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ERK6, a mitogen-activated protein kinase involved in C2C12 myoblast differentiation

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ABSTRACT **ERK6, a mitogen-activated protein (MAP) kinase-related serine/threonine kinase, is highly expressed in human skeletal muscle and appears to function as a signal transducer during differentiation of myoblasts to myotubes. In transfected 293 cells, activation of the 45-kDa enzyme results in tyrosine-phosphorylated 46- and 56-kDa forms, which phosphorylate myelin basic protein. Overexpression of wild-type ERK6 or the inactive mutant Y185F has no effect on fibroblast and myoblast proliferation, but it enhances or inhibits C2C12 cell differentiation to myotubes, respectively. Our findings suggest ERK6 to be a tissue-specific, differentiation signal-transducing factor that is connected to phosphotyrosine-mediated signaling pathways distinct from those activating other members of the MAP kinase family such as ERK1 and ERK2.**

Mitogens, tumor promoters, and cell differentiation-inducing agents trigger intracellular signaling cascades, which lead to phosphorylation and activation of mitogen-activated protein (MAP) kinases [MAPKs; also known as extracellular signal-regulated kinases (ERKs)] (reviewed in refs. 1 and 2). These proline site-directed, serine/threonine kinases in turn phosphorylate transcription factors such as p65TCF/Elk-1 (3, 4), c-jun, and c-myc (reviewed in ref. 1) and thus appear to play a crucial role in signal transduction by converting extracellular stimuli into transcriptional activation of specific genes.

While originally it was thought that the signaling cascade involving SOS, ras, raf, MEK (MAP kinase/ERK kinase), and MAPK (ERK1/2) represents a generic mitogenic pathway, recent observations with the PC12 cell neuronal differentiation system suggest that negative regulation mechanisms determining temporal parameters of MAPK activation are critical for the definition of growth versus differentiation signals (5, 6). In analogous investigations aimed at the characterization of molecular events involved in myogenic differentiation signals, we isolated a human skeletal muscle cDNA encoding a novel serine/threonine kinase of the ERK family, designated ERK6.‡ Here we report structural and functional properties of this novel signaling pathway component.

MATERIALS AND METHODS

cDNA Cloning of ERK6. A human cDNA library from skeletal muscle prepared as described by Okayama and Berg (7, 8) was screened with a set of radiolabeled oligonucleotides with the following sequences: AAG GGT TTT ACC ATG GCA GAG AAA (E10), TTA ACT TGT CGA CTA CGT CAG CAG (E11), and A(CT) AT(GT) TGG (GT)CT G(CT)(AG) GGC TGC ATC (E13). Oligonucleotide E10 corresponds to the 5′ region, including the first in-frame start codon, and E11 to the 3′ region, including the first in-frame stop codon of the rat ERK3 sequence (9). Oligonucleotide E13 was based on the rat ERK3 sequence within the conserved region in subdomain IX (9) and includes the codons for the homologous amino acids of rat ERK1 and ERK2. Oligonucleotides were mixed in equimolar amounts and 5′ labeled with ^{32}P using T4 polynucleotide kinase (10). Screening was performed essentially as described in Sambrook *et al.* (10).

Sequence Analysis. Sequencing was performed according to Sanger *et al.* (11) with the sequenase kit (United States Biochemical).

Preparation of RNA and Northern Analysis. Total RNA was extracted from normal human tissues (obtained from the National Disease Research Interchange, Philadelphia) or C2C12 cells according to Puissant and Houdebine (12). Poly(A)$^+$ RNA was prepared as described by Aviv and Leder (13). A total of 0.5 μg of poly(A)$^+$ RNA per lane was loaded on a 1.2% agarose/2.2 M formaldehyde gel and, after separation, blotted onto nitrocellulose (10). A 1200-bp *Bam*HI fragment of ERK6 cDNA was used as a hybridization probe for labeling with ^{32}P using the random-primed DNA labeling kit (Boehringer Mannheim).

Preparation of Antisera. Antisera were generated against two different glutathione-*S*-transferase fusion proteins with (*i*) the C-terminal 238 amino acids of ERK6 protein and (*ii*) the complete ERK6 protein (367 amino acids), respectively, using the pGEX3X vector (Pharmacia). Purified fusion proteins were used to immunize rabbits. Antiserum raised against the C-terminal portion of ERK6 was used for Western blot analysis (dilution 1:5000). Immunoprecipitation of ERK6 was performed with antiserum against the complete ERK6 protein (dilution 1:300).

Mutagenesis. To introduce an amino acid change of one of the two regulatory phosphorylation sites of MAPK, codon 185 of the ERK6 cDNA (TAC, encoding Y) was changed on the second position to TTC (encoding F) by PCR with specific oligonucleotides carrying one mismatch at the indicated position.

Overexpression of ERK6 in 293 Cells. For transient expression in human embryonal kidney fibroblasts (293 cells) (14), the ERK6 wild-type (wt) and Y185F mutant cDNAs were cloned in a cytomegalovirus promoter-driven expression vector (15). Transfection was performed with cesium chloride-purified DNA. Cells were then incubated for 16 h at 3% CO_2 and 35°C followed by removal of the medium and replacement by Dulbecco's modified Eagle's medium (DMEM) supplemented either with 0.5% fetal calf serum (FCS) to starve cells for 24 h before stimulation with insulin or with 10% FCS. Insulin stimulation was performed when cells had been cotransfected with an expression vector encoding the human IR A-type (16), with a final insulin concentration of 10^{-8} M for

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Abbreviations: MAP, mitogen-activated protein; MAPK, MAP kinase; ERK, extracellular signal-regulated kinase; wt, wild type; FCS, fetal calf serum; MBP, myelin basic protein; TPA, phorbol 12-tetradecanoate 13-acetate; IR, insulin receptor.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. X79483).

10 min. For metabolic labeling, growth medium was replaced by methionine-free minimal essential medium (MEM) (GIBCO) containing 60 μCi of [^{35}S]methionine (Amersham; 1 Ci = 37 GBq) per ml and dialyzed FCS at a concentration of 0.5% and 10%, respectively, 16 h before lysis with Triton X-100, immunoprecipitation, and gel analysis.

Kinase Assay in Myelin Basic Protein (MBP)-Containing Polyacrylamide Gels. Unlabeled anti-ERK6-immunoprecipitates of transfected 293 cells were electrophoresed on an SDS/10% polyacrylamide gel containing 0.5 mg of MBP per ml from bovine brain (Sigma). The assay was performed according to the procedure described in Lee *et al.* (17).

Stable Overexpression of ERK6 wt and Y185F Mutant in NIH 3T3 and C2C12 Cells. To obtain stably ERK6-overexpressing C2C12 cells, recombinant retroviruses conferring expression of wt ERK6 or Y185F mutant were generated in PA317/GP+E86 cells by using the pLXSN vector (18). NIH 3T3 and C2C12 cells were infected with retrovirus-containing supernatant of the producer cell line and selected for expression of ERK6.

Stimulation of ERK6 wt and Y185F in Infected NIH 3T3 and C2C12 Cells. For activation studies, retrovirus-infected NIH 3T3 (not shown) and C2C12 cells stably expressing wt or Y185F ERK6 were grown in 10% FCS medium to subconfluency, starved for 24 h in 0.5% FCS, and treated either with 2 mM sodium orthovanadate (2 h) without or with 10% FCS (last 10 min), 1 mM phorbol 12-tetradecanoate 13-acetate (TPA) (10 min) without or with 2 mM sodium orthovanadate (110 min before addition of TPA), 1 nM okadaic acid (2 h) alone or with either 1 mM TPA (last 10 min) or 10% FCS (last 10 min), or treated with 10^{-7} M and 10^{-8} M insulin, respectively, or left untreated. For lysis, cells were put on ice, washed once with cold phosphate-buffered saline (PBS), and resuspended in Laemmli buffer, electrophoresed on an SDS/10% polyacrylamide gel, and blotted onto nitrocellulose. Immunodetection of ERK6 was performed with antiserum against the C-terminal portion of ERK6; endogenous ERK1 and ERK2 were visualized with a specific polyclonal rabbit antiserum (Santa Cruz Biotechnologies) that recognizes both MAPK isoforms. Reacting proteins were visualized using horseradish-peroxidase-coupled goat anti-rabbit IgG antibodies (Bio-Rad) and the ECL kit (Amersham).

Differentiation Experiments in Infected C2C12 Myoblasts. C2C12 cells stably expressing wt ERK6 or Y185F were seeded in proliferation medium at a density of 12,000 cells per cm^2 and grown for 48 h to confluency. Cells were washed once with serum-free medium and induced to fuse in medium containing 2% horse serum (differentiation medium). The state of fusion was documented 3, 5, and 7 days after change to differentiation medium.

RESULTS AND DISCUSSION

cDNA Cloning of MAPK-Related Sequences from Human Skeletal Muscle. By screening a human skeletal muscle cDNA library with a set of oligonucleotides based on the rat ERK3

A

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                                                           I                   II
ERK6    MSSPPPGGSGFYRQEVTKTAWEVRAVYRDLQPVGSGAYGAVCSAVDGRTGAKVAIKKLYRPFQSELFAKL   70
CSBP1   ..Q---ERPT.....LN..I...PER.QN.S........S..A.F.TK..LR..V...S.....IIH..R   67
ERK2    .AA--AAAAGAGPEM.RGQVFD.GPR.IN.SYI.E....M....Y.NVNKVR.....IS-..EHQTYCQR   67
JNK1    ..R-SKRDNN..SV.IGDSTFT.LKR.QN.K.I....Q.I..A.Y.AILERN......S....NQTH..R   69

          III         IV                        V
ERK6    AYRELRLLKHMRHENVIGLLDVFTPDETLDDFTDFYLVMPFMGTDLGKLMKHEKLGEDRIQFLVYQMMKG  140
CSBP1   T..........K.............ARS.EE.N.V...THL..A..NNIV.CQ..TD.HV...I..ILR.  137
ERK2    TL..IKI.LRF....I..IN.II-QAP.IEQMK.V.I.QDL.E...Y..L.TQH.SN.HICYFL..ILR.  136
JNK1    .....V.M.CVN.K.I....N....QKS.EE.Q.V.I..EL.DAN.CQVIQM.-.DHE.MSY.L...LC.  138

         VI                          VII                  * *  VIII
ERK6    LRYIHAAGIIHRDLKPGNLAVNEDCELKILDFGLARQADSE------MTGYVVTRWYRAPEVILNWIAYT  204
CSBP1   .K...S.D........S...................HT.D.------.....A........IM...MH.N  201
ERK2    .K...S.NVL......S..LL.TT.D...C......V..PDHDHTGFL.E..A........IM..SKG..  206
JNK1    IKHL.S..........S.IV.KS..T..........T.GTS----FM..P.....Y........G-MG.K  203

               IX                               X
ERK6    QTVDIWSVGCIMAEMITGKTLFKGSDHLDQLKEIMKVTGTPPAEFVQRLQS------DEAKNYMKGLPEL  268
CSBP1   ..............LL..R...P.T..IN..QQ..RL......YLIN.MP.------H..R..IQ-SLTQ  264
ERK2    KSI........L...LSNRPI.PGKHY....NH.LGIL.S.SQ.DLNCIIN------LK.R..LL-SLPH  269
JNK1    EN..L.......G..VCH.I..P.R.YI..WNKVIEQL...CP..MKK..PTVRTYVENRPK.AGYSF.K  273

                                  XI
ERK6    EKKDFASILTNAS-----PLAVNLLEKMLVLDADIRLTAGEFLSHPYFESLHDTEDEPQVQKYDDSFDYF  333
CSBP1   M-PKMNFANVFIGAN---....D.........S.K.I..AQA.A.A..AQY..PD...VADP..Q..ESR  330
ERK2    K-NKVPWNRLFPNAD---SK.LD..D...TFNPHK.IEVEQA.A...LEQYY.PS...IAEAPFKFDMEL  335
JNK1    LFP.VLFPADSEHNKLKASQ.RD..S....I..SK.ISVD.A.Q...INVWY.PSEAEAPPPKIPDKQLD  343

ERK6    DRTLDEWKRVTYKEVLSFKPPRQLGARVSKETPL                                     367
CSBP1   .LLI....SL..D..I..V..PLDQEE                                            357
ERK2    .DLPK.KLKELIF.ETARFQ.GYRS                                              360
JNK1    E.EHTIEEWKELIYKEVMDLEERTKNG.IRGQ.SPLAQVQQ                              384
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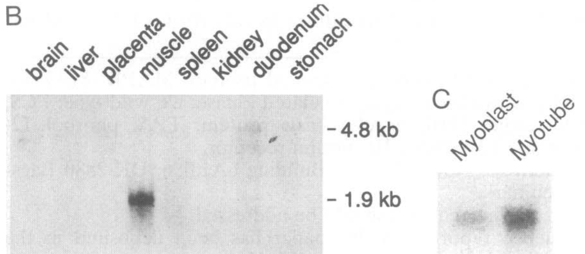

FIG. 1. (*A*) ERK6 sequence and comparison with members of the human MAPK family. Roman numerals indicate the 11 conserved protein kinase regions (19). Conserved serine/threonine kinase domains are underlined. Asterisks mark regulatory residues that are required for the activity of MAPKs (11). Residues that differ from ERK6 are shown. Identical residues are indicated by dots. Hyphens indicate spaces introduced for optimal alignment. Northern blot analysis of poly(A)$^+$ RNA from normal human tissues (*B*) and C2C12 myoblasts and myotubes (*C*) (exposure time, 3 days).

sequence (9), we isolated a cDNA clone containing a 1101-nucleotide open reading frame that defines a protein of M_r = 40,300 (Fig. 1*A*). Sequence comparison revealed 63% identity with the human CSBP subfamily of MAPKs, 44% with human JNK1, 41% with ERK1, 39% with ERK2, and 33% with ERK3 (20–22). Conservation of three amino acid residues that are essential for ERK activity (23–25), including a lysine in subdomain II (19) at position 52 or 57 in mouse or *Xenopus* ERK2, respectively, and threonine-183 and tyrosine-185 in subdomain VIII, indicated that our cDNA clone encoded a previously unknown member of the MAPK superfamily. We designated this novel gene product ERK6. Northern blot analysis of poly(A)$^+$ RNAs revealed a 1.9-kb ERK6 mRNA, which was highly expressed in skeletal muscle (Fig. 1*B*) but was not detected in other human adult tissues tested (Fig. 1*B*). Moreover, as shown in Fig. 1*C*, ERK6 mRNA expression appeared to be induced in differentiated C2C12 cells over the level present in undifferentiated C2C12 myoblasts, which suggested a role of this serine/threonine kinase in the myogenic differentiation signal.

Phosphorylation of ERK6 in Transfected 293 Cells. The enzymatic activity and phosphorylation characteristics of ERK6 were determined in human 293 cells after transfection with expression vectors for the wt enzyme or a Y185F mutant. Mutation of tyrosine residue 185 to phenylalanine was expected to yield a kinase-impaired ERK6 due to the loss of one of two regulatory phosphorylation sites previously identified in ERK2 (24). Cells were metabolically labeled with [^{35}S]methionine, lysed, and immunoprecipitated with ERK6 antiserum. SDS/PAGE of the precipitate and autoradiography revealed the ERK6 expression product as a double band of 45/46 kDa (p45/pp45) as well as a weaker doublet of 56 kDa (Fig. 2*A Left*).

To investigate whether any of the bands represented ERK6 phosphorylation states that could be induced, as shown for MAPK, by an extracellular signal such as insulin (9, 26, 27), 293 cells were cotransfected with either wt ERK6 or Y185F mutant expression vectors and a plasmid conferring expression of human IR. After starvation for 24 h before stimulation with insulin, the cells were metabolically labeled with [^{35}S]methi-

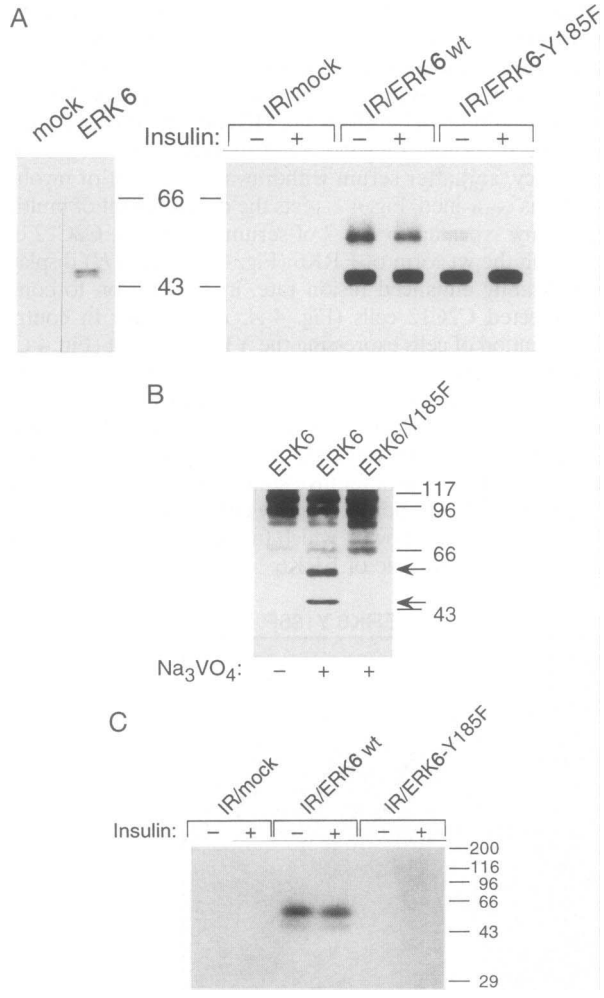

FIG. 2. (*A*) Transient expression of ERK6 in 293 cells. Cells were grown in 10% FCS (*Left*) or starved in 0.5% FCS and stimulated with insulin (*Right*). Anti-ERK6 immunoprecipitates of ^{35}S-labeled cells were separated on SDS/10% PAGE. (*B*) Anti-phosphotyrosine immunoblot analysis of transiently transfected 293 cells. Cells were transfected with ERK6 wt cDNA (lanes 1 and 2) or the Y185F mutant (lane 3). Before lysis, cells were treated with orthovanadate (2 mM for 2 h) and TPA (1 mM for 10 min) (lanes 2 and 3) or left untreated (lane 1). Phosphorylated forms of ERK6 are marked by arrows. (*C*) ERK6 catalytic activity. 293 cells were transfected with IR and ERK6 cDNA expression plasmids, starved, and stimulated with insulin. The unlabeled anti-ERK6 immunoprecipitate of transfected 293 cells was electrophoresed on an SDS/10% polyacrylamide gel containing 0.5 mg/ml MBP as substrate.

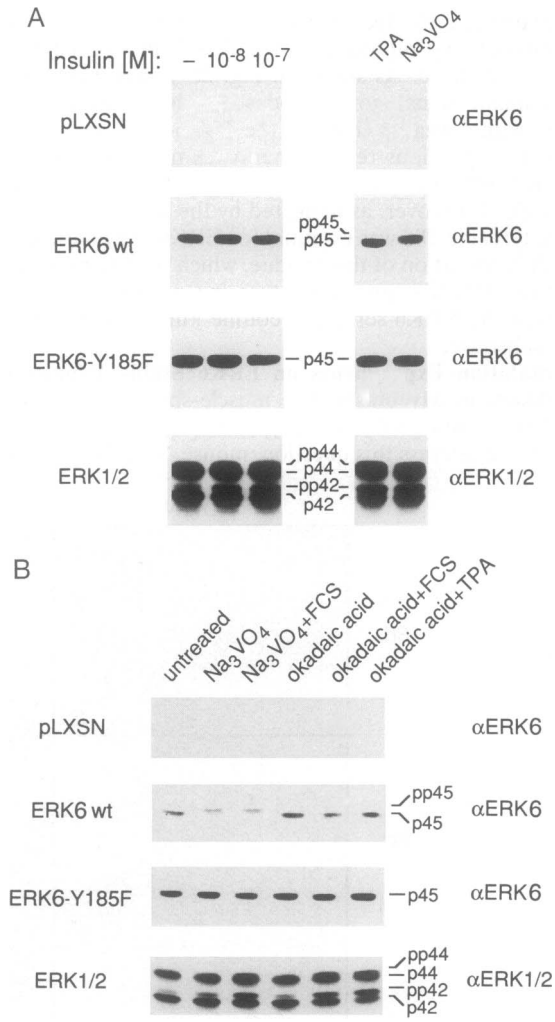

FIG. 3. Activation of ERKs by insulin, TPA, and vanadate (*A*) and by phosphorylation agents (*B*). The left-hand columns indicate the C2C12 derivative cell lines used, except for the ERK1/2 experiment, which was performed on parental C2C12 cells. The right-hand columns indicate the blotting antibodies. All immunoblots were performed on whole-cell lysates. p42, p44, and p45 indicate the positions of unphosphorylated proteins, and pp42, pp44, and pp45 indicate phosphorylated proteins.

onine, lysed, and analyzed by anti-ERK6 immunoprecipitation, SDS/PAGE, and autoradiography. As shown in Fig. 2*A Right* (lanes 3 and 4), major bands of 45/46 kDa were detected as well as the 56-kDa band, which was also detected in crude lysates of transfected 293 cells (Fig. 2*A Left*). Insulin stimulation had no significant effect on this expression pattern. In infected NIH 3T3 or C2C12 cells, with lower levels of ERK6, the 56-kDa band was proportionally weaker than the 45/46-kDa doublet, indicating that its appearance correlated with the level of ERK6 overexpression. Interestingly, the shift from p45 to pp45 and p56 to pp56 involved phosphorylation on tyrosines, as demonstrated by treating transfected 293 cells with orthovanadate and immunoblotting of crude lysates with antiphosphotyrosine monoclonal antibody 5E2 (Fig. 2*B*) and reduction of the ERK6 band size by treatment of orthovanadate-stimulated lysates of transfected NIH 3T3 cells with alkaline phosphatase (data not shown). Similarly, in human primary myoblasts, a tyrosine-phosphorylated 45-kDa ERK6 band was detected (data not shown). Mutation of tyrosine-185 to phenylalanine inhibited this shift of ERK6 in both insulin- and vanadate-treated cells and prevented tyrosine phosphorylation (Fig. 2 *A* and *B*).

Enzymatic Activity of ERK6. Substrate phosphorylation activity was determined by analyzing lysates of transfected 293 cells expressing the IR and wt or mutant ERK6 by immunoprecipitation and subsequent SDS/PAGE in a gel containing MBP as substrate. As shown in Fig. 2*C* (lanes 3 and 4), the predominant signal corresponded to the 56-kDa form of ERK6, with a weaker signal at the 45/46-kDa position. Consistent with previous results, there was no significant difference in MBP kinase activity between stimulated or unstimulated cells. Moreover, as indicated by the lack of ^{32}P incorporation, tyrosine-185 was essential for ERK6 enzymatic activity (Fig. 2*C*). Mutation of this residue, which is conserved among all active MAPKs, prevented tyrosine phosphorylation and rendered the ERK6 serine/threonine kinase inactive for the MBP substrate.

Stimulation Experiments on ERK6 Stably Expressed in Fibroblasts or Myoblasts. The muscle-specific expression of ERK6 suggested a role in cell type-characteristic signal transduction. To address this question, mouse NIH 3T3 fibroblasts and C2C12 myoblasts were infected with a wt ERK6 or Y185F mutant virus and, after starvation for 24 h in 0.5% FCS, subconfluent cells expressing human ERK6 were treated with orthovanadate, TPA, okadaic acid, insulin, or FCS. Immunodetection of blotted total lysates after SDS/PAGE with ERK6 or ERK1/2 antiserum revealed essentially the same picture for both cell types. Whereas endogenous ERK1 and ERK2 shifted upon insulin, TPA, orthovanadate, and FCS treatment, the electrophoretic mobility of ERK6 was reduced only after orthovanadate treatment and was not affected by treatment with insulin, okadaic acid, or FCS (Fig. 3). Furthermore, overexpression of neither wt ERK6 nor the Y185F mutant altered the activation of endogenous ERK1 and ERK2. This result demonstrated that ERK6 and ERK1/2 do not share the same activating signaling cascade or regulatory systems in both NIH 3T3 fibroblasts and C2C12 myoblasts. It also demonstrates, however, that ERK6 activation presumably involves one or more upstream counteracting phosphotyrosine phosphatases that are sensitive to orthovanadate.

Stimulation of C2C12 Myoblast Differentiation by ERK6. Involvement of ERK6 in myogenic differentiation was further investigated in mouse C2C12 myoblasts, which form multinucleated myotubes upon serum withdrawal under high cell density conditions. Therefore, C2C12 cells stably expressing wt ERK6 or the kinase-negative Y185F mutant were grown to confluency, and after serum withdrawal the extent of myoblast fusion was examined. Fig. 4 depicts the development of multinucleated myotubes up to day 7 of serum starvation. C2C12 cells expressing the wt form of ERK6 (Fig. 4 *B, E*, and *H*) displayed a significantly enhanced fusion rate, in comparison to control virus-infected C2C12 cells (Fig. 4 *A, D*, and *G*). In contrast, differentiation of cells expressing the Y185F mutant (Fig. 4 *C, F*, and *I*) was inhibited and, rather than fusing and forming myotubes, they continued to proliferate in multiple layers. Thus, the dominant-negative effect of ERK6-Y185F causes the escape of C2C12 myoblasts from cell cycle arrest required for fusion and retains the cells in a proliferative state. Interestingly, the growth of NIH 3T3 cells was not affected by either wt or Y185F mutant overexpression (not shown), which further confirmed the signaling pathway-specific role of ERK6.

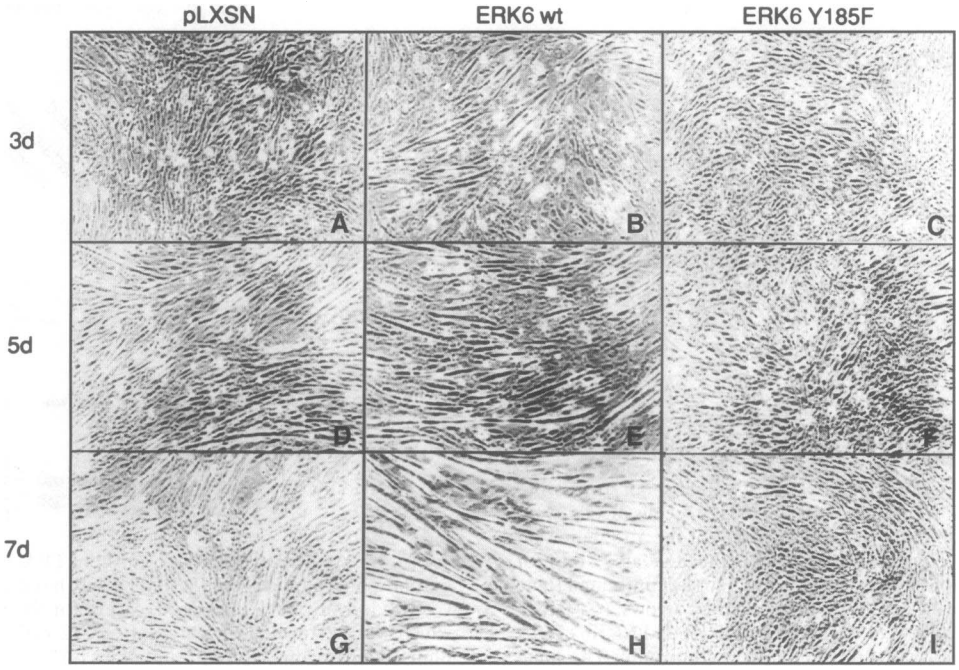

FIG. 4. Differentiation of retrovirus-infected C2C12 cells expressing wt or Y185F ERK6 at 3, 5, and 7 days (d) after change to differentiation medium.

In summary, definition of differentiation signals in C2C12 cells may involve a mechanism that is distinct from that proposed for the neuronal PC12 system (5, 6), in which negative regulatory elements specify the duration of the ras/MAPK signal and thereby determine a proliferative or differentiation response of the cell. Instead, this process requires the ERK6 serine/threonine kinase, a cell type-specific, signal-transducing protein that, upon activation through tyrosine phosphorylation, may play a role in activation of transcription factors necessary for differentiation of myoblasts to myotubes. The specific role of this novel signal transducer, including the mechanism of its activation as well as its substrates and other interacting proteins, is currently under investigation.

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1. Davis, R. J. (1993) *J. Biol. Chem.* **268,** 14553–14556.
2. Blenis, J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5889–5892.
3. Gille, H., Sharrocks, A. D. & Shaw, P. E. (1992) *Nature (London)* **358,** 414–417.
4. Marais, R., Wynne, J. & Treisman, R. (1993) *Cell* **73,** 381–393.
5. Traverse, S., Seedorf, K., Paterson, H., Marshall, C. J., Cohen, P. & Ullrich, A. (1994) *Curr. Biol.* **4,** 694–701.
6. Dikic, I., Schlessinger, J. & Lax, I. (1994) *Curr. Biol.* **4,** 702–708.
7. Okayama, H. & Berg, P. (1982) *Mol. Cell. Biol.* **2,** 161–170.
8. Okayama, H. & Berg, P. (1983) *Mol. Cell. Biol.* **3,** 280–289.
9. Boulton, T. G., Nye, S. K., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. & Yancopoulos, G. D. (1991) *Cell* **65,** 663–675.
10. Sambrook, J., Fritsch, E. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
11. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
12. Puissant, C. & Houdebine, L.-M. (1990) *Biotechniques* **8,** 148–149.
13. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1408–1412.
14. Graham, F. L., Smiley, J., Russell, W. C. & Nairu, R. (1977) *J. Gen. Virol.* **36,** 59–77.
15. Gorman, C. M., Gies, D., McCray, G. & Huang, M. (1989) *Virology* **171,** 377–385.
16. Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M. & Ramachandran, J. (1985) *Nature (London)* **313,** 756–761.
17. Lee, H., Ghose-Dastidar, J., Winawer, S. & Friedman, E. (1993) *J. Biol. Chem.* **268,** 5255–5263.
18. Miller, A. D. & Rosman, G. J. (1989) *Biotechniques* **7,** 980–990.
19. Hanks, S. K., Quinn, A. M. & Hunter, T. (1988) *Science* **241,** 42–52.
20. Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E. & Gustin, M. C. (1993) *Science* **259,** 1760–1763.
21. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M. & Davis, R. J. (1994) *Cell* **76,** 1025–1037.
22. Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W., Strickler, J. E., McLaughlin, M. M., Siemens, I. R., Fisher, S. M., Livi, G. P., White, J. R., Adams, J. L. & Young, P. R. (1994) *Nature (London)* **372,** 739–746.
23. Her, J.-H., Wu, J., Rall, T. B., Sturgill, T. W. & Weber, M. J. (1991) *Nucleic Acids Res.* **19,** 3743.
24. Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J.-H., Shabanowitz, J., Hunt, D. F., Weber, M. J. & Sturgill, T. W. (1991) *EMBO J.* **10,** 885–892.
25. Posada, J. & Cooper, J. A. (1992) *Science* **255,** 212–215.
26. Ray, L. B. & Sturgill, T. W. (1988) *J. Biol. Chem.* **263,** 12721–12727.
27. Rossomando, A. J., Payne, D. M., Weber, M. J. & Sturgill, T. W. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6940–6943.