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An Analysis of Mek1 Signaling in Cell Proliferation and Transformation*

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The Mek1 dual specificity protein kinase phosphorylates and activates the mitogen-activated protein kinases Erk1 and Erk2 in response to mitogenic stimulation. The molecular events downstream of Mek and Erk necessary to promote cell cycle entry are largely undefined. In order to study signals emanating from Mek independent of upstream proteins capable of activating multiple signaling pathways, we fused the hormone-binding domain of the estrogen receptor (ER) to the C terminus of constitutively activated Mek1 phosphorylation site mutants. Although 4-OH-tamoxifen stimulation of NIH-3T3 cells expressing constitutively activated Mek-ER resulted in only a small increase in specific activity of the fusion protein, a 5–10 fold increase in total cellular Mek activity was observed over a period of 1–2 days due to an accumulation of fusion protein. Induction of constitutively activated Mek-ER in NIH-3T3 cells resulted in accelerated S phase entry, proliferation in low serum, morphological transformation, and anchorage independent growth. Endogenous Erk1 and Erk2 were phosphorylated with kinetics similar to the elevation of Mek-ER activity. However, elevated Mek-ER activity attenuated subsequent stimulation of Erk1 and Erk2 by serum. 4-OH-tamoxifen stimulation of Mek-ER-expressing fibroblasts also resulted in up-regulation of cyclin D1 expression and down-regulation of p27^{Kip1} expression, establishing a direct link between Mek1 and the cell cycle machinery.

The transduction of mitogenic signals from the cell membrane to the nucleus involves a cascade of protein binding events and modifications, including a series of phosphorylations resulting in the successive activation of several protein kinases. The intensively studied Ras-MAP¹ kinase pathway exemplifies these signaling cascades. Activation of growth factor receptors stimulates nucleotide exchange on the Ras low molecular weight GTP-binding protein (1–3), which then participates in activation of the Raf-1 family of serine/threonine kinases (4). Activated Raf phosphorylates and activates the

Mek1 and Mek2 dual specificity kinases, shown to be responsible for phosphorylating the MAP kinases Erk1 and Erk2 on threonine and tyrosine, thus activating them in response to mitogenic stimulation (5–7).

Stimulation of the Ras-MAP kinase pathway ultimately leads to cell proliferation. Ras transformation has been linked to the cell cycle machinery by elevation of cyclin D1 levels in G₁, concomitant with down-regulation of the cyclin-dependent kinase inhibitor p27^{Kip1} (8, 9). It has been shown that overexpression of D-type cyclins can accelerate G₁ and contribute to fibroblast transformation (10, 11). Consistent with these results, the requirement for Ras function in induction of cell proliferation in response to mitogenic signaling can be obviated by overexpression of cyclin D1 (9). Elevated cyclin D1 levels were observed in fibroblasts expressing activated c-Raf-1 (12, 13), as well as constitutively activated Mek1.² However, the molecular events that lead to elevated levels of cyclin D following Erk activation remain murky.

Mek1 and Mek2 (14–16), which share 81% identity, consist almost entirely of a conserved kinase domain flanked by short sequences of lesser homology. Raf-1 activates Mek1 and Mek2 by phosphorylation of 2 serine residues, amino acids 218 and 222 in Mek1 (17–20). Replacement of these two serines with acidic residues results in constitutive activation of Mek enzymatic activity, and overexpression of constitutively active phosphorylation site mutants results in fibroblast transformation (21–26). Phosphorylation of Erk1 and Erk2 on threonine and tyrosine residues in the conserved sequence “TEY” located in the Erk catalytic domain (27, 28) accompanies Mek-induced transformation in most but not all (26) systems.

One limitation of the studies done thus far is that they involve constitutive expression of the activated forms of Mek1. Prolonged passage of cells in the presence of a growth- and transformation-promoting protein raises the issues of autocrine loops, accumulated growth-promoting mutations, and acclimation to the transformed milieu, complicating interpretation of results. In an attempt to circumvent some of these problems, we explored a system that permits the regulation of activated Mek1 expression. A modified estrogen receptor hormone-binding domain (ER HBD) containing a point mutation rendering the HBD unable to bind estrogen while retaining affinity for the synthetic ligand 4-hydroxytamoxifen (4-OH-tamoxifen) has been used to conditionally regulate heterologous proteins (29, 30). In this study, the mutated ER HBD was fused to the C terminus of constitutively activated forms of Mek1 to facilitate investigation of early events in cell proliferation initiated by Mek1 in isolation of parallel pathways that may be activated by extracellular mitogens.

MATERIALS AND METHODS

DNA Construction—200 nucleotides from the 5' end of the cDNA encoding the hormone-binding domain (HBD) of the murine estrogen

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¹ The abbreviations used are: MAP, mitogen-activated protein; ER, estrogen receptor; HBD, hormone-binding domain; GST, glutathione S-transferase; bp, base pair; Mek, MAP kinase/extracellular signal-regulated kinase kinase; TM, tamoxifen mutant.

² Q. Hou and R. Erikson, unpublished data.

receptor (ER) containing the "tamoxifen mutant" (TM) point mutation (30) was amplified by polymerase chain reaction using the following primers: 5'-AATATGCTAGCATGGGTGCTTCAGGAGACA-3' and 5'-CACACAGTCGACGGGTCTAGAAGGATCATA-3', introducing novel *NheI*, *SalI*, and *XbaI* sites, underlined in that order. 140 nucleotides from the 3' end of the cDNA encoding wild-type murine Mek1 in SK⁺Mek 4-3A (5) were amplified by polymerase chain reaction using the following primers: 5'-AACCTGCAGAGAGAGCA-3' and 5'-TTATCGATGCTAGCTCCGATGCTGGCAGCGTGGGT-3', introducing novel *PstI*, *ClaI*, and *NheI* sites, underlined in that order. The 140-base pair (bp) Mek1 fragment was digested with *PstI* and *ClaI* and inserted into Bluescript SK-(Stratagene). This construct was digested with *NheI* and *SalI*, into which the *NheI*-*SalI*-cut 200-bp fragment of the ER HBD was subcloned, resulting in the insertion of Gly-Ala-Ser between Ile³⁹² of Mek1 and Met²⁸⁴ of the ER HBD. This construct was confirmed by sequence analysis and digested with *SmaI* and *PstI*, into which the N-terminal 300-bp blunt/*PstI* fragment of Mek1 was inserted, followed by digestion with *PstI* and insertion of the DS (Asp²¹⁸) or DD (Asp²¹⁸, Asp²²²) Mek1 phosphorylation site mutant (23) 700-bp *PstI* fragments. Restriction sites between the polylinker *SpeI* and *NotI* were removed, and the resulting construct was cut with *SalI* and *XbaI*, into which site the 3' 800-bp of the ER HBD (*SalI*-*XbaI* fragment) was inserted. Finally, the entire cDNAs encoding the Mek1-ER fusions were cut out of Bluescript with *BamHI* and *SalI* and inserted into *BamHI*-*SalI*-digested pBabe-puro.

Cell Culture and Transfection—NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. Calcium phosphate transfections were done using a buffer kit from 5 Prime → 3 Prime, Inc., Boulder, CO. Transfected cells were selected in 2 μ g/ml puromycin (Sigma) for 12 days, and individual drug-resistant colonies were isolated. Fusion constructs were induced using 100 nM 4-hydroxy-tamoxifen (Research Biochemicals), typically for 24 h. Unless otherwise noted, cell count experiments were performed by seeding 6-cm plates with 6.5×10^4 cells in DMEM supplemented with 10% calf serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin, incubating for 2 days, replacing medium with that containing 0.5% calf serum with or without 100 nM 4-OH-tamoxifen, then counting and/or photographing cells over the subsequent 3 days.

Colony formation was assayed by suspending 5×10^5 cells in DMEM containing 10% calf serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, 100 nM tamoxifen, and 0.25% agar, plated on a layer of DMEM containing 10% calf serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 0.5% agar in 6-cm tissue culture plates. Plates were incubated at 37 °C for 2 weeks, and then colonies were arbitrarily divided into small (<0.5 mm) or large (≥ 0.5 mm) categories, and colonies in 5 randomly chosen 1-cm² areas were counted.

Cells were lysed in a buffer containing 10 mM Tris, pH 7.6, 1% Triton X-100, 50 mM NaCl, 30 mM NaPP_i, 50 mM NaF, 1 mM EGTA, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and 20 μ g/ml aprotinin. Protein concentrations were measured by the Bradford assay using the Bio-Rad reagent.

Flow Cytometry—Cells were placed in medium containing 0.5% calf serum simultaneously with addition of 100 nM 4-OH-tamoxifen. 24 h later, cells were trypsinized and resuspended in 100 μ l of cold phosphate-buffered saline containing 0.1% dextrose, to which 3 ml of 70% ethanol was added, incubated on ice for 30 min, and spun down. Pellets were resuspended in 40 mM sodium citrate, pH 7.4, containing 70 μ M propidium iodide and 100 μ g/ml RNase, incubated at 37 °C for 30 min, and analyzed by fluorescence-activated cell sorting using the Cellquest program (Becton Dickinson).

Immunoblotting—Whole cell lysates or immunoprecipitates separated by SDS-polyacrylamide gel electrophoresis were electrophoretically transferred to polyvinylidene difluoride membranes, blocked in Tris-buffered saline (20 mM Tris, pH 7.4, 150 mM NaCl) containing 0.1% Tween 20 and 5% bovine serum albumin, probed with the relevant antibody (all secondary antibody incubations were done in Tris-buffered saline containing 0.1% Tween 20 and 5% milk), and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). Mek-ER fusion proteins were detected with monoclonal anti-ER Ab-1 (Oncogene Research/Calbiochem) or monoclonal anti-Mek1 3D9 (Zymed); endogenous Mek1 was detected with monoclonal anti-Mek1 3D9 (Zymed). Activation-specific phosphorylated species of Erk1 and Erk2 were detected with polyclonal phospho-specific Erk (New England Biolabs), cyclin D1 with monoclonal anti-cyclin D1 72-13G (Santa Cruz), and Kip1 with monoclonal anti-p27 Kip1 (Transduction Laboratories).

Immunoprecipitation—Mek-ER fusion proteins were immunopre-

cipitated from lysates prepared as described above with anti-ER Ab-1 (Oncogene Research/Calbiochem) and protein G-agarose (Zymed or Santa Cruz).

Immunocomplex Kinase Assays—Mek-ER fusion protein kinase activity was measured as follows. Anti-ER immunoprecipitates (generally from 100 μ g of cell lysate) were washed twice with lysis buffer and once with kinase buffer (50 mM Tris, pH 8.0, 10 mM MgCl₂, 1 mM EGTA, 5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin) and split into two tubes. Half of each immunoprecipitate was used for immunoblotting, and half of each immunoprecipitate was incubated at 30 °C for 15 min in 20 μ l of kinase buffer containing 10 μ Ci [γ -³²P]ATP, 25 μ M cold ATP, and 1 μ g GST-Erk1 K63M per sample. Reactions were terminated by addition of an equal volume of SDS sample buffer containing 50 mM EDTA, boiled for 5 min, and separated by 8% SDS-polyacrylamide gel electrophoresis. Gels were Coomassie-stained, dried, and exposed to film. Gel sections containing Coomassie-stained substrate and autophosphorylated fusion protein were quantitated by scintillation counting.

RESULTS

Biochemical Analysis of Fibroblasts Expressing Mek1-ER Fusion Proteins—The constitutively activated Mek1 phosphorylation site mutants Asp²¹⁸ (DS) and Asp²¹⁸,Asp²²² (DD) exhibit elevated kinase activity toward kinase-inactive GST-Erk1 and transform fibroblasts in tissue culture (23, 26). The DD form is more active and more transforming than Mek1-DS, consistent with the introduction of a constitutive negative charge at the two sites required to be phosphorylated for full activation of wild-type Mek1. In order to conditionally regulate activity of these Mek1 phosphorylation site mutants, fusion constructs were engineered in which the coding sequence for the TM mutant (30) of the estrogen receptor hormone-binding domain (ER HBD) was placed in frame at the 3' end of the Mek1 cDNAs (construction described under "Materials and Methods"). These fusion constructs were then inserted into the expression vector pBabe-puro, creating pBp Mek1-DSER and pBp Mek1-DDER. NIH-3T3 cells were transfected with pBp Mek1-DSER, pBp Mek1-DDER, or empty pBp vector and selected in puromycin. Stable clonal lines were isolated and screened for basal levels of fusion protein expression by whole cell lysate immunoblot analysis using an anti-ER antibody (data not shown). Representative lines exhibiting high levels of expression were used in the following experiments.

Cells expressing empty vector, Mek1-DSER or Mek1-DDER, were treated for 24 h with 100 nM 4-OH-tamoxifen and lysed. Fusion protein kinase activity was measured in the absence of endogenous Mek1 by anti-ER immune complex kinase assay, using kinase inactive GST-Erk1 K63M as a substrate (Fig. 1A). A 5–10-fold increase in fusion protein kinase activity toward kinase-inactive GST-Erk1 was observed after tamoxifen treatment; in the experiment shown, Mek1-DSER activity was increased 7-fold, and Mek1-DDER activity was increased 6.5-fold. A concomitant increase in fusion protein autophosphorylation was also observed. Treatment with an equal volume of ethanol vehicle had no effect on fusion protein kinase activity (data not shown). Half of each immunoprecipitate used in the kinase assay in Fig. 1A was immunoblotted with anti-ER to detect protein levels in each immunoprecipitate. Significantly, the 4-OH-tamoxifen-stimulated lysates exhibited higher levels of precipitable fusion protein contributing to the observed kinase activity (Fig. 1B), suggesting that the increase in observed kinase activity was not due to an increase in specific activity.

The increase in fusion protein expression was confirmed by whole cell lysate immunoblot analysis (Fig. 1C). Elevation of ER fusion protein levels in response to hormone induction, possibly due to protein stabilization, has been previously described (31, 32). Because the expression levels of Mek1-DDER are slightly higher than those of Mek1-DSER, the experiment was repeated normalizing for the amount of fusion protein in

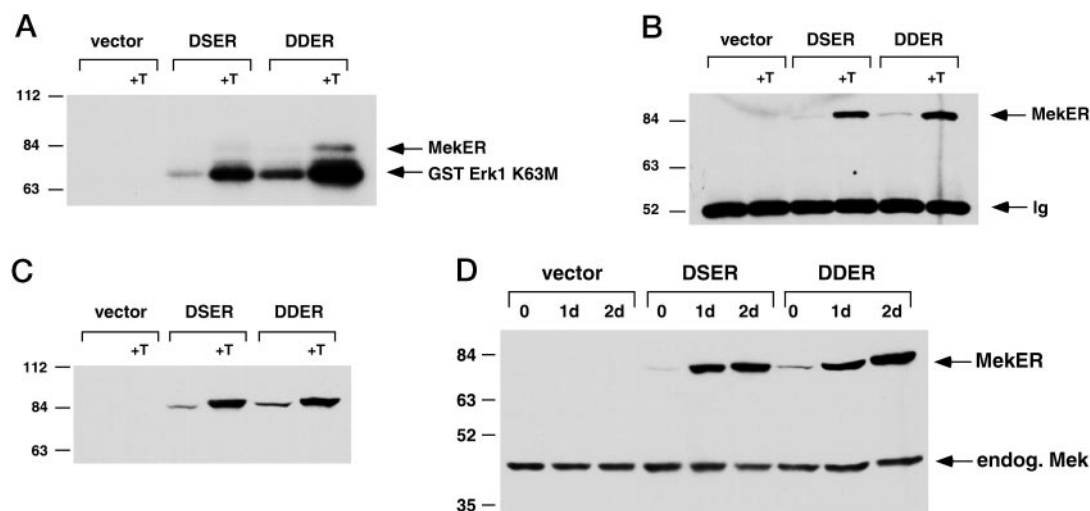


FIG. 1. Tamoxifen treatment induces Mek-ER activity in proportion to the increase in fusion protein levels. A, NIH-3T3 cells expressing Mek1-DSER, Mek1-DDER, or empty vector were stimulated 24 h with 100 nM 4-OH-tamoxifen (+T). Cells were lysed, Mek-ER proteins were immunoprecipitated with anti-ER, and immunoprecipitates were split into two tubes. Half of each immunoprecipitate was used in an *in vitro* kinase assay on kinase-inactive GST-Erk1-K63M, and B, the other half was subjected to immunoblot analysis with anti-ER. C, the stimulated lysates described in A were used in a whole cell lysate anti-ER immunoblot. D, whole cell lysate anti-Mek immunoblot. NIH-3T3 expressing Mek1-DSER, Mek1-DDER, or empty vector were stimulated for the indicated number of days with 100 nM 4-OH-tamoxifen. Ig, immunoglobulin; d, days; endog., endogenous.

each immunoprecipitate. The resulting basal Mek1-DDER activity was 4-fold higher than basal Mek1-DSER activity, whereas 4-OH-tamoxifen-stimulated Mek1-DDER activity was 2.5-fold higher than stimulated Mek1-DSER activity (data not shown). Basal and induced levels of fusion protein expression were also compared with that of endogenous Mek1 by whole cell lysate anti-Mek1 immunoblot analysis (Fig. 1D). Whereas the uninduced Mek1-DSER and Mek1-DDER proteins were expressed at only about 10% the level of endogenous Mek1 protein, 24–48 h of tamoxifen stimulation led to an increase in fusion protein expression to levels 3–5-fold greater than that of endogenous Mek1, comparable to levels in previously described fibroblasts constitutively expressing these activated Mek1 phosphorylation site mutants (26).

The kinetics of induction of fusion protein levels and activity upon addition of 100 nM 4-OH-tamoxifen were then examined. No large increases in kinase activity were observed until 6–12 h after hormone addition (Fig. 2A), at which time the levels of fusion protein began to increase as well (Fig. 2B). However, a slight but reproducible increase in specific activity, about 1.5-fold, was detected as early as 15 min after 4-OH-tamoxifen stimulation (data not shown). Thus, unlike previous reports using ER HBD fusion proteins (12, 32), we observed only a barely detectable immediate increase in fusion protein-specific activity, followed by a more robust delayed response due to fusion protein accumulation. The levels of fusion protein induced by 4-OH-tamoxifen addition peaked 48 h after hormone addition and remained elevated for at least 12 weeks, the latest time point tested (Fig. 2C and data not shown). Anti-ER-precipitable kinase activity remained elevated as well (Fig. 2D and data not shown).

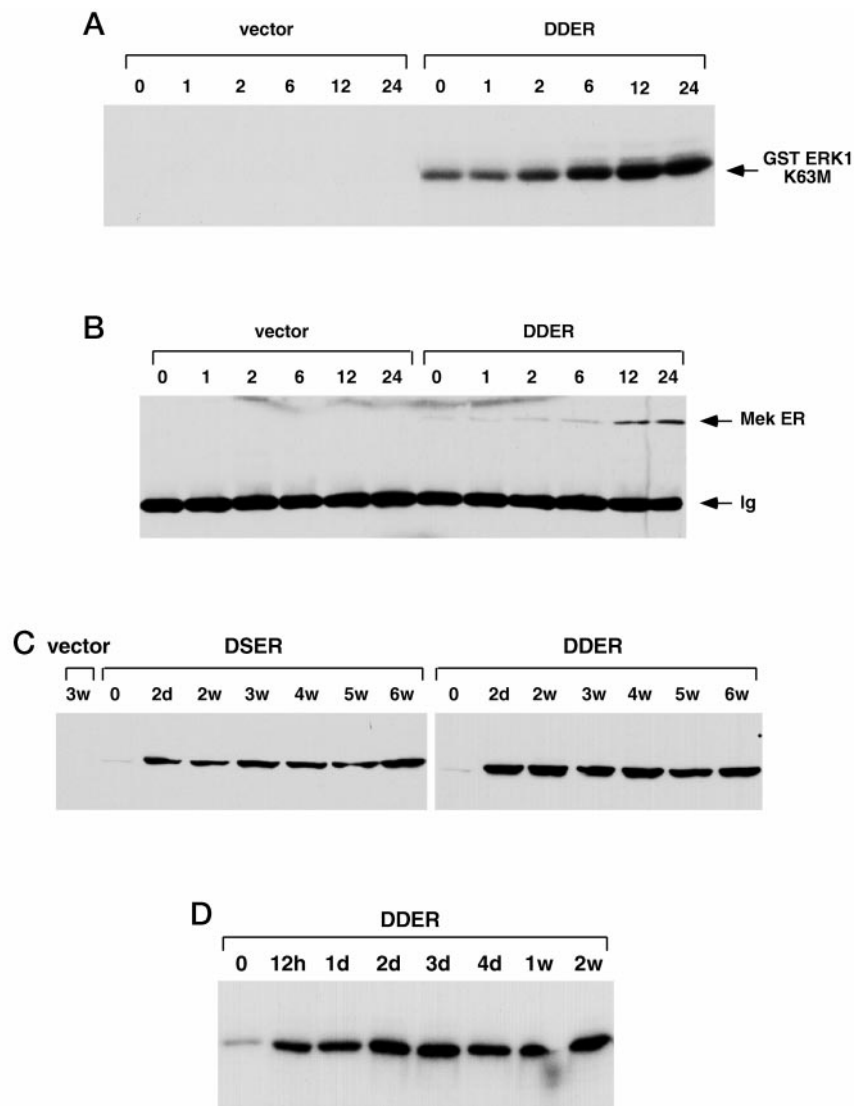
4-OH-tamoxifen titration indicated that maximal stimulation of fusion protein expression and kinase activity occurred between 50 and 100 nM (data not shown). Subsequent experiments therefore involved stimulation with 100 nM 4-OH-tamoxifen unless otherwise noted. Removal of 4-OH-tamoxifen from the medium resulted in complete elimination of the induced fusion protein over a 2-day period, and it should be noted that there was no further elevation of fusion protein levels or kinase activity by repeated addition of hormone once a day for 3 days as compared with a single addition for 3 days (data not shown).

Effects of Induction of Mek-ER on Cell Growth—The cells were first examined for the ability to proliferate in low serum. 6×10^4 cells transfected with vector or Mek1-DDER were plated and grown for 2 days in complete medium containing 10% calf serum. The cells were then placed in medium containing low (0.5%) calf serum and treated with 100 nM 4-OH-tamoxifen or an equal volume of ethanol vehicle for 24 h and then counted each of the following 3 days. Whereas the number of vector-transfected and unstimulated Mek1-DDER cells peaked at day 3 and then decreased, the number of 4-OH-tamoxifen-stimulated Mek1-DDER cells continued to increase until day 5 (Fig. 3A). At no time during this representative experiment did any of the plates reach confluence, indicating that the inhibition of growth was not due to contact inhibition (Fig. 3B). Stimulation of Mek1-DSER cells with 4-OH-tamoxifen resulted in an intermediate growth phenotype (Fig. 3C), while untreated Mek1-DDER cells grown continuously in 10% calf serum proliferated three times as fast as Mek1-DDER cells treated with 4-OH-tamoxifen but grown in low serum (data not shown).

The effects of Mek1-DDER on the cell cycle were analyzed by flow cytometry (Fig. 4). Cells were placed in 0.5% serum and treated with 100 nM 4-OH-tamoxifen or ethanol vehicle for 24 h prior to analysis. Although no significant effect of Mek1-DDER induction on G₂/M was detected, a 3–4-fold increase in the percentage of cells in S phase was observed following 4-OH-tamoxifen treatment. The percentage of Mek1-DDER cells in S phase peaked after 18 h of 4-OH-tamoxifen treatment and slowly declined over a period of 48 h (data not shown).

Characterization of Fusion Protein-induced Transformation—Constitutive expression of activated Mek1 phosphorylation site mutants has been previously shown to transform fibroblasts; however, the kinetics of cell transformation by these activated kinases were in some cases delayed, raising the issue of whether secondary effects were involved (21, 22, 25, 26). The ability of activated Mek1-ER to transform cells upon 4-OH-tamoxifen stimulation was therefore examined. When grown in complete medium containing 10% calf serum, the cells expressing Mek1-DDER exhibited morphological alteration and were more refractile within 24 h of hormone addition (Fig. 5A), a time at which significant levels of fusion protein and anti-ER-precipitable kinase activity have accumulated (Fig. 2,

FIG. 2. Kinetics of induction of Mek-ER fusion protein activity. *A*, anti-ER immune complex kinase assay on kinase-inactive GST-Erk1. NIH-3T3 cells expressing Mek1-DDER or empty vector were stimulated for the indicated number of hours with 100 nM 4-OH-tamoxifen. *B*, anti-ER immunoblot on the anti-ER immunoprecipitates described in *A*. *C*, whole cell lysate anti-ER immunoblot. NIH-3T3 cells expressing Mek1-DSEr, Mek1-DDER, or empty vector were stimulated for the indicated length of time with 100 nM 4-OH-tamoxifen. *D*, anti-ER immune complex kinase assay on kinase-inactive GST-Erk1. Cells were stimulated for the indicated length of time with 100 nM 4-OH-tamoxifen. *Ig*, immunoglobulin; *d*, days; *w*, weeks.



A and *B*). No morphological alterations were observed in the Mek1-DSEr expressing cells after 24 h 4-OH-tamoxifen stimulation (Fig. 5A), consistent with Mek1-DSEr activity in these cells at this time point, 2-fold greater than that of unstimulated Mek1-DDER (data not shown). However, prolonged treatment of Mek1-DSEr-expressing NIH-3T3 cells with 100 nM 4-OH-tamoxifen did result in detectable morphological changes, although not to the extent observed in Mek1-DDER-expressing NIH-3T3 cells (Fig. 5B). Mek1-DDER cells exhibited a similar change in morphology when placed in medium containing low serum simultaneously with 4-OH-tamoxifen addition, in stark contrast to the flattened, quiescent control cells (Fig. 3B).

The ability of the Mek1-ER expressing cells to grow in an anchorage-independent manner was then analyzed by colony formation in soft agar. NIH-3T3 cells transfected with vector alone, Mek1-DSEr, or Mek1-DDER were stimulated for 24 h with 100 nM 4-OH-tamoxifen or left unstimulated and were plated in soft agar medium with or without 100 nM 4-OH-tamoxifen. Plates were incubated 12 days, then the number of colonies were counted. No colonies were observed on vector or Mek1-DSEr plates in the absence of hormone stimulation (Table I), although a low level of background colony formation was seen on the Mek1-DDER plate, probably due to the basal level of anti-ER-precipitable kinase activity in these cells (Fig. 1A). The number of colonies on the 4-OH-tamoxifen-stimulated Mek1-DDER plate was several orders of magnitude greater

than that on the unstimulated plate. Only a small number of colonies was observed on the 4-OH-tamoxifen-stimulated Mek1-DSEr plate, comparable to the unstimulated Mek1-DDER plate.

Fusion Protein Induction of Erk Phosphorylation—One potential advantage of this inducible system is in examination of the kinetics of downstream signaling protein activation in response to induction of activated Mek. Activation of the known substrates of Mek1, the MAP kinases Erk1 and Erk2 (5–7), was first examined. As expected, time course analysis of activation-specific Erk phosphorylation indicated that the appearance of phosphorylated endogenous Erk2 coincided with accumulation of Mek1-DDER, approximately 6 h after stimulation with 4-OH-tamoxifen (Fig. 6A). Erk2 activation-specific phosphorylation peaked 2 days after hormone stimulation of Mek1-DDER and remained elevated for at least 12 weeks, the last time point examined (Fig. 6B and data not shown). This is in contrast to results from a set of cell lines constitutively expressing activated Mek1-DD, in which basal levels of endogenous Erk activity are comparable to levels in vector-transfected cells (26). Induction of Mek1-DSEr resulted in only a slight increase in Erk2 phosphorylation after 2 days of hormone treatment. Erk1 phosphorylation is less sensitive in this assay and is only observed when exceptionally high levels of Erk2 phosphorylation are present (such as the *last lane* in the *middle panel* of Fig. 6B).

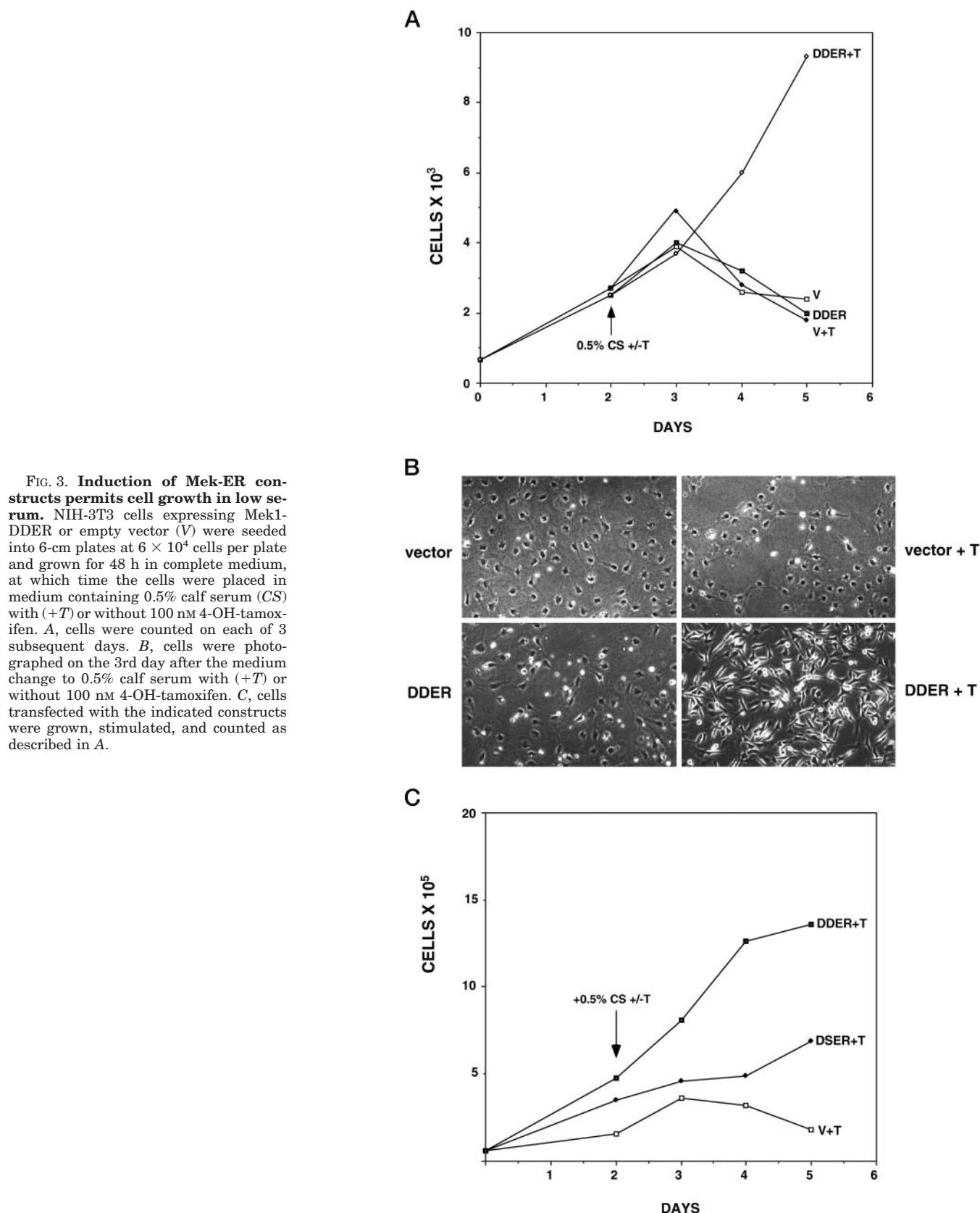


FIG. 3. Induction of Mek-ER constructs permits cell growth in low serum. NIH-3T3 cells expressing Mek1-DDER or empty vector (V) were seeded into 6-cm plates at 6×10^4 cells per plate and grown for 48 h in complete medium, at which time the cells were placed in medium containing 0.5% calf serum (CS) with (+T) or without 100 nM 4-OH-tamoxifen. **A**, cells were counted on each of 3 subsequent days. **B**, cells were photographed on the 3rd day after the medium change to 0.5% calf serum with (+T) or without 100 nM 4-OH-tamoxifen. **C**, cells transfected with the indicated constructs were grown, stimulated, and counted as described in **A**.

Because serum stimulation results in activation of wild-type Mek via a signal transduction cascade culminating in activation of the Mek activator c-Raf-1 (33), serum stimulation of cells expressing the Mek1-ER fusion proteins was analyzed. NIH-3T3 cells transfected with empty vector, Mek1-DSER or Mek1-DDER, were placed in medium containing low serum for

24 h with simultaneous addition of 100 nM 4-OH-tamoxifen ("+T") or an equal volume of ethanol vehicle in duplicate, of which half were stimulated with medium containing 15% calf serum for 10 min ("+S") following the 24-h tamoxifen incubation, and all were lysed. When assayed for fusion protein expression by whole cell lysate anti-ER immunoblot analysis,

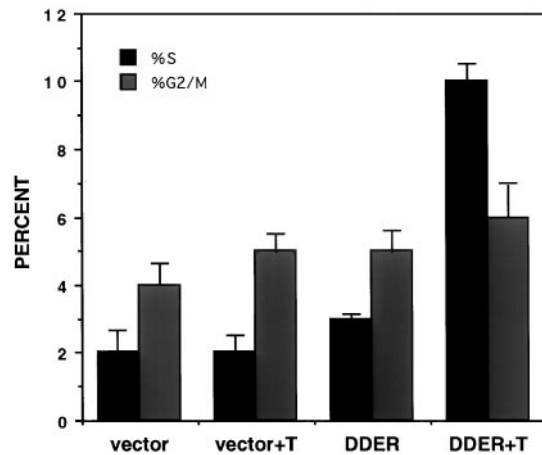


FIG. 4. Increase in S phase population in response to stimulation of Mek-ER. NIH-3T3 cells expressing Mek1-DDER or empty vector were placed in medium containing 0.5% calf serum with (+T) or without 100 nM 4-OH-tamoxifen for 24 h, stained with propidium iodide, and analyzed by flow cytometry. Results shown are the average of quadruplicate samples. Black bars, percent S phase; gray bars, percent G₂/M phase.

only the tamoxifen-treated cells exhibited elevated levels of Mek1-DSEER and Mek1-DDER (Fig. 7A, upper panel, lanes 7, 8, 11, and 12). No elevation of fusion protein levels was induced by serum stimulation. Basal levels of fusion protein expression were visible in ethanol-treated cells upon longer exposure (data not shown).

In order to examine fusion protein kinase activity in isolation of endogenous Mek1, an anti-ER immune complex kinase assay was performed on the lysates described above. No ER-precipitable kinase activity capable of phosphorylating kinase-inactive GST-Erk1 was observed in the vector-transfected cells, with or without serum or 4-OH-tamoxifen addition (Fig. 7A, lower panel, lanes 1–4). Basal levels of fusion protein activity in untreated Mek1-DSEER and Mek1-DDER cells (Fig. 7A, lanes 5 and 9) and levels of fusion protein activity in Mek1-DSEER and Mek1-DDER cells treated for 24 h with 100 nM 4-OH-tamoxifen alone (Fig. 7A, lanes 7 and 11) were consistent with previous experiments (compare with Fig. 1A). Subsequent serum stimulation increased both basal levels and hormone-stimulated levels of Mek1-DSEER specific activity (Fig. 7A, lanes 6 and 8) but not Mek1-DDER specific activity (Fig. 7A, lanes 10 and 12), consistent with the availability of a single phosphorylation site in Mek1-DSEER and the lack of remaining activation-specific phosphorylation sites in Mek1-DDER. These data suggest that phosphorylation is more efficient than acidic residue substitution for Mek1 activation, in agreement with previously reported results (34).

The activation state of endogenous Erk1 and Erk2 in response to serum stimulation of 4-OH-tamoxifen-treated cells was examined by anti-phospho-Erk immunoblot. 24-h hormone induction of Mek1-DDER but not Mek1-DSEER caused a small increase in endogenous Erk2 phosphorylation (Fig. 7B, lanes 11 and 7), consistent with the data shown in Fig. 6B. Serum stimulation elevated Erk phosphorylation above basal, unstimulated levels in all three cell types (Fig. 7B, lanes 2, 6 and 10), as well as 4-OH-tamoxifen-stimulated levels in vector-transfected and Mek1-DSEER cells (Fig. 7B, lanes 4 and 8), as expected. However, little or no additional phosphorylation of Erk was detected upon serum stimulation of the Mek1-DDER cells treated with 4-OH-tamoxifen (Fig. 7B, lane 12). Similar results were obtained by anti-Erk1 immune complex myelin basic protein kinase assay (data not shown). These data indicate the possible induction of a feedback inhibition mechanism by activated Mek1.

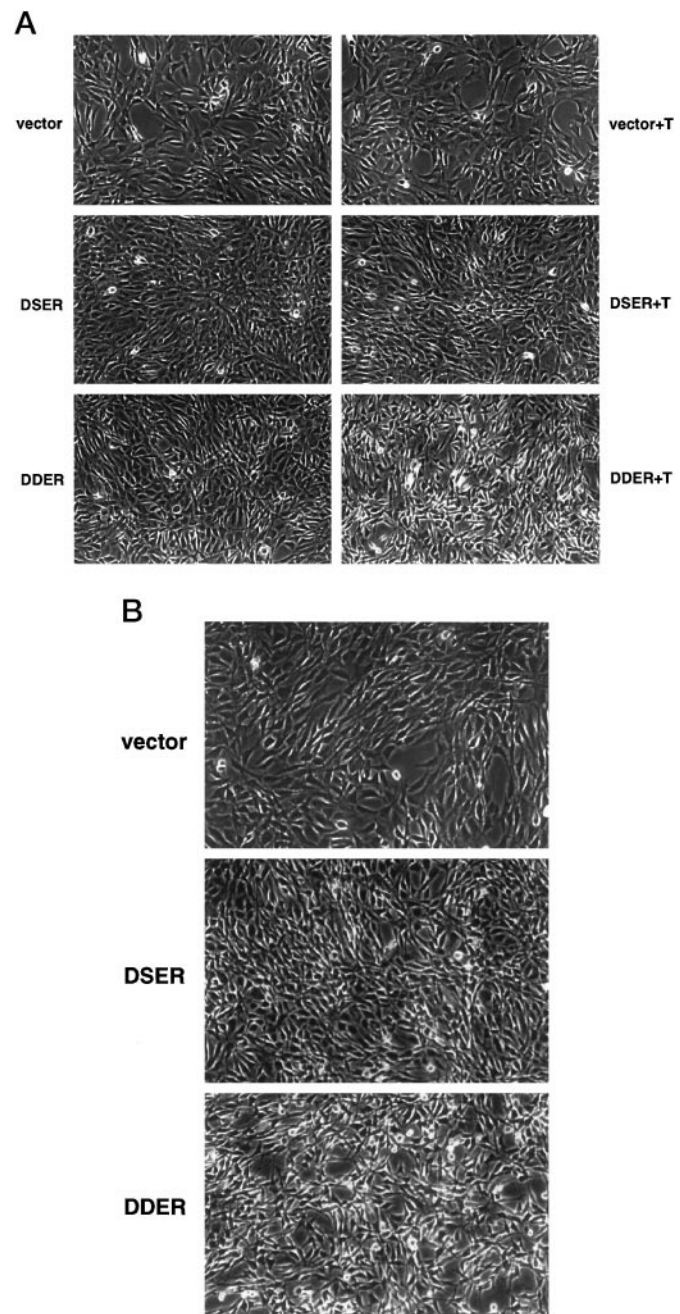


FIG. 5. Morphological transformation induced by Mek-ER. A, NIH-3T3 cells expressing the indicated construct were stimulated with 100 nM 4-OH-tamoxifen (+T) for 24 h and then photographed. B, NIH-3T3 expressing the indicated construct were stimulated with 100 nM 4-OH-tamoxifen for 1 week and then photographed.

Analysis of Cell Cycle Proteins—Effects of Ras pathway activation on cell cycle components involved in G₁ progression have recently been described (8, 9, 12, 13, 31). D-type cyclins are responsive to growth factor levels and are required for S phase entry (35, 36). Because we observed an increase in the S phase population of Mek1-DDER cells following stimulation with 4-OH-tamoxifen (Fig. 4), we examined whether Mek1-DDER affects proteins involved in cell cycle entry. Anti-cyclin D1 immunoblot analysis revealed that induction of Mek1-DDER but not Mek1-DSEER caused an elevation of cyclin D1 protein after 2 days 4-OH-tamoxifen treatment (Fig. 8A). Cyclin D1 levels remained elevated upon prolonged treatment of the Mek1-DDER cells with 4-OH-tamoxifen, whereas no detectable elevation of cyclin D1 levels was observed after prolonged

TABLE I

Growth in soft agar of tamoxifen-treated NIH-3T3 cells expressing Mek-ER fusion proteins

	<0.5 mm	>0.5 mm
Vector	0 ^a	0
Vector + T ^b	0	0
DSER	0	0
DSER + T	4	0
DDER	9	0
DDER + T	4160	480

^a Number of colonies counted in five randomly chosen 1-cm² fields.

^b T, 100 nM 4-OH-tamoxifen.

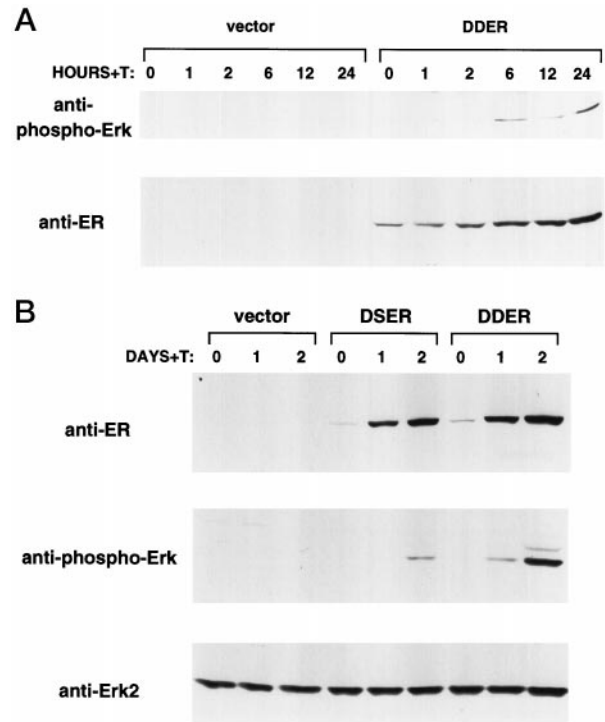


FIG. 6. Induction of Mek-ER constructs increases the phosphorylation levels of Erk. A, NIH-3T3 cells expressing Mek1-DDER or empty vector were grown in complete medium containing 10% calf serum, stimulated for the indicated number of hours with 100 nM 4-OH-tamoxifen (+T), and lysed. Upper panel, anti-phospho-Erk whole cell lysate immunoblot. Lower panel, filter was reprobed with anti-ER. B, NIH-3T3 cells expressing Mek1-DSER, Mek1-DDER, or empty vector were placed in medium containing 0.5% calf serum, stimulated for the indicated number of days with 4-OH-tamoxifen (+T), and lysed. Upper panel, whole cell lysate anti-ER immunoblot. Middle panel, filter was reprobed with anti-phospho-Erk. Lower panel, filter was reprobed with anti-Erk2.

treatment of the Mek1-DSER cells (Fig. 8B).

The cyclin-dependent kinase inhibitor p27^{Kip1} was identified by its capacity to bind cyclin D-Cdk4 and cyclin E-Cdk2 (37, 38) and appears to participate in arresting the cell cycle in response to growth factor deprivation and contact inhibition (39–41). The effects of Mek1-ER fusion protein induction on p27^{Kip1} protein levels were examined by immunoblot analysis of cells stimulated as described for Fig. 7. While a 10-min stimulation with 15% serum had no effect on p27^{Kip1} levels (Fig. 8C, lanes 2, 6 and 10), treatment of the Mek1-DSER and Mek1-DDER cells for 24 h with 4-OH-tamoxifen (Fig. 8C, lanes 7, 8, 11 and 12) reduced p27^{Kip1} Triton-soluble protein levels. These data are consistent with a reduction of p27^{Kip1} levels reported upon conditional induction of constitutively activated c-Raf-1 (12).

DISCUSSION

We have utilized an inducible system to study the downstream effects of Mek activation and its role in stimulating cell

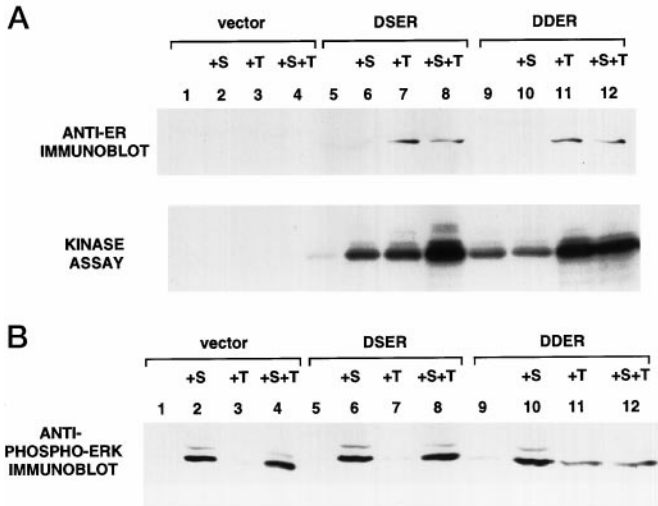


FIG. 7. Serum stimulation of cells expressing Mek-ER constructs. NIH-3T3 cells expressing the indicated construct were placed in medium containing 0.5% calf serum with (+T) or without 100 nM 4-OH-tamoxifen for 24 h. Half of the cells were then stimulated with medium containing 15% calf serum (+S) for 10 min, and all plates were lysed. A, fusion protein activity relative to expression levels. Upper panel, whole cell lysate anti-ER immunoblot. Lower panel, anti-ER immune complex kinase assay using GST-Erk1-K63M as a substrate. B, whole cell lysate anti-phospho-Erk immunoblot. Data shown are representative of four identical experiments.

growth and transformation. We focused our attention on Mek1 mutants that are constitutively active as the result of substitution of aspartate residues for serines normally phosphorylated by activated Raf. We reasoned that fusion of such mutants to the hormone-binding domain of the murine estrogen receptor would permit rapid induction of the active kinases upon treatment with 4-OH-tamoxifen. In this study only a small (1.5-fold) immediate increase in specific activity was obtained by 4-OH-tamoxifen treatment of fibroblasts expressing these fusion constructs. This small elevation of Mek1 activity did not produce any detectable change in cellular phenotype at early time points (Fig. 6A and data not shown). Within 24 h, however, an increase in fusion protein levels was observed, accompanied by a proportional increase in total cellular anti-ER-precipitable kinase activity (Fig. 1). This elevation of Mek1-ER kinase activity permitted the initiation of studies to evaluate the molecular mechanisms by which Mek1 influences the cell cycle.

24 h after 4-OH-tamoxifen addition, fusion protein levels were 3–5-fold greater than that of endogenous Mek1 (Fig. 1D), comparable to levels that have been previously described to transform fibroblasts when constitutively expressed (26). Hormone stimulation of NIH-3T3 cells expressing the doubly substituted Mek1-DDER caused morphological transformation within 24 h (Fig. 5A). Elevation of fusion protein levels and protein kinase activity was detected 6–12 h after initiation of treatment. Thus, the time necessary for Mek1 to elicit cell transformation events is no greater than 12–18 h. Stimulated Mek1-DDER cells can also grow in an anchorage-independent manner (Table I), but the kinetics of this phenotype are more difficult to analyze for technical reasons. Induction of singly substituted Mek1-DSER is only weakly transforming (Fig. 5, A and B, and Table I), consistent with its poor ability to activate Erk1 and Erk2, compared with that of Mek1-DDER (Fig. 6B).

The ability of the Mek1-DDER expressing NIH-3T3 cells to grow in low serum upon treatment with 4-OH-tamoxifen provides a further measure of a transformed phenotype (Fig. 3). As in the previous assays, Mek1-DSER induction caused only a weak cell growth response compared with Mek1-DDER. It should be noted that untreated Mek1-DDER cells maintained

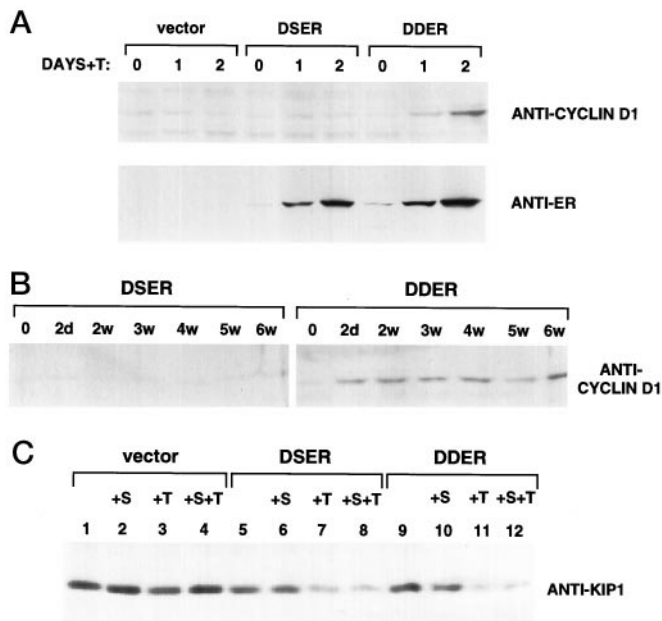


FIG. 8. Induction of Mek-ER constructs causes an elevation of cyclin D1 levels. A, NIH-3T3 cells expressing Mek1-DSER, Mek1-DDER, or empty vector were stimulated for the indicated number of days with 100 nM 4-OH-tamoxifen (+T) and lysed. Upper panel, whole cell lysate anti-cyclin D1 immunoblot. Lower panel, filter was reprobed with anti-ER. B, the cells described in A were stimulated for the indicated length of time with 4-OH-tamoxifen, lysed, and used in a whole cell lysate anti-cyclin D1 immunoblot. d, days; w, weeks. C, NIH-3T3 expressing the indicated constructs were placed in medium containing 0.5% calf serum with (+T) or without 100 nM 4-OH-tamoxifen for 24 h. Half of the cells were then stimulated with medium containing 15% calf serum (+S) for 10 min, and all plates were lysed and used in a whole cell lysate anti-p27^{Kip1} immunoblot.

in complete medium containing 10% calf serum grew three times as fast as the Mek1-DDER cells placed in low serum containing 4-OH-tamoxifen (data not shown), indicating that Mek1 activation cannot completely substitute for the growth factors provided by serum. Serum may act in two ways to cause a greater response as follows: it may stimulate phosphorylation of endogenous Mek1 on both serines 218 and 222, which apparently activates Mek1 more efficiently than substitution of these two residues with aspartate (48 and Fig. 7A); or it may activate other pathways in parallel to Mek1 signaling that synergize to promote further cell growth and transformation. It is therefore unlikely that Mek1 activation is sufficient for maximal stimulation of cell growth. However, the question of whether all of the signals emanating from activated Raf are dependent on Mek1 and/or Mek2 remains: is Mek1 activation functionally equivalent to Raf activation in fibroblast transformation?

Another issue is whether Mek1 activation is sufficient for cell cycle entry or oncogenic transformation. It has been reported that heparin-binding epidermal growth factor expression is induced by activated c-Raf-1 or Ras and activates the Jnk pathway in the process of stimulating proliferation (12, 42). Although the kinetics of Erk phosphorylation between 24 and 48 h (Fig. 6B) do not correlate in a linear fashion, implicating either delayed activation of another kinase capable of phosphorylating Erk or an autocrine loop that becomes fully activated only after 24 h of 4-OH-tamoxifen treatment, we detected no activation of Jnk1 (data not shown). The kinetics of both S phase entry and cell transformation are relatively rapid, as described above, and induction of Mek1-DDER causes up-regulation of cyclin D1 and down-regulation of p27^{Kip1}, two hallmarks of G₁ progression (Fig. 8). Although one could argue that

the cells in the experiment shown in Fig. 3A were still cycling at the time of hormone addition, this experiment has been repeated with cells placed in low serum 24 h prior to 4-OH-tamoxifen stimulation; the stimulated Mek1-DDER cells still continued to proliferate (data not shown). These data are consistent with the hypothesis that activation of Mek1 is sufficient to drive cell cycle progression.

Serum stimulation of endogenous Erk phosphorylation is attenuated in cells expressing elevated levels of Mek1-DDER (Fig. 7, lanes 4, 8, and 12), which is consistent with previously reported results which demonstrated a delay or attenuation of serum stimulation of Erk activity in oncogene-transformed cells (43–45). Although constitutively activated Mek1-DDER is expressed for the duration of the serum stimulation, any serum-driven increase in Erk activity in the Mek1-DDER cells would have had to be mediated by endogenous Mek, as the activity of Mek1-DDER is not affected by serum. Northern analysis for the expression of MKP-1, a dual specificity phosphatase normally induced by serum and reported to inactivate Erk1 and Erk2 (46), revealed that MKP-1 message levels are not stimulated by serum in Mek1-DDER expressing fibroblasts (data not shown). The mechanism of attenuation could therefore involve another Erk-specific phosphatase, a protein that prevents interaction of Mek and Erk, or a feedback mechanism that inhibits an upstream component of the signal transduction pathway leading to activation of endogenous Mek in response to serum stimulation.

Modulation of cell cycle components was also observed in response to induction of constitutively activated Mek1-ER. Two events associated with G₁ progression, up-regulation of cyclin D1 levels and down-regulation of p27^{Kip1} levels (35, 36, 39–41), were shown to occur within 24 h of treatment with 4-OH-tamoxifen (Fig. 8). High level induction of activated c-Raf-1 or activated Ras has been demonstrated to up-regulate expression of the cyclin-dependent kinase inhibitor p21^{Cip1}, accompanied by cell cycle arrest (13, 47, 48). We did not observe cell cycle arrest in response to hormone induction of Mek1-DDER (Fig. 3), although it is possible that the levels of activity attained were not sufficient to induce p21^{Cip1}. Future studies will focus on the influence of activated Mek1 on p21^{Cip1} expression, the question of the respective effects of Mek1 and c-Raf-1 on the cell cycle, and the role of feedback control in this pathway.

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An Analysis of Mek1 Signaling in Cell Proliferation and Transformation

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