FEBS 22865 FEBS Letters 461 (1999) 107–110

MyoD binds to Mos and inhibits the Mos/MAP kinase pathway

Brigitte Solhonne[1], Jean Luc Lenormand[2], Karine Pelpel, Marie Pierre Leibovitch, Serge A. Leibovitch*

Laboratoire de Génétique Oncologique UMR 1599 CNRS, Institut Gustave Roussy, 39, rue Camille Desmoulins, 94800 Villejuif, France

Received 27 September 1999

Abstract When ectopically expressed, the serine/threonine kinase Mos can induce oncogenic transformation of somatic cells by direct phosphorylation of MAP kinase/ERK kinase (MEK1), activating the mitogen-activated protein kinases ERK1 and ERK2. On the other hand, overexpression of Mos in C2C12 myoblasts is not transforming. Mos activates myogenic differentiation by promoting heterodimerization of the MyoD/E12 proteins, increasing the expression of myogenic markers and the positive autoregulatory loop of MyoD. In this study, we show that in myogenic cells, the mitogenic and oncogenic signalling from the Mos/MEK/ERK pathway is suppressed by MyoD through the formation of a heterotrimeric complex.

Key words: Mos transformation; MAP kinase pathway; MyoD; Muscle differentiation

1. Introduction

In recent efforts to identify and characterize accessory factors that impinge on MyoD activity, we reported that in C2C12 myoblasts, ectopic conditional expression of the protooncogene *c-mos*^{rat} activated muscle differentiation while inhibition of endogenous Mos expression by RNA antisense resulted in reversible blockage of myogenesis [1], suggesting that Mos may interact with the muscle regulatory factors. Indeed, we showed that unphosphorylated MyoD but not its E12 partner interacts physically with the Mos kinase [2]. Mutational analysis of the proteins demonstrated that a highly conserved region of Mos proteins that shares sequence homologies with the ubiquitous E-protein class of b-HLH associates with the helix 2 domain of MyoD. Furthermore, phosphorylation of the COOH domain of MyoD by Mos inhibits the DNA-binding activity of MyoD homodimers but promotes the formation and DNA-binding activity of MyoD-E12 heterodimers [2]. Recent data indicated that Mos favors the capacity of MyoD to up-regulate its own expression and promotes the levels of MyoD products, suggesting that Mos participates in the positive autoregulation of MyoD and thus in activation of muscle differentiation [3].

The biological functions of Mos in germ cells correlate well with the activation of the mitogen-activated protein kinase (ERKs) [4,5]. Mos associates with and phosphorylates MEK1 at Ser-218 and Ser-222 to activate MEK1 kinase [6]. Consistent with the persistent ERK activation in Mos-transformed cells, Mos also activates MEK1, suggesting that Mos may utilize the MEK1/ERK pathway to transform cells. Indeed, it has been shown that the MEK1/ERK pathway is essential for oncogenic transformation of NIH 3T3 cells by Mos [7]. The Mos MAP kinase (MAPK) pathway stabilizes c-Fos by phosphorylation and augments its transforming activity in NIH 3T3 cells [8]. In this study, we attempted to elucidate the molecular mechanism that leads to the absence of transformation by Mos in the myogenic cells [2]. We demonstrate heterotrimeric complex forms between MyoD/Mos/MEK1 in vitro and in cultured cells. The formation of this complex suppresses the Mos/MEK/ERK kinase pathway and could be one of the means by which Mos activity is deviated from proliferative/transforming genes to differentiating genes upon early myogenic differentiation.

2. Materials and methods

2.1. Cell cultures, DNA transfections and luciferase assays

The fibroblastic cell line 10T1/2 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (FCS). The 10T1/2 cells were transfected using polyethylenimine (Sigma) essentially as described [9]. The total amount of DNA used for each plate was normalized with the respective empty expression vector. Forty-eight hours after transfection, cells were harvested and luciferase activity determined on aliquots of cell extracts containing equivalent amounts of proteins with a luminometer (Lumat LB 9507-DLA, Berthold). Plasmid pCH110 (Pharmacia) was included in the assays as an internal control for transfection efficiency. Experiments were performed in triplicate and repeated at least twice.

2.2. Plasmid constructions

Expression vectors pEMSV-MyoD, pGEX2T-Mos wild-type (Mos^{wt}), pCMV-HA-Mos^{wt} and pCMV-HA-kinase inactive Mos (Mos^{KM}) were previously described [3]. Plasmids pet-MyoD, pVP16-MyoD, pVP16-E12, pM-MyoD, pM-Mos and pM-E12 were constructed as previously described [2]. pM-MEK1 was generated after excising the *Xho*I-*Pvu*II insert from pSp64-MEK1. Insert was filled in with the Klenow DNA polymerase and inserted in the pM vector restricted with *Bam*HI, filled in with the Klenow polymerase. These constructs were controlled by sequencing across the junctions of the fusion genes.

2.3. Mammalian two and triple hybrid assay

10T1/2 cells were maintained and transfected as described previously [2]. Then, 4×10^5 cells were transfected with 2 μg of the Gal-dependent reporter plasmid pG5E1b-luciferase and 10 μg of each of the indicated plasmids and brought to 25 μg total DNA with empty vector. Following transfection, cells were maintained in growth medium for 48 h before harvesting. Luciferase assays were performed as previously described [2]. Experiments were done in triplicate and repeated at least three times.

2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting

Cells were lysed and total proteins solubilized in RIPA buffer containing 10 mM EGTA and processed as previously described [10].

*Corresponding author. Fax: (33) (1) 42 11 64 42.
E-mail: leibovit@igr.fr

[1] Present address: INSERM U99, Hôpital Henry Mondor, 94010 Créteil, France.
[2] Present address: Laboratoire des Protéines du Cytosquelette, 41, rue Jules Horowitz, 38000 Grenoble, France.

PII: S0014-5793(99)01430-1

After electrophoretic transfer of proteins onto nitrocellulose membranes, immunodetection was performed with the 12CA5 monoclonal antibody (dilution: 1/1000, Boehringer) or the MyoD polyclonal antibody (dilution: 1/1000, clone C-20, Santa Cruz) or the EKR1/ERK2 polyclonal antibody (1/1000, clone K23, Santa Cruz). After exposure to the secondary antibody, horseradish peroxidase-linked sheep anti-rabbit or anti-mouse immunoglobulin G (Sigma), antigen-antibody complexes were revealed by the enhanced chemoluminescence system (ECL, Amersham). Exposure was performed with Agfa Curix RP2 films.

2.5. Protein expression, purification and GST pull-down assay

Bacterial expression of proteins was performed in *Escherichia coli* BL21. Protein induction, cell lysis and affinity purification with glutathione-agarose beads (Sigma) were done as described previously [2]. GST-Mos^{wt} fusion protein was not eluted but washed four times at 4°C in NTEN buffer (20 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing protease inhibitors and phosphatase inhibitors. The GST and GST fusion proteins were collected on glutathione-Sepharose 4B (Pharmacia), analyzed by SDS-PAGE and their purity was estimated to be 70–80% by Coomassie brilliant blue staining of the gels. ^{35}S-labelled proteins were prepared by coupled in vitro transcription-translation using the TnT-coupled rabbit reticulocyte lysate system (Promega). GST pull-down assays were performed as described previously [2]. The programmed lysates (1–9 μl) were incubated with GST alone and GST fusion proteins for 2 h at 4°C. Beads were washed four times in NTEN buffer at room temperature and then mixed with one volume of 2×SDS loading buffer and bound proteins were analyzed by SDS-PAGE by using standard procedures.

For the competition/association assays, GST-Mos^{wt}-covered beads were first incubated with ^{35}S-labelled in vitro-translated MyoD or MEK1 for 2 h at 4°C and then washed by three wash cycles of binding buffer. Increasing amounts of labelled MyoD or MEK1 were then added to the binding reactions and the resulting mixtures were subjected to a GST pull-down assay. The reaction products were separated on SDS-PAGE. Bound proteins were detected by autoradiofluorography and quantified by using a phosphorimager.

3. Results and discussion

3.1. MEK1 and MyoD physically interact with Mos in 10T1/2 cells

Previous studies have shown that the NH_2-terminal domain of Mos and particularly the Ser-3 is important for Mos interaction with MEK1 [11]. On the other hand, a highly conserved sequence in Mos^{rat} (covering amino acids 184–209), related to the helix 2 of the E-protein class of b-HLH factors, mediates interaction with the helix 2 of MyoD [2]. Altogether, these data indicate that MEK1 and MyoD interact with two different domains of Mos. To determine whether MEK1 or MyoD interaction with Mos is exclusive or not in vivo, we employed the triple hybrid approach in 10T1/2 cells. We used the GAL4 DNA-binding domain (DBD)-dependent reporter system, in which Mos, MEK1 or MyoD (this latter is deleted of its transcription activation domain to avoid a high background) were fused to the DBD of GAL4 (pM vector) and MyoD, E12 or Mos were fused to the VP16 activation domain (pVP16AD vector). We next introduced into the same 10T1/2 cells, three expression vectors encoding a fusion protein between the GAL4 DBD and MEK1, a fusion protein between the VP16 activation domain and Mos and increasing amounts of pEMSV-MyoD expression vector along with the pG5E1b-luciferase reporter vector. As illustrated in Fig. 1, expression of either pM-MyoD, pM-MEK1 or VP16-MyoD, VP16-E12 or VP16-Mos polypeptides failed to induce a significant luciferase activity in transfected 10T1/2 cells. Co-expression of pM-MyoD and pVP16-E12 generated a large increase in luciferase activity to levels 500-fold those found with the empty pM and pVP16AD vectors. These data show that heterodimers are well formed in vivo through a stable interaction between the b-HLH domains of MyoD and E12. Under the same conditions, experiments with pM-MyoD, pM-MEK1 and VP16-Mos or VP16-MyoD revealed in vivo association between Mos and MEK1 (Fig. 1, lane 3), Mos and MyoD (Fig. 1, lanes 9 and 12), but not with MyoD and MEK1 (Fig. 1, lane 4). Surprisingly, in the presence of increasing amounts of MyoD, no significant decrease in luciferase activity was observed, indicating that interaction between pM-MEK1 and VP16-Mos was not modified by MyoD (Fig. 1, lanes 5–8). These data demonstrate the ability of Mos to interact with MEK1 independently of MyoD and suggest that MyoD does not suppress Mos-MEK1 interaction although the magnitude of activation of luciferase activity observed with pM-MEK1 interaction was less than Mos-MyoD. This difference probably reflects a surprising weaker affinity between Mos and MEK1 than Mos and MyoD (compare lanes 3 and 9).

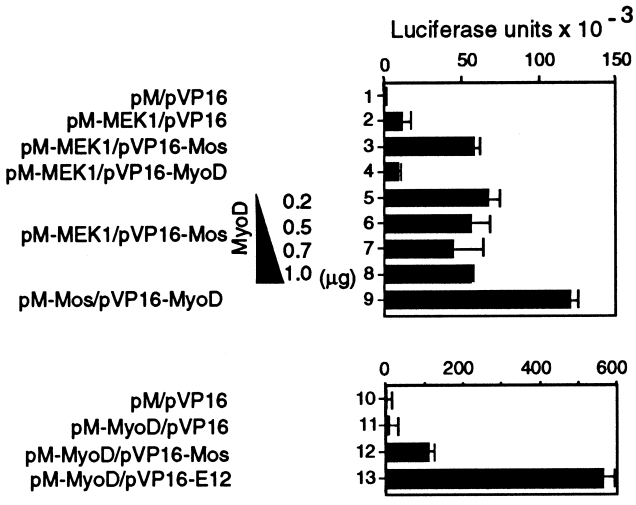

Fig. 1. Ex vivo interactions between Mos, MyoD and MEK1. 10T1/2 cells were transiently transfected with 2 μg of the pG5E1b-luciferase reporter plasmid and 10 μg of each expression plasmid. Forty-eight hours following transfection, cells maintained in DMEM supplemented with 15% FCS were harvested and luciferase activity was assayed in an aliquot equivalent to 10 μg of whole cell extracts. Differences in transfection efficiencies were corrected based upon the level of β-galactosidase activity from the co-transfected pCH110 plasmid. Each bar represents the average of at least three independent transfections.

3.2. Interaction between MyoD and Mos is not competed by MEK1

While MEK1 and MyoD could interact with the NH_2 and COOH domain of Mos, respectively, the experiments described above suggest that MyoD and MEK1 could bind independently to each other with Mos. To test this hypothesis, we exploited an in vitro association/competition assay. GST-Mos-covered beads were first incubated with ^{35}S-labelled in vitro-translated MyoD for 2 h at 4°C (Fig. 2A), then, increasing amounts of labelled MEK1 were added to the binding reactions (Fig. 2A, lanes 9–12) and the resulting mixtures were subjected to a GST pull-down assay. Increasing amounts of MEK1 did not affect the level of MyoD bound to GST-Mos. In the converse experiment, GST-Mos-covered beads were first incubated with ^{35}S-labelled in vitro-translated

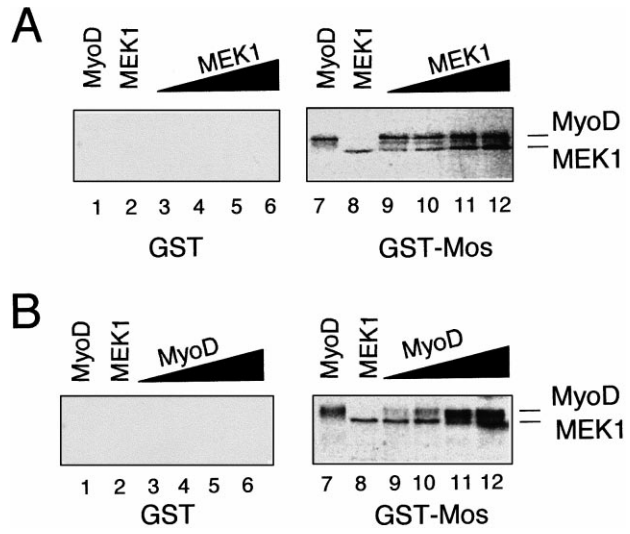

Fig. 2. MyoD does not inhibit MEK1 association with Mos. A: MyoD and MEK1 were separately in vitro-translated and 5 μl of each reaction was pre-incubated with GST alone (lanes 1 and 2) or GST-Mos fusion protein (lane 7 and 8). Five μl of MEK1 was also pre-incubated for 2 h at 4°C with GST alone (lane 3–6) or GST-Mos (lane 8–12) and then, 1, 2, 5 and 9 μl of in vitro-translated MyoD was added to the binding reaction and incubation was further continued for 1 h. After washing, binding proteins were analyzed by SDS-PAGE and autofluorography. B: The experiment was carried out as in A except that 5 μl of in vitro-translated MyoD was first incubated with GST alone or GST-Mos fusion protein. Then, increasing amounts (1–9 μl) of in vitro-translated MEK1 were added.

MEK1 (Fig. 2B) and increasing amounts of labelled MyoD were added to the binding reaction (Fig. 2B, lanes 9–12). When increasing amounts of MyoD were added to the mixture, GST-Mos-MEK1 complexes were not disrupted (Fig. 2B, lanes 9–12). Even if probably, a portion of GST-Mos is not fully saturated by MyoD (Fig. 2A) or MEK1 (Fig. 2B)

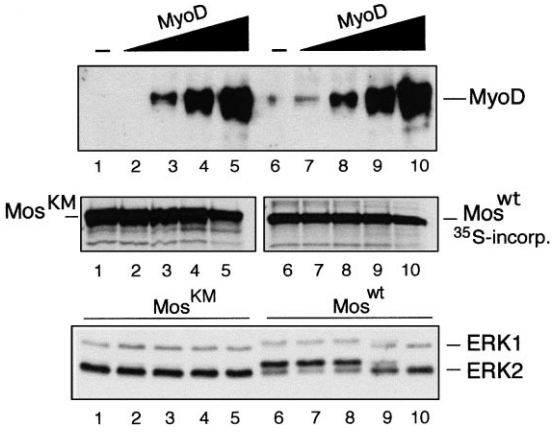

Fig. 3. Inhibitory effect of MyoD on Mos-mediated MAPK phosphorylation in vitro. Mos^{KM} (lanes 1–5) or Mos^{wt} (lanes 6–10) proteins were synthesized in rabbit reticulocyte lysates in the absence (lanes 1 and 6) or in the presence of increasing amounts of bacterially produced MyoD (1.5 ng–45 ng/ml) (lanes 2–5 and 7–10) at 30°C for 1 h. Samples (2 μl) were analyzed by blotting with antibodies against MyoD or ERK1 and ERK2 MAPKs. In vitro-translated Mos proteins were detected by SDS-PAGE and autoradiography.

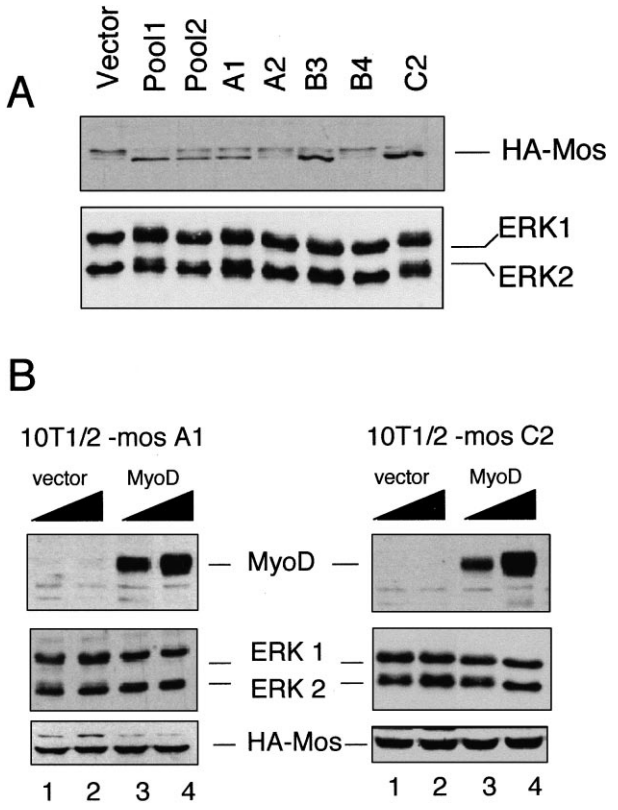

Fig. 4. MAPK activity in 10T1/2-mos clones after transfection of MyoD. A: 10T1/2 cells were stably transfected with pCMC-HA-Mos^{wt} and whole cell lysates (10 μg) were analyzed by a Western blot for Mos expression and activation of the MEK/ERK kinase pathway. B: 10T1/2-mos clones A1 and C2 were transiently transfected with 0.25 and 1 μg of pEMSV vector (lanes 1 and 2) or 0.25 and 1 μg of pEMSV-MyoD (lanes 3 and 4). Protein samples were separated by SDS-PAGE, transferred to nitrocellulose and analyzed for expression of exogenous HA-Mos^{wt}, MyoD and ERK1/ERK2 by Western blots using anti-HA monoclonal antibody, anti-MyoD or anti-EKR1/ERK2 polyclonal antibodies and visualized by ECL (Amersham).

and can associate with increasing amounts of MEK1 (Fig. 2A) or MyoD (Fig. 2B). Altogether, our results strongly suggest that MEK1 and MyoD do not compete for Mos interaction.

3.3. *MyoD inhibits Mos-mediated MEK1 and MAPK activation*

To study the effects of MyoD on Mos-mediated MEK and MAPK activation, we used an in vitro kinase assay. The translation of *mos* mRNA in rabbit reticulocyte lysates induces the phosphorylation and activation of endogenous MAPK family members, ERK1 and ERK2, detected by their reduced electrophoretic mobilities on SDS-PAGE (bandshifts) [11,12]. Increasing amounts of purified bacterially produced MyoD protein were added to reticulocytes in which Mos protein was translated. Samples were removed after incubation and assayed for ERK1 and ERK2 bandshifts by anti-ERK1/ERK2 immunoblotting. Mos proteins were efficiently translated and ERK1/ERK2 bandshifts were observed with Mos^{wt} but not with Mos^{KM} [2]. In contrast, ERK1 and ERK2 bandshifts were inhibited in the presence of increasing amounts of MyoD (Fig. 3, lanes 6–10). The reduction in ERK phospho-

rylation caused by MyoD was not due to a decrease in Mos translation nor an inhibition in Mos kinase activity as evidenced by the reduced mobility of MyoD (Fig. 3, lane 10). Moreover, we previously showed that MyoD is a substrate for phosphorylation by the Mos^{wt} kinase protein and the ability of Mos^{wt} to phosphorylate MyoD was correlated with the amount of Mos protein being found in the reticulocyte lysates [2]. These data strongly suggest that MyoD inhibits Mos-mediated MAPK phosphorylation by interacting with Mos.

To assay the effect of MyoD on Mos kinase activity in vivo, Mos was first transfected in 10T1/2 cells. Stably transfected cells expressed a biologically active Mos protein as evidenced by ERK1/ERK2 bandshifts (Fig. 4A). To assess the role of MyoD in the repression of the Mos/MAPK pathway, the 10T1/2-mos cell lines stably expressing Mos protein (clones A1 and C2) were transiently transfected with a MyoD expression vector and the phosphorylation and activation of endogenous MAPK family members, ERK1 and ERK2, were detected by their reduced electrophoretic mobilities on SDS-PAGE. As shown in Fig. 4B, increasing amounts of empty vector did not modify the Mos-mediated MAPK phosphorylation (lanes 1 and 2). In contrast, MyoD repressed MAPK activation in a dose-dependent manner (lanes 3 and 4) while the expression of Mos was not modified in both clones.

In contrast to Ras and Raf oncoprotein transformation that used multiple signalling pathways [13,14], the MEK1/MAPK pathway is necessary and sufficient for transformation by Mos [7]. Our results indicate that MyoD is able to form a heterotrimeric complex with Mos and MEK1 both in vitro and in vivo leading to the inhibition of MEK1 kinase. The existence of this complex could explain how Mos is converted into a muscle specific regulator on myogenic cell differentiation.

Acknowledgements: Mrs. K. Pelpel is a fellow of the Ministère de la Recherche et de la Technologie (MRT). This work was supported by the Institut National de la Santé et de la Recherche Médicale, The Centre National de la Recherche Scientifique and grants from Association Française contre les Myopathies (AFM), Ligue Nationale contre le Cancer, Association pour la Recherche sur le Cancer (Grant no. 6829) and the Institut Gustave Roussy.

References

[1] Leibovitch, M.P., Solhonne, B., Guillier, M., Verelle, P. and Leibovitch, S.A. (1995) Oncogene 10, 251–260.
[2] Lenormand, J.L., Benayoun, B., Guillier, M., Vandromme, M., Leibovitch, M.P. and Leibovitch, S.A. (1997) Mol. Cell. Biol. 17, 584–593.
[3] Benayoun, B., Pelpel, K., Solhonne, B., Guillier, M. and Leibovitch, S.A. (1998) FEBS Lett. 437, 39–43.
[4] Nebrada, A.R. and Hunt, T. (1993) EMBO J. 12, 1979–1986.
[5] Posada, J., Yew, N., Ahn, N., Vande Woude, G.F. and Cooper, J.A. (1993) Mol. Cell. Biol. 13, 2546–2553.
[6] Resing, K.A., Mansour, S.J., Hermann, A.S., Johnson, R.S., Candia, J.M., Fukasawa, K., Vande Woude, G.F. and Ahn, N.G. (1995) Biochemistry 34, 2610–2620.
[7] Okasaki, K. and Sagata, N., Oncogene 10, 1149–1157.
[8] Okasaki, K. and Sagata, N. (1995) EMBO J. 14, 5048–5059.
[9] Boussif, O., Lezoualc'h, F.M., Zanta, A., Mergy, M.D., Scherman, D., Demeix, B. and Behr, J.P. (1995) Proc. Natl. Acad. Sci. USA 92, 7297–7301.
[10] Leibovitch, S.A., Guillier, M., Lenormand, J.L. and Leibovitch, M.P. (1991) Oncogene 6, 1617–1622.
[11] Chen, M. and Cooper, J.A. (1995) Mol. Cell. Biol. 15, 4727–4734.
[12] Shibuya, E.K., Morris, J., Rapp, U.R. and Ruderman, J.V. (1996) Cell. Growth Differ. 7, 235–241.
[13] Weyman, C.M., Ramocki, M.B., Taparowsky, E.J. and Wolfman, A. (1997) Oncogene 13, 697–704.
[14] Ramocki, M.B., Johnson, S.E., White, M.A., Ashendel, C.L., Konieczny, S.F. and Taparowsky, E.J. (1997) Mol. Cell. Biol. 7, 3547–3555.