a single transcription factor that promotes muscle cell differentiation of a rhabdomyosarcoma-derived tumor cell line.

EXPERIMENTAL PROCEDURES

Materials—TPA (Sigma P-8139) was dissolved in Me_2SO to a concentration of 3 mM and was added directly to the differentiation medium to a final concentration of 100 nM. Insulin and transferrin were purchased from Sigma. SB202190 was purchased from Calbiochem.

Antibodies—Anti-JDP2 polyclonal antibody has been described previously (14). Anti-p38 (catalog no. 9212), anti-phospho-p38 (catalog no. 9211), and anti-CREB (catalog no. 9192) antibodies were purchased from Cell Signaling Technology Inc. Anti-MHC monoclonal antibody MF-20 was a gift from Dr. S. J. Tapscott. Anti-BrdUrd monoclonal antibody (catalog no. BMC9318) was purchased from Roche Molecular Biochemicals. Anti-p21 antibody was obtained from Transduction Laboratories. Anti-cyclin D1 monoclonal antibody A-12 (catalog no. sc-304), anti-c-Jun polyclonal antibody (catalog no. sc-1698), and anti-myogenin antibody F5D (catalog no. sc-12732) were purchased from Santa Cruz Biotechnology. Anti-MyoD1 monoclonal antibody (catalog no. 5.8A) was purchased from Castra Novo, and anti-GFP antibody (catalog no. A-6455) was supplied by Molecular Probes, Inc.

Plasmids—Myogenin-luciferase was a generous gift of Dr. S. J. Tapscott. p21-luciferase was as described (20). Cyclin D1-luciferase was a generous gift of Dr. R. G. Pestell. The full-length *jdp2* cDNA was inserted into the pCLBabe retroviral expression plasmid (21) by *Eco*RI digestion.

Generation of Stable JDP2-expressing Cells—C2 and RD myoblast cells were infected with replication-defective retroviruses to generate C2 cell lines overexpressing JDP2. Retroviruses expressing the different proteins were generated by cotransfection of retroviral expression vectors with an expression vector encoding the vesicular stomatitis virus glycoprotein gene into the viral packaging cell line 293gp, expressing the *gag* and *pol* genes (21). The medium of transfected 293gp cells containing retroviruses was used to infect C2 or RD cells. Infected cells were selected with puromycin (3 μg/ml), and resistant clones were pooled together 1 week later. Cells were kept in culture for no more than 3 weeks.

Cell Culture—Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 15% calf serum (Hyclone Laboratories), penicillin, and streptomycin (growth medium (GM)). To induce differentiation, cells were grown in Dulbecco's modified Eagle's medium containing 10 μg/ml insulin and 10 μg/ml transferrin (differentiation medium (DM)). C2 cells were a gift of Dr. David Yaffe (22).

Transient Transfection Assays—Transfections were performed by calcium phosphate precipitation for 293gp cells or using X-tremeGENE transfection reagent (10 μl/60-mm plate; Roche Molecular Biochemicals) according to the manufacturer's instructions for C2 cell lines. Myoblast cells were transfected with 3 μg of total plasmid DNA mixture composed of 2 μg of the corresponding luciferase reporter plasmid and 1 μg of the transfection control CMV-β-galactosidase reporter plasmid. Following transfection, the medium was replaced with DM or GM for 24–48 h. Total protein lysates were prepared and used to measure luciferase activities using the Promega luciferase assay system. CMV-β-galactosidase was used to normalize differences in transfection efficiency between plates. The luciferase activity obtained in each transfection was divided by the β-galactosidase activity obtained in each transfection.

Immunohistochemical Staining—Cells were fixed and immunostained as described (23). The primary antibody used was anti-MHC monoclonal antibody MF-20. The immunochemically stained cells were visualized using an Olympus Model BX50 fluorescence microscope at a magnification of ×200 and were photographed with a digital camera.

Western Blot Analysis—Cells were lysed, and whole cell extracts were collected as described (23). Nuclear extract was prepared as described (19). Equal amounts of extracted proteins (50 μg) were loaded, separated by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were incubated in blocking buffer containing 20 mM

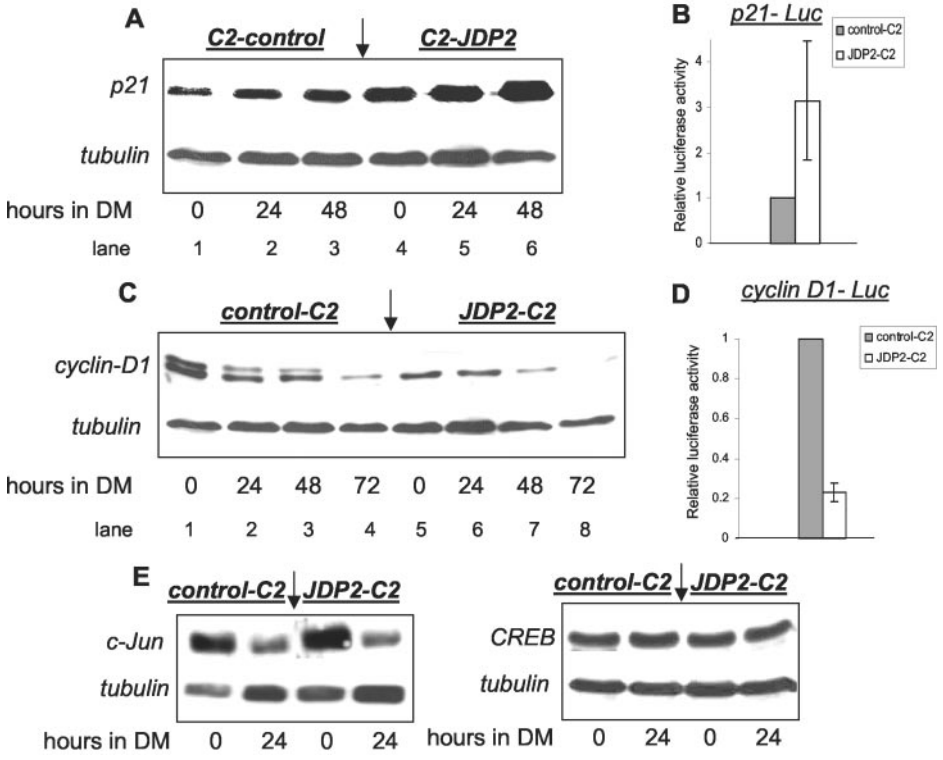

FIG. 2. **JDP2-dependent inhibition of cell cycle regulatory markers in C2 cells.** Whole cell extract (50 μg) (*A* and *C*) or nuclear extract (*E*) was derived from either JDP2-C2 or control-C2 cells grown in GM or for the indicated times in DM, separated by SDS-PAGE, and analyzed by Western blotting. The levels of $p21^{waf1}$ (*A*), cyclin D1 (*C*), CREB (*E*, *right panel*), and c-Jun (*E*, *left panel*) were determined with the appropriate antibodies. The level of tubulin served as a loading control using anti-tubulin antibody. In *B* and *D*, JDP2-C2 and control-C2 cells were co-transfected with p21-luciferase (*Luc*) and cyclin D1-luciferase reporter genes, respectively, together with the CMV-β-galactosidase reporter gene as described under "Experimental Procedures." Following transfection, cells were grown in GM for 24–48 h. Transfected cells were harvested, and the β-galactosidase levels were determined and used to normalize the luciferase activity obtained. The resulting normalized activity of control-C2 cells was set as 1, and the activity obtained with JDP2-C2 cells was calculated accordingly. The results represent the mean ± S.E. of three independent experiments.

Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20, and 2% (w/v) nonfat dry milk. All incubations and washes were performed with blocking buffer. Primary antibody incubations were performed at 1:1000 dilution, unless otherwise indicated. The following antibodies were used: anti-JDP2 (1:1000 dilution), anti-c-Jun (1:1000), anti-myogenin (1:500), anti-cyclin D1 (1:500), anti-p21 (1:500), anti-phospho-p38 (1:500), anti-p38 (1:1000), anti-MHC (1:2.5), anti-CREB (1:200), and anti-tubulin (1:1000). Proteins were visualized using enhanced chemiluminescence reagent (Pierce) according to the manufacturer's instructions.

BrdUrd Staining—BrdUrd staining was performed with cells grown on coverslips as previously described (23). In principle, BrdUrd was added to cell media at 10 μM for 2–3 h. The cells were then washed with PBS, fixed with methanol (10 min), and permeabilized in 0.25% Triton X-100 (10 min). Following a wash with PBS, the cells were incubated in 2 N HCl solution for 1 h, followed by a neutralization step composed of washing the cells three times with 0.1 M borate buffer (pH 8.5). Subsequently, the cells were incubated with 6 mg/ml anti-BrdUrd antibody in PBS containing 0.1% bovine serum albumin for 1.5 h.

GFP and BrdUrd Co-staining—Staining was performed as described above, but prior to the acid incubation step, cells were incubated with rabbit anti-GFP polyclonal antibody in PBS containing 0.1% bovine serum albumin for 1.5 h. Following three washes with PBS, the cells were incubated for 1.5 h with fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). Subsequently, cells ware fixed with 2% paraformaldehyde (10 min), and after extensive washing with PBS, staining was performed as described above.

RESULTS

JDP2 Reduces Cell Proliferation of C2 Cells—The AP-1 transcription factor plays a major role in mediating cell response to growth stimulation (10). Because JDP2 was shown to repress AP-1 activity (14, 15), we tested the ability of JDP2 to inhibit cell proliferation. C2 myoblast cells were infected either with retroviruses encoding the JDP2 protein (JDP2-C2 cells) or with a noncoding control retrovirus (control-C2 cells). Puromycin-resistant cells were selected, and their proliferation profile was measured using BrdUrd incorporation assay. The number of BrdUrd-incorporating nuclei was calculated relative to the total number of DAPI-stained nuclei. Fewer JDP2-C2 cells in S phase were observed relative to control-C2 cells growing in GM (45% *versus* 60%, respectively) (Fig. 1, *A* and *B*). Interestingly, the nuclei of the JDP2-expressing cells were of smaller dimensions relative to the those of the control-C2 cells (Fig. 1*A*). As expected, following transfer of C2 cells to DM for 24 h, the number of cells in S phase was reduced dramatically (25–30%) (Fig. 1*C*, *left panel*), indicating their withdrawal from the cell cycle. In JDP2-C2 cells, a smaller fraction of nuclei were stained, and their staining was significantly less intense compared with the nuclei of control-C2 cells (Fig. 1*C*, *right panel*), indicating a slower cell cycle progression. To investigate how JDP2 may inhibit cell proliferation, we examined the expression levels of two major cell cycle regulatory markers, the cyclin-dependent kinase inhibitor p21 and cyclin D1, by Western blot analysis (Fig. 2, *A* and *C*) and reporter gene assay (Fig. 2, *B* and *D*). Control-C2 cells grown in GM exhibited lower p21 levels compared with JDP2-C2 cells (Fig. 2*A*, compare *lanes 1* and *4*). Following transfer of cells to DM, p21 expression levels gradually increased in control-C2 cells with time of incubation, whereas the already high p21 levels in JDP2-C2 cells increased only modestly (Fig. 2*A*, compare *lanes 2–3* and *5–6*). To analyze whether this effect is mediated at the transcriptional level, we transfected the stable cell lines with the p21-luciferase reporter plasmid and assessed the luciferase activity of cells growing in GM (Fig. 2*B*). The expression of the p21-luciferase reporter gene was significantly higher in the JDP2-C2 cells relative to the control-C2 cells (Fig. 2*B*), thus suggesting that JDP2 caused an increase in p21 transcription. A similar analysis was performed for cyclin D1; however, whereas control-C2 cells expressed high levels of cyclin D1 protein when grown in GM, JDP2-C2 cells expressed significantly reduced levels of cyclin D1 (Fig. 2*C*, compare *lanes 1* and *5*). Following transfer of cells to DM, cyclin D1 levels declined in both cell lines, yet its levels were always lower in JDP2-expressing cells relative to control cells at each time point in DM (Fig. 2*C*, compare *lanes 2–4* and *lanes 6–8*). To examine whether JDP2 affected cyclin D1 transcription, we measured the expression of the luciferase reporter gene regulated by the cyclin D1 promoter elements. JDP2-C2 and control-C2 cells were transiently transfected with the cyclin D1-luciferase reporter plasmid and grown for 24–48 h in

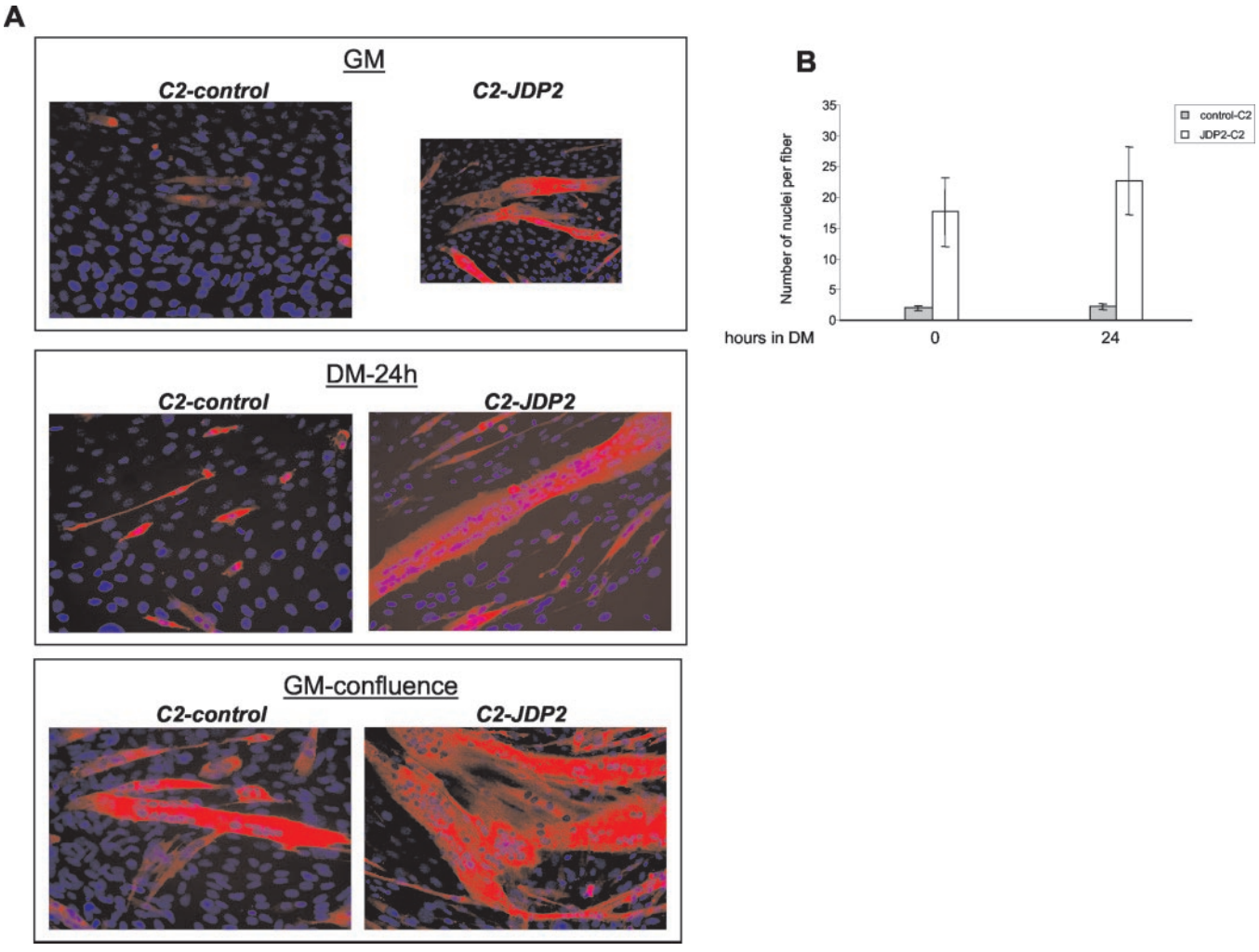

FIG. 3. **JDP2 promotes induction of C2 myotube formation and strong expression of major myogenic markers.** *A*, JDP2-C2 and control-C2 cells were grown in GM (*upper panels*), DM for 24 h (*middle panels*), or GM for 72–96 h (*lower panels*); fixed; and stained with anti-MHC antibody (*red* staining). DAPI staining was used to visualize cell nuclei (*blue* staining). Representative co-stained fields are shown. *B*, the number of DAPI-stained nuclei present in MHC-positive cells was determined. The results represent the mean ± S.E. of three independent experiments.

GM. The expression of cyclin D1-luciferase was significantly lower in JDP2-C2 cells relative to control-C2 cells (Fig. 2*D*). These results suggest that JDP2 interferes with cell cycle progression by modulating the expression of two key regulators, cyclin D1 and p21. The cyclin D1 promoter is regulated by both AP-1 and CRE DNA elements (24, 25) found in its promoter and their corresponding DNA-binding transcription factors c-Jun and CREB. Because JDP2 is able to bind both CRE and TRE DNA elements (14), we decided to test whether JDP2 effects on the cyclin D1 promoter are direct or through a decrease in c-Jun and/or CREB protein levels. We performed Western blot analysis on stable C2 cell lines with anti-CREB and anti-c-Jun antibodies. No differences in CREB and c-Jun protein expression levels were observed in control-C2 and JDP2-C2 cells (Fig. 2*E*). This result suggests that cyclin D1 inhibition may represent a direct effect mediated by JDP2.

Collectively, these data indicate that JDP2 inhibits cell cycle progression at least in part by reducing the expression levels of cyclin D1 and increasing the levels of the p21 cyclin-dependent kinase inhibitor. These two opposite effects are mediated at least in part at the transcriptional level, coordinately resulting in down-regulation of cell cycle progression.

JDP2 Induces Terminal Muscle Cell Differentiation in C2 Myoblasts—The fact that JDP2 expression reduced cell cycle progression prompted us to test the ability of JDP2-C2 cells to induce muscle cell differentiation. JDP2-C2 and control-C2 cells were grown in GM at subconfluent density, followed by fixation and staining with anti-MHC antibody. Even under these growth conditions, we observed that JDP2-C2 cells formed multiple multinucleated myotubes consisting of 5–17 nuclei/myotube that stained positively for MHC (Fig. 3, *A*, *upper right panel*; and *B*). Control-C2 cells grown under identical conditions formed only a small number of multinucleated myotube cells consisting of one to three nuclei/myotube. These differences were even more pronounced after 24 h of growth in DM (Fig. 3, *A*, *middle panels*; and *B*). The number of nuclei/myotube was ~10-fold higher in JDP2-C2 expressing cells than in control-C2 cells (Fig. 3*B*). In addition, myotubes formed in JDP2-C2 cells exhibited an elongated morphology and were significantly larger in size. Control-C2 cells were able to spontaneously differentiate in GM when grown to high density for 72–96 h, resulting in hypertrophic multinucleated myotubes (Fig. 3*A*, *lower left panel*). JDP2-C2 cells grown at identical density in GM formed multi-giant hypertrophic myotubes that bundled into large skeletal fibers (Fig. 3*A*, *lower right panel*).

To examine the myogenic activity induced by JDP2, we transiently transfected JDP2-C2 and control-C2 cells with a luciferase reporter plasmid placed under the control of the promoter of the early myogenic marker myogenin (myogenin-luciferase). The myogenin reporter activity in JDP2-C2 cells was ~10-fold

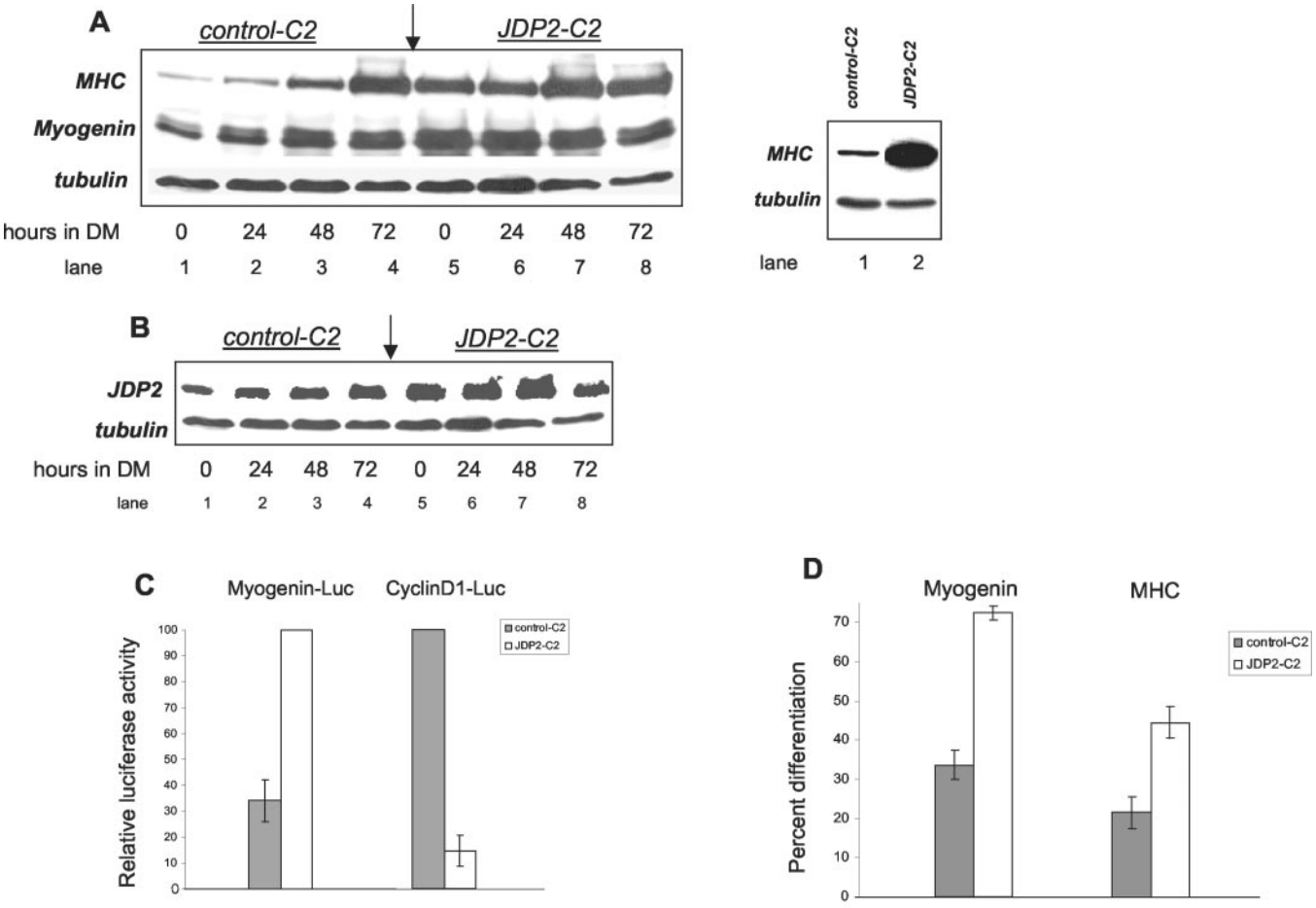

FIG. 4. **JDP2 induces the expression of myogenic markers.** *A* and *B*, whole cell extract (50 μg) derived from either subconfluent (*A*, *left panel*; and *B*) or confluent (*A*, *right panel*) JDP2-C2 and control-C2 cells grown in GM and for the indicated times in DM was separated by SDS-PAGE and analyzed by Western blotting. The levels of MHC and myogenin (*A*) and JDP2 (*B*) were determined with the appropriate antibodies as indicated. The level of tubulin served as a loading control using anti-tubulin antibody. *C*, C2 cells were cotransfected with a luciferase reporter plasmid under the control of either the myogenin-luciferase (*Luc*) or cyclin D1 promoter together with either control or JDP2 expression plasmids. The CMV-β-galactosidase reporter plasmid served to normalize transfection efficiency as described in the legend to Fig. 2*B*. Following transfection, cells were grown in DM for 24 h, and luciferase and β-galactosidase activities were determined. The results obtained with myogenin-luciferase and the JDP2 expression plasmid were set as 100%, and the vector control activity was calculated accordingly, whereas the results obtained with the cyclin D1 reporter gene and vector control transfection were set as 100%, and JDP2 transfections were calculated accordingly. The results represent the mean ± S.E. of three independent experiments. *D*, C2 cells were transiently transfected with the pBabe-GFP expression plasmid coexpressing either the control or JDP2 protein. Following transfection, cells were transferred to DM for 40 h, fixed, and co-stained for GFP and either myogenin or MHC with appropriate antibodies. The results presented were derived from at least 250 counted cells and represent the mean ± S.E. of three independent experiments.

higher than that in control-C2 cells grown in either GM or DM (data not shown). To study the expression of endogenous early and late muscle-specific markers, we performed Western blot analysis with anti-myogenin and anti-MHC antibodies (Fig. 4*A*). In control-C2 cells, the expression level of the muscle-specific markers was low when cells were grown in GM and gradually increased following their growth in DM. By contrast, in JDP2-C2 cells, myogenin and MHC expression levels were already high in GM and increased only modestly during growth in DM (Fig. 4*A*, *left panel*). JDP2-C2 cells grown in GM at high density, which exhibited the hypertrophic phenotype (Fig. 3*A*), also displayed a marked increase in MHC expression compared with control-C2 cells (Fig. 4*A*, *right panel*). To examine the expression levels of JDP2 during the differentiation of myoblasts, we performed Western blot analysis with anti-JDP2 antibody on nuclear extract derived from differentiated control-C2 and JDP2-C2 cells. JDP2 levels was mildly increased during differentiation in control-C2 cells (Fig. 4*B*). The expression of JDP2 in JDP2-C2 cells was ~3-fold higher than in control-C2 cells grown in GM. This level of JDP2 expression was not significantly changed during differentiation. Together, these data indicate that ectopic expression of JDP2 strongly promotes both morphological as well as biochemical differentiation of C2 myoblasts.

To exclude the possibility that the JDP2 effects were due to clonal selection of the stable cell lines, we examined the ability of JDP2 to induce muscle differentiation of C2 cells in transient transfections assays. Toward this end, C2 cells were cotransfected with either control or JDP2 expression plasmids together with a reporter plasmid under the control of either the myogenin or cyclin D1 promoter (Fig. 4*C*). Consistent with the results in stable cell lines, transient expression of JDP2 increased myogenin reporter activity by 3-fold and reduced cyclin D1 activity by 10-fold. In addition, we transiently transfected JDP2 into C2 cells, followed by cell fixation and staining with either anti-myogenin or anti-MHC antibody (Fig. 4*D*). JDP2-transfected cells exhibited 40 and 27% increases in myogenin and MHC staining, respectively, compared with control plasmid-transfected cells.

JDP2 Restores Muscle Differentiation in Rhabdomyosarcoma Cells—RD cells are tumor cells of muscle origin that, even in the absence of mitogens, fail to withdraw from the cell cycle and

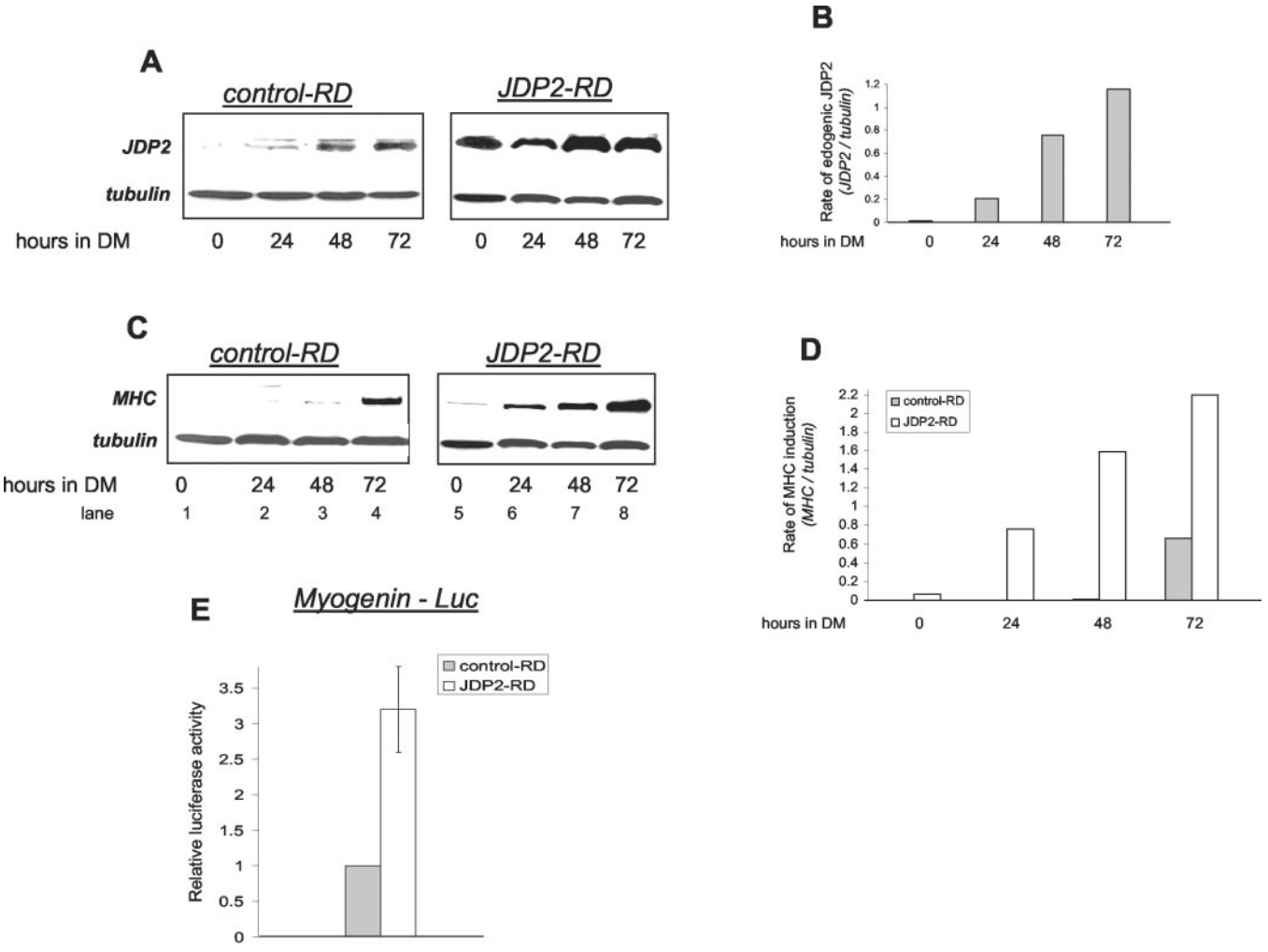

FIG. 5. **JDP2-dependent induction of myogenic markers in RD cells.** *A* and *C*, whole cell extract (50 μg) derived from either control-RD or JDP2-RD cells grown in GM or at the indicated times following transfer to DM was separated by SDS-PAGE and analyzed by Western blotting. The levels of JDP2 (*A*) and MHC (*C*) were determined with the appropriate antibodies as indicated. The level of tubulin served as a loading control using anti-tubulin antibody. *B* and *D*, shown are the results from densitometric analysis of the autoradiograms shown in *A* and *C*, respectively. The expression levels of JDP2 relative to tubulin levels were determined in control-RD cells grown in GM and for the indicated times in DM (*B*). The expression levels of MHC relative to tubulin levels were determined in either control-RD or JDP2-RD cells grown in GM and for the indicated times in DM (*D*). *E*, control-RD and JDP2-RD cells were cotransfected with myogenin-luciferase (*Luc*) and CMV-β-galactosidase reporter genes. Following transfection, cells were grown in DM for 48 h. Transfected cells were harvested, and the β-galactosidase levels were determined and used to normalize the luciferase activity obtained. The resulting normalized activity of control-C2 cells was set as 1, and the activity obtained with JDP2-C2 cells was calculated accordingly. The results represent the mean ± S.E. of three independent experiments.

to undergo terminal differentiation to myotubes. To test the possible role of JDP2 in the rescue of RD cell differentiation, we first analyzed the expression level of JDP2 protein in control-RD cells grown in GM and DM (Fig. 5, *A*, *left panel*, and *B*). The JDP2 protein could hardly be detected in control-RD cells grown in GM; however, its expression was markedly increased following 48- and 72-h growth in DM. The increase in JDP2 was followed by a subsequent increase in the muscle-specific MHC protein marker, which was detected 72 h following growth of control-RD cells in DM (Fig. 5, *C*, *left panel*; and *D*). To test the possibility that the expression of the JDP2 protein may induce further differentiation of these cells, we infected RD cells with JDP2-expressing retroviruses (JDP2-RD cells) and selected for puromycin-resistant cells. The expression of JDP2 in JDP2-RD cells was ~10-fold higher than in control-RD cells (Fig. 5*A*, *right panel*). Surprisingly, JDP2-RD cells expressed detectable levels of MHC even in GM, and its expression was induced significantly during their growth in DM. The expression of MHC in JDP2-RD cells was significantly higher than in control-RD cells at any time point examined (Fig. 5, *C*, compare *lanes 1–4* and *5–8*; and *D*). To compare muscle-specific transcriptional activity of JDP2-RD with control-RD cells, we transiently transfected these cell lines with a myogenin-luciferase reporter plasmid. Luciferase activity was measured 48 h following transfection and growth in DM (Fig. 5*E*). The transcriptional activity of the myogenin reporter gene in JDP2-RD cells was 3.7-fold higher than in control-RD cells (Fig. 5*E*).

The ability of JDP2 to promote the induction of early and late muscle-specific genes prompted us to examine its effect on morphological differentiation of RD cells. Both control-RD and JDP2-RD cell lines grown in DM for 48 and 72 h were fixed and stained with anti-MHC antibody (Fig. 6*A*). Whereas control-RD cells poorly differentiated and rarely fused to form myotubes, JDP2-RD cells formed multinucleated myotubes that were intensely stained with anti-MHC antibody (Fig. 6, *A* and *B*). These results are consistent with the observed increase in MHC protein levels and myogenin promoter transcription (Fig. 5, *C* and *E*). Moreover, the effect of JDP2 expression was even more pronounced in RD cells grown in GM to high density (Fig. 6*C*). Although MHC staining was undetectable in control-RD cells, JDP2-RD cells were differentiated into multinucleated myotubes and intensely stained for MHC. Therefore, ectopic

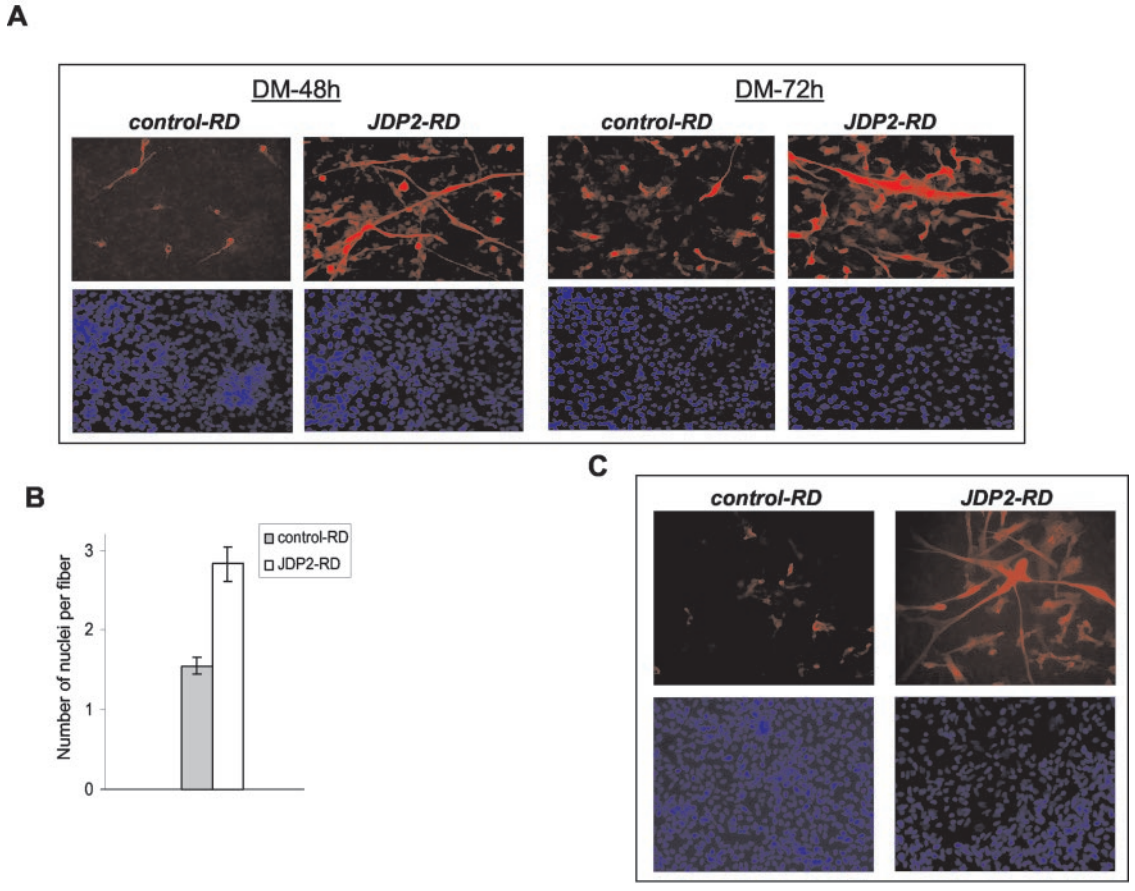

FIG. 6. **JDP2 restores myotube formation in rhabdomyosarcoma cells.** *A*, JDP2-RD and control-RD cells were incubated in DM for either 48 or 72 h, followed by cell fixation and staining with anti-MHC antibody (*upper panels*, *red* staining). DAPI staining was used to visualize cell nuclei (*lower panels*, *blue* staining). Representative stained fields are shown. *B*, the number of DAPI-stained nuclei present in MHC-positive cells was determined. The results represent the mean ± S.E. derived from at least 200 fibers, representing four independent experiments. *C*, RD-JDP2 and control-RD cells were grown at high density for 72–96 h in GM, followed by cell fixation and staining with anti-MHC antibody (*upper panels*, *red* staining). DAPI staining was used to visualize cell nuclei (*lower panels*, *blue* staining). Representative stained fields are shown.

expression of JDP2 in rhabdomyosarcoma cells induces the expression of muscle-specific genes as well as the fusion of these cells into multinucleated myotubes.

JDP2 Inhibits Rhabdomyosarcoma Cell Growth—To test the effect of JDP2 expression on cell cycle progression, we used a transient transfection assay with a JDP2 expression vector cotransfected with a GFP expression plasmid. Transfected cells were grown in DM for 48 h, followed by labeling with BrdUrd to detect cells in S phase of the cell cycle (Fig. 7*A*). Only a marginal fraction of cells transfected with the JDP2 expression vector also stained for BrdUrd (3%), whereas a significant portion of cells (43%) transfected with a control empty vector were also BrdUrd-stained (Fig. 7, *A* and *B*). Most of the nuclei of JDP2-transfected cells that did not stain for BrdUrd displayed myotube morphology (Fig. 7*A*). GFP-negative cells in both JDP2- and control vector-transfected cells exhibited an identical fraction of BrdUrd-stained cells (40%), suggesting that the reduced BrdUrd staining of JDP2-transfected cells was not a consequence of the transfection itself. The proliferative capacity was also analyzed in stable JDP2-RD cells grown in DM for 24 and 72 h. JDP2-RD cells exhibited 25 and 41% lower BrdUrd staining compared with control-RD cells grown for 24 and 72 h in DM, respectively (Fig. 7, *C* and *D*). Thus, the expression of JDP2 significantly reduced proliferation of RD cells. In addition, whereas control-RD cells grown to high density did not display growth contact inhibition and grew in piles (Fig. 8*A*, *left panel*), JDP2-RD cells displayed a flattened morphology, and their contact inhibitory growth was restored (*right panel*). Thus, JDP2 expression significantly reduced the uncontrolled growth of the RD cells. In addition, JDP2-RD cells grown at high density displayed elongated myotubes even in the presence of mitogens (Fig. 8*A*, *right panel*).

To test whether the reduced cell proliferation of RD cells expressing JDP2 is mediated via inhibition of cyclin D1 expression, we examined the activity of the cyclin D1-luciferase reporter plasmid in RD cells grown in DM for 72 h. The activity obtained in JDP2-RD cells was 70% lower than that in control-RD cells (Fig. 8*B*). Indeed, the results from Western blot analyses using anti-cyclin D1 antibody were consistent with the activity obtained using the cyclin D1-luciferase reporter gene assay (Fig. 8, *C* and *D*). Collectively, these data indicate that ectopic expression of JDP2 in RD cells reduces their proliferation potential, restores contact growth inhibition, and dramatically increases their differentiation to multinucleated myotubes.

JDP2 Synergizes with TPA-induced Differentiation of RD Cells—Prolonged TPA treatment was shown to partially induce RD cell differentiation. We examined JDP2 protein levels in RD cells treated with TPA and observed no change in JDP2 levels (data not shown). To test whether or not JDP2 is able to potentiate the differentiation of RD cells by TPA, we treated JDP2-RD or control-RD cells grown in DM with TPA for 48 h. TPA-treated control-RD cells exhibited only very low MHC staining compared with untreated JDP2-RD cells (Fig. 9*A*). Surprisingly, JDP2-RD cells treated with TPA exhibited significantly stronger MHC staining compared with untreated JDP2-RD cells. In addition, treated JDP2-RD cells displayed thicker myotubes compared with untreated JDP2-RD cells. The

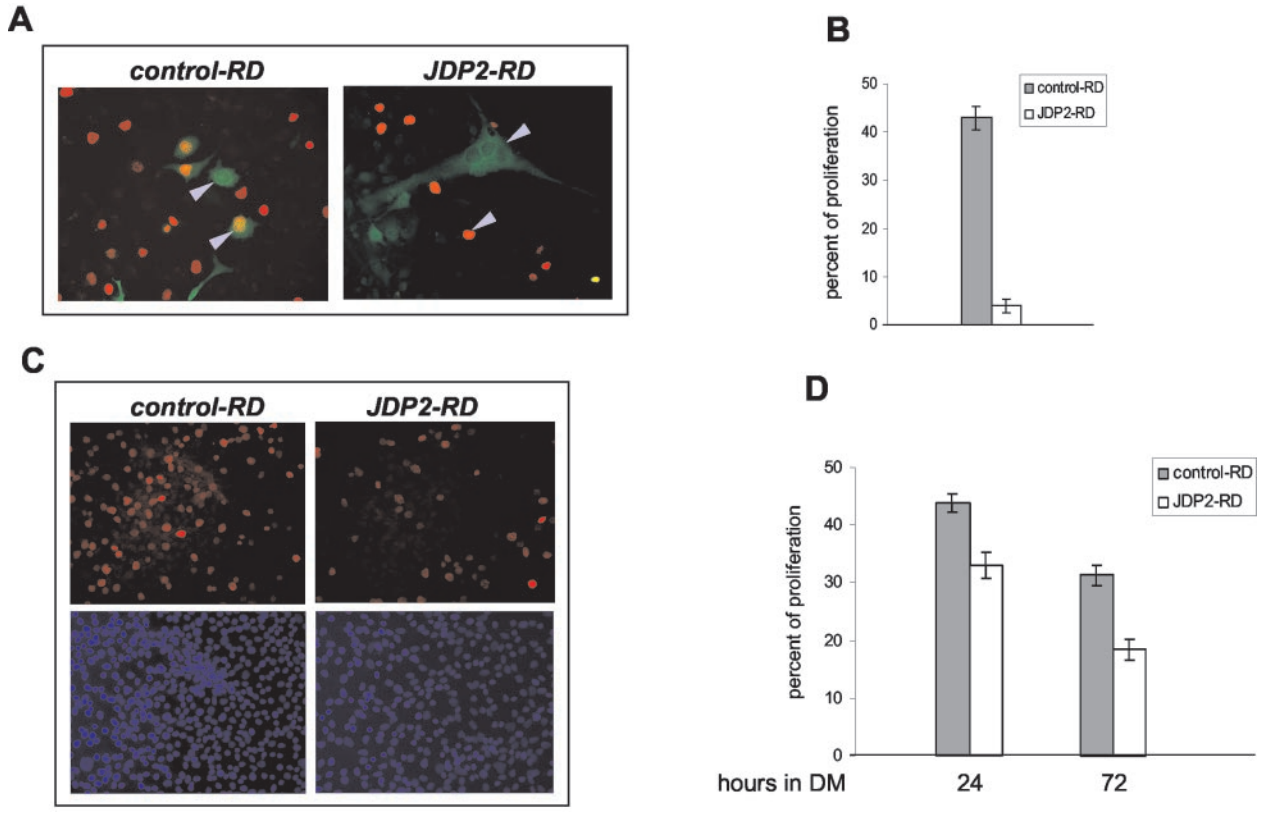

FIG. 7. **JDP2 suppresses cell cycle progression in RD cells.** *A*, rhabdomyosarcoma cells were transiently cotransfected with expression plasmids coexpressing the GFP protein with either the JDP2 protein (JDP2-RD cells) or a control empty expression vector (control-RD cells). Cells were grown in DM for 48 h, followed by exposure to BrdUrd for 3 h prior to cell fixation and immunostaining for BrdUrd (*red* staining) and GFP (*green* staining). Representative co-stained fields are shown. *Arrowheads* indicate BrdUrd-positive (*red*) and GFP-positive (*green*) cells. *B*, GFP-positive cells were counted, and the percentage of BrdUrd-positive cells was determined. The results represent the mean ± S.E. of at least 80–100 of the GFP-positive cells derived from two independent experiments. *C* and *D*, stably transfected JDP2-RD and control-RD cells were stained for BrdUrd incorporation (*upper panels*), and cell nuclei were visualized with DAPI (*lower panels*). Representative stained fields are shown (*C*). The percentage of BrdUrd-incorporating cells was determined (*D*).

levels of MHC as measured by Western blot analysis were consistent with the immunostaining results, suggesting that JDP2 expression significantly enhances TPA-induced differentiation (Fig. 9, *B* and *C*). Nevertheless, JDP2-RD cells treated with TPA failed to enhance the level of cell fusion. These results suggest that TPA and JDP2 possibly function via parallel pathways.

JDP2 Promotes the Activation of the p38 MAPK Cascade in Both RD and C2 Cells—Recently, it was shown that the p38 MAPK cascade is actively involved as an inducer of the myogenic program. p38 activates MyoD activity and directly phosphorylates myocyte enhancer factor-2C, resulting in the induction of their transcriptional activity. Because our findings illustrate that JDP2 promotes muscle cell differentiation in both C2 and RD cell lines, we examined whether the expression of JDP2 affects the activity of the p38 pathway by analyzing p38 phosphorylation by Western blot analysis using anti-phospho-p38 antibody (Fig. 10, *A* and *B*). In both C2 and RD cell lines expressing JDP2, the level of phosphorylated p38 was higher than that in the parental cell lines grown in GM (Fig. 10, *A* and *B*). Phosphorylated p38 levels remained high in the cell lines expressing JDP2 following transfer to DM, whereas phosphorylated p38 was significantly increased in differentiating parental C2 cells, reaching similar levels compared with JDP2-C2 cells grown for 48 h in DM (Fig. 10*A*). Consistent with previous results (6), RD cells failed to induce p38 phosphorylation following transfer to DM. In contrast, in JDP2-RD cells, p38 phosphorylation was significantly elevated (Fig. 10*B*). It was previously shown that the p38 kinase inhibitor SB202190 blocks the formation of MHC-positive myotubes in C2 cells grown in DM (20), thus, we tested the effect of the p38 inhibitor on control-C2 and JDP2-C2 cells. As expected, SB202190 treatment dramatically reduced the staining of MHC-positive cells in control-C2 cells; however, in JDP2-C2 cells, SB202190 treatment did not completely abolish their differentiation phenotype (Fig. 10*C*). Thus, it seems likely that JDP2 expression in differentiated muscle cells specifically activates p38 kinase; however, JDP2 potentiation of muscle cell differentiation acts in a p38-independent manner as well.

DISCUSSION

The muscle-specific transcription factors from the myogenic response factor and myocyte enhancer factor-2 families play a major role in the determination and differentiation processes, respectively. However, it is becoming evident that, although their activity is necessary, multiple signaling circuits are engaged and required to act in concert for the execution of the differentiation program of muscle cells (1, 26). Over the last few years, several signaling cascades have been shown to actively participate in muscle differentiation. For example, the ERK MAPK was shown to have a dual functioning role in cell differentiation (20, 23), whereas the p38 MAPK (20, 27) and phosphatidylinositol 3-kinase (28) signaling cascades were proven to promote differentiation. By contrast, the activity of numerous proteins was shown to inhibit muscle cell differentiation. These include members of the helix-loop-helix protein family Id, Twist, MyoR, Mist-1, oncogenic Ras, and activated Rac (29) and c-Jun, a member of the AP-1 family of transcription factors (reviewed in Ref. 1). The Jun protein that plays a major role in cell proliferation was shown to directly associate

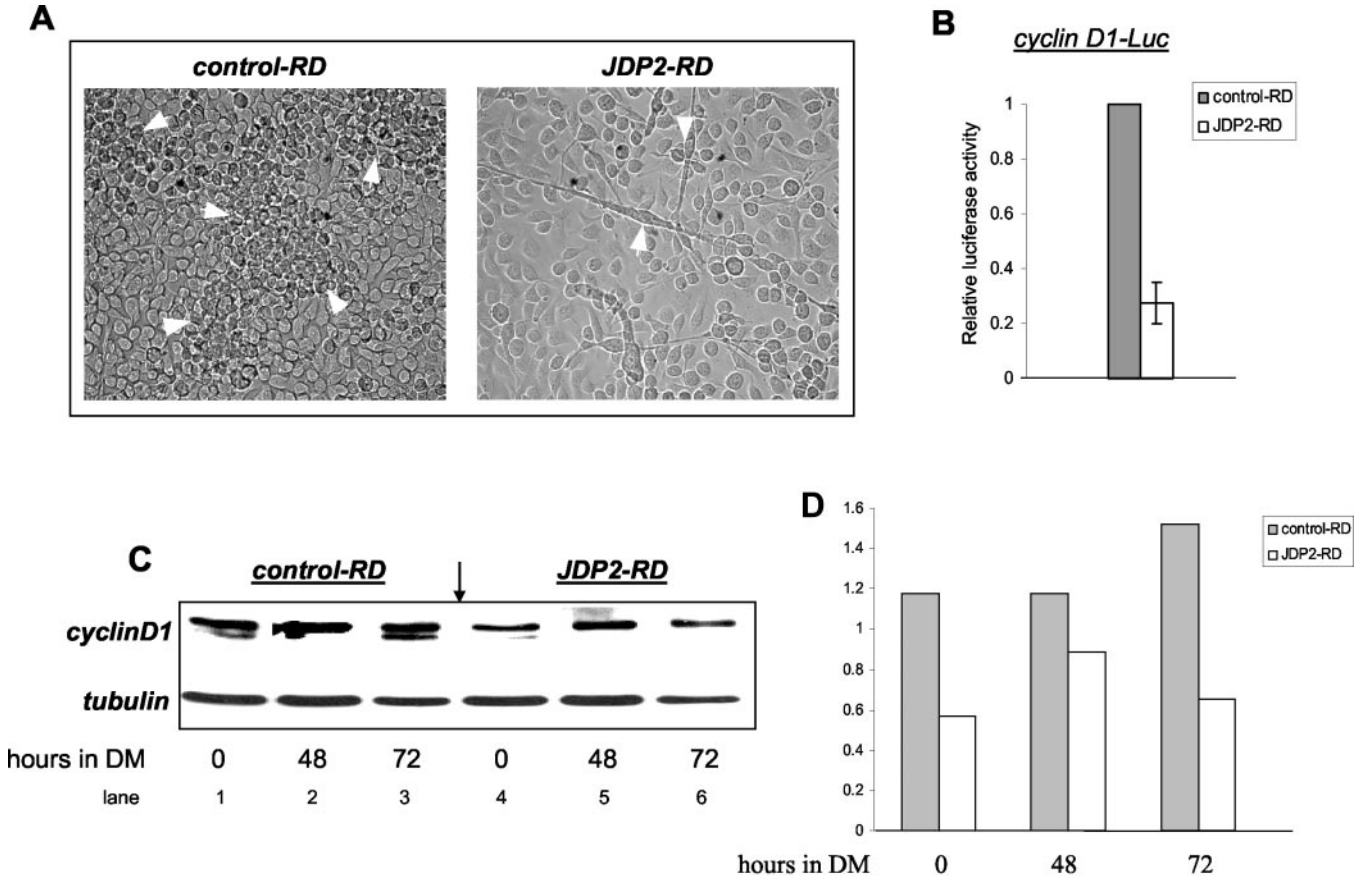

FIG. 8. **JDP2 inhibits tumorigenicity and cell proliferation parameters of RD cells.** *A*, JDP2-RD and control-RD cells were grown to high density in DM for 48 h. Cells were washed with PBS and photographed with a phase microscope at ×20 magnification. Representative fields are shown. *B*, JDP2-RD and control-RD cells were transiently cotransfected with the cyclin D1-luciferase reporter gene together with the CMV-β-galactosidase reporter gene. Following transfection, cells were transferred to DM for 72 h, and β-galactosidase and luciferase activities were determined. β-Galactosidase levels were used to normalize the luciferase activity obtained. The resulting normalized activity of control-C2 cells was set as 1, and the activity obtained with JDP2-C2 cells was calculated accordingly. The results represent the mean ± S.E. of three independent experiments. *C*, whole cell extract (50 μg) derived from either JDP2-RD or control-RD cells grown in GM or for the indicated times in DM was separated by SDS-PAGE and analyzed by Western blotting. The levels of cyclin D1 and tubulin were determined with the appropriate antibodies. *D*, shown are the results from densitometric analyses of the autoradiograms shown in *C*. The expression levels of cyclin D1 relative to tubulin levels were determined in control-RD cells grown in GM and for the indicated times in DM.

and inhibit MyoD activity. In this study, we have shown that the AP-1 repressor protein JDP2 plays a positive role in terminal differentiation of C2 myoblast cells. In addition, constitutive expression of JDP2 restores terminal myogenic differentiation in RD tumor cell lines.

In differentiating C2 cells, the endogenous JDP2 level increased concomitantly with the rise in the early myogenic markers myogenin (Fig. 4*A*) and p21 (Fig. 2*A*) and prior to the late marker MHC (Fig. 4*A*). In RD cells, defective in the differentiation program, the endogenous JDP2 level was very low compared with that in C2 cells (compare Figs. 4*B* and 5*A*). Nevertheless, 48–72 h following serum withdrawal, endogenous JDP2 levels were markedly increased. A rise in JDP2 occurred 24 h prior to the expression of MHC in RD cells. Ectopic expression of JDP2 in C2 cells resulted in only 3-fold higher levels compared with endogenous JDP2 levels in C2 cells grown in GM. In RD cells, exogenous JDP2 levels were >10-fold higher than endogenous JDP2 levels, but similar to endogenous levels in C2 cells. Thus, overexpression of JDP2 in RD cells mimics only the relevant physiological levels found in differentiating myoblasts.

JDP2 is a ubiquitously expressed protein, and unlike other members of the AP-1 family, JDP2 protein levels remain constant in response to a large variety of cell stimuli. Therefore, its induction during muscle differentiation is of great interest. One possibility is that either myogenic response factor and/or myocyte enhancer factor-2 family members directly regulate the *jdp2* promoter. Alternatively, the half-life of the *JDP2* protein may be extended during differentiation. JDP2 undergoes phosphorylation by JNK at threonine 148 (19); however, the precise role of JDP2 phosphorylation in JDP2 activity is not known. It will be interesting to examine the state of JDP2 phosphorylation during muscle cell differentiation. The ectopic and sustained expression of JDP2 bypasses the normal regulation of endogenous JDP2 and therefore induces an early and robust appearance of myogenic markers in C2 and RD cells. Further studies toward understanding JDP2 regulation in differentiating muscle cells will be necessary and are beyond the scope of this study.

JDP2 overexpression either in transient transfection or in stably transfected C2 cells overcomes the inhibitory effect of serum on muscle differentiation and stimulates myotube formation in the presence of mitogens. In the absence of mitogens, ectopic expression of JDP2 in C2 cells caused an earlier and higher expression of muscle-specific genes compared with the parental cells. Ectopic expression of JDP2 in RD cells induced full myogenesis of these cells, including the expression of the late myogenic marker MHC (Fig. 5*C*) and myotube formation both in GM and DM (Fig. 6). Prolonged TPA treatment was previously described to partially induce RD cell differentiation by down-regulation of protein kinase C, thus reducing the phosphorylation of MyoD at its inhibitory site, threonine 185

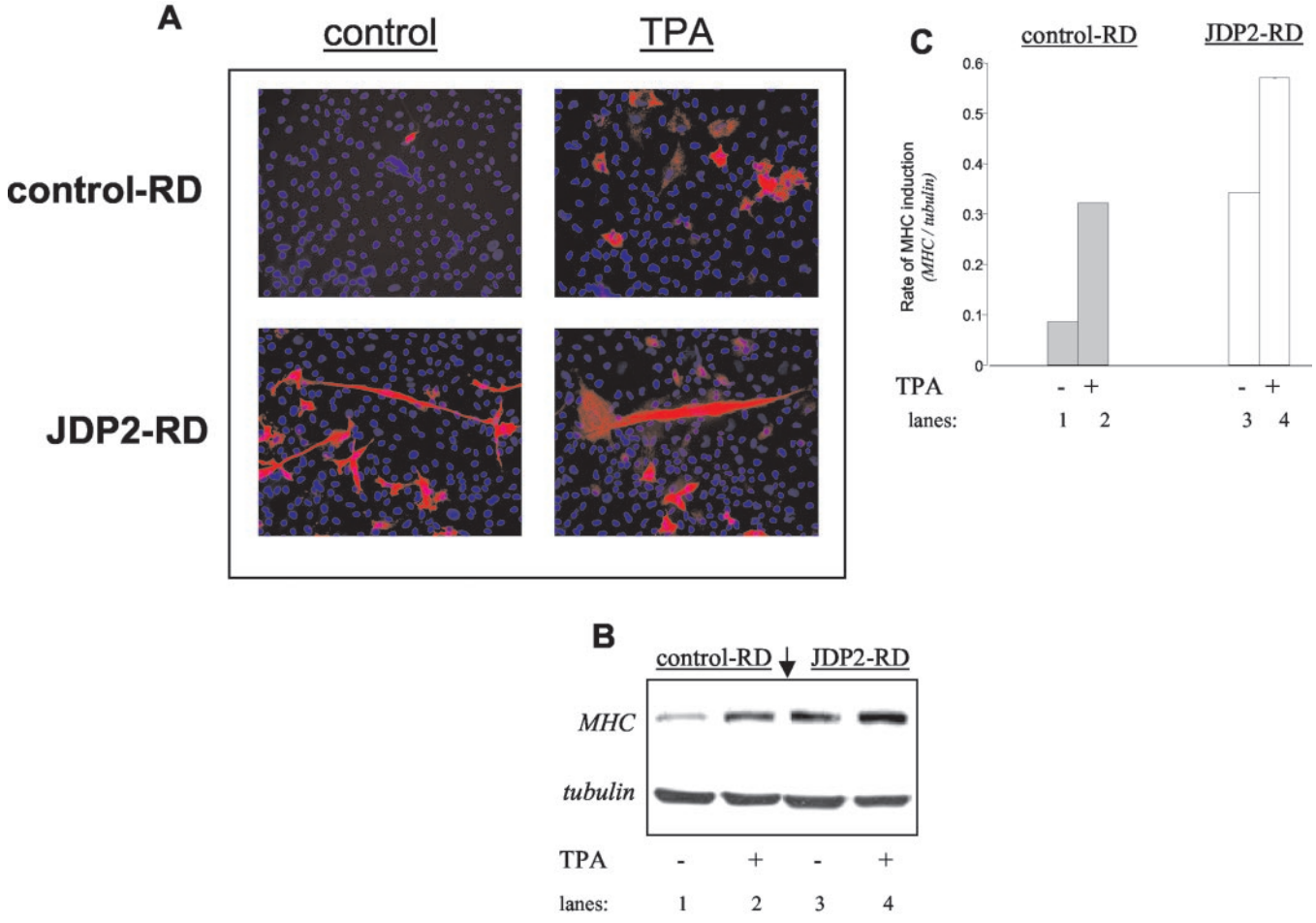

FIG. 9. **JDP2 cooperates with TPA-induced differentiation in rhabdomyosarcoma cells.** *A*, control-RD and JDP2-RD cells were treated with TPA (100 nM) or left untreated and grown in DM for 30 h prior to cell fixation and immunostaining with anti-MHC antibody (*red* staining). DAPI staining was used to visualize cell nuclei (*blue* staining). Representative co-stained fields are shown. *B*, shown are the results from Western blot analysis of cell extract derived from either JDP2-RD or control-RD cells treated with TPA (+) or left untreated (−) as described for *A*. The levels of MHC and tubulin were determined with the appropriate antibodies. *C*, shown are the results from densitometric analyses of the autoradiograms shown in *B*. The expression levels of MHC relative to tubulin levels were determined in either TPA-treated (+) or untreated (−) control-RD and JDP2-RD cells grown in DM.

(30). We have shown that MyoD activation following TPA treatment together with ectopic expression of JDP2 in RD cells clearly resulted in significant enhanced differentiation compared with either of the treatments alone. Prolonged TPA treatment in RD cells did not result in increase of JDP2 protein levels (data not shown), suggesting that MyoD is not directly involved in the regulation of JDP2 levels. The fact that ectopic JDP2 expression together with protein kinase C down-regulation induced muscle cell differentiation strongly indicates that JDP2 plays an important role in this process.

Cell Cycle Withdrawal—To differentiate, myoblasts must first withdraw from the cell cycle. To try to unravel the molecular mechanisms by which JDP2 functions to promote myoblast differentiation, we tested the effect of JDP2 on cell cycle progression. Overexpression of JDP2 significantly reduced the transcription of a cyclin D1 promoter reporter gene (Figs. 2*D* and 8*B*). The cyclin D1 promoter consists of both TRE and CRE sites (25), which are targets of JDP2 repression.[2] A decrease in cyclin D1 CDK4/6 activity reduces the levels of retinoblastoma phosphorylation, resulting in cell cycle arrest. In addition, lower cyclin-dependent phosphorylation of MyoD at serine 200 results in its stabilization (31). Indeed, we observed accumulation of the MyoD protein in JDP2-C2 cells (data not shown). Recently, it was shown that JDP2 inhibits retinoic acid-induced differentiation of F9 cells by inhibition of c-Jun transcription (18). Here we have shown that the protein level of either c-Jun or CREB is not affected by JDP2, thus suggesting that JDP2 represses cyclin D1 expression either directly or indirectly by inducing MyoD activity. Moreover, we observed a 3-fold increase in the transcription of the cyclin-dependent kinase inhibitor p21 in JDP2-C2 cells. This increase in transcription may be mediated by MyoD, which directly regulates the p21 promoter. The concomitant increase in p21 and decrease in cyclin D1 expression is most probably the cause for the reduced number of C2 and RD cells in S phase. Additionally, the ectopic expression of JDP2 in RD cells restored contact inhibition to these cells, suggesting that it reduced their tumorigenicity (Fig. 8*A*).

Although irreversible withdrawal from the cell cycle is absolutely essential for muscle cell differentiation (26), deliberate expression of p16, p27, or p21 in RD cells is not sufficient to induce muscle cell differentiation (6, 32). The above findings suggest that the mechanism of JDP2 promotion of muscle cell differentiation is mediated by additional function(s). One possibility is that JDP2 counteracts c-Jun action via heterodimerization, which results in the repression of c-Jun transcriptional activity and/or competes the binding of c-Jun to MyoD, thereby relieving inhibition of MyoD. A second potential MyoD activa-

[2] A. Aronheim, unpublished data.

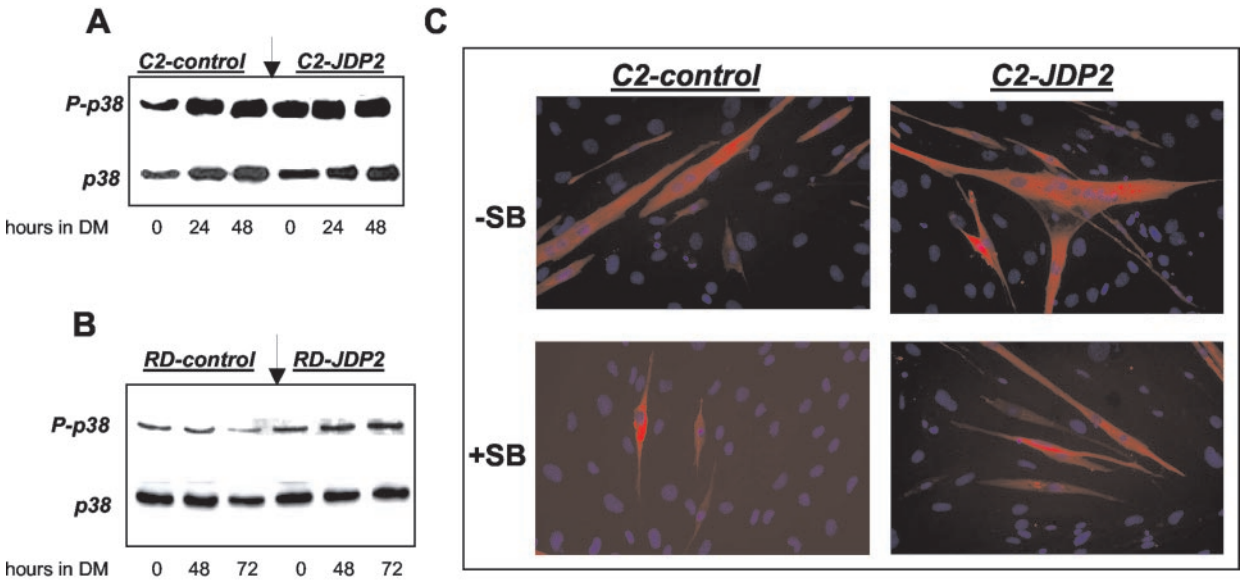

FIG. 10. **JDP2 promotes the activation of the p38 cascade in both RD and C2 cell lines.** *A* and *B*, whole cell extract (50 μg) derived from either control-C2 and JDP2-C2 cells (*A*) or control-RD and JDP2-RD cells (*B*) grown in GM or for the indicated times following transfer to DM was separated by SDS-PAGE and analyzed by Western blotting. The levels of phospho-p38 (*P-p38*) and total p38 were determined using the appropriate antibodies. *C*, C2-control and C2-JDP2 cells were grown in DM for 48 h in the absence or presence of SB202190 (*SB*; 20 μM) for 48 h. Cells were fixed immunostained with anti-MHC antibody (*red* staining). DAPI staining was used to visualize cell nuclei (*blue* staining). Representative co-stained fields are shown.

tion may result from down-regulation of the helix-loop-helix inhibitory protein Id. However, we have not observed change in Id levels following JDP2 expression (data not shown). Alternatively, JDP2 may directly potentiate MyoD activity. Recently, JDP2 has been shown to interact with and activate the progesterone receptor by serving as a transcription coactivator (17). Although c-Jun and MyoD proteins are members of different protein families (the bZIP and the helix-loop-helix families, respectively), their association has already been described (11). Therefore, we cannot exclude the possibility that JDP2 directly associates with MyoD and induces its myogenic activity.

Paracrine/Autocrine Pathways—Growth factors such as basic fibroblast growth factor and transforming growth factor-β have been shown to inhibit muscle cell differentiation; therefore, JDP2 expression could repress the autocrine secretion of an inhibitory factor or induce a positive growth factor. This possibility is unlikely because in our transient transfection assays, untransfected cells did not exhibit any muscle-specific phenotype or cell cycle arrest compared with the neighboring JDP2-expressing cells (Fig. 7*A*). Therefore, it is reasonable to assume that JDP2 functions by inhibiting intracellular regulatory circuits and does not affect the secretion of factors into the extracellular milieu.

Although significant, the consequence of JDP2 overexpression in RD cells was different from that in C2 cells. Differentiated JDP2-RD cells contained smaller myotubes with fewer nuclei/myotube compared with differentiated C2 cells, suggesting that JDP2 was unable to completely rescue all aspects of muscle cell differentiation. Because RD cells contain multiple lesions that prevent their differentiation, JDP2 can only partially drive differentiation in these cells. Additional factors, yet to be discovered, are required to fully execute the myogenic program. Indeed, TPA treatment together with JDP2 overexpression synergistically induced RD cell differentiation.

Recently, it was shown that the sustained activation of the p38 MAPK pathway is capable of inducing terminal differentiation in RD cells (20). We observed that expression of JDP2 in both RD and C2 cells potentiated p38 kinase activity. In contrast, we tested the ERK and phosphatidylinositol 3-kinase activity in these cells using anti-phospho-ERK and anti-phospho-protein kinase B antibodies, respectively and observed no change in their activity.[3] In addition, unlike in control-C2 cells, cell treatment with the p38 kinase inhibitor SB202190 did not completely abolish differentiation of JDP2-C2 cells grown in DM. In JDP2-C2 cells, SB202190 treatment strongly inhibited the formation of multinucleated cells; however, a significant number of MHC-positive cells bypassed the inhibition of the p38 kinase inhibitor. The precise signal that activates p38 following serum withdrawal is not known. It has been suggested that mitogen-dependent factor(s) might be involved in silencing the p38 kinase. One possibility is that this factor could be regulated by the AP-1 complex. In this work, we found that the expression of JDP2 induced p38 activity (Fig. 10). Thus, JDP2 may be the factor that relieves the inhibitory function of the AP-1 complex. Alternatively, p38-specific phosphatase has been suggested to inhibit the activation of p38 under mitogenic conditions (20). It will be interesting to study whether the transcription of such a p38-specific phosphatase is directly regulated by JDP2. In summary, our study strongly demonstrates the important role for JDP2, a member of the AP-1 family, in the terminal differentiation of skeletal muscle.

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