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Conditional Transformation of Cells and Rapid Activation of the Mitogen-Activated Protein Kinase Cascade by an Estradiol-Dependent Human Raf-1 Protein Kinase

MICHAEL L. SAMUELS,[1] MICHAEL J. WEBER,[2] J. MICHAEL BISHOP,[3] AND MARTIN McMAHON[1]*

DNAX Research Institute of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, California 94304[1]; Department of Microbiology and Cancer Center, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908[2]; and Department of Microbiology and Immunology and the G. W. Hooper Foundation, University of California, San Francisco, California 94143[3]

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We report a strategy for regulating the activity of a cytoplasmic signaling molecule, the protein kinase encoded by *raf-1*. Retroviruses encoding a gene fusion between an oncogenic form of human p74^{raf-1} and the hormone-binding domain of the human estrogen receptor (hrafER) were constructed. The fusion protein was nontransforming in the absence of estradiol but could be reversibly activated by the addition or removal of estradiol from the growth media. Activation of hrafER was accompanied in C7 3T3 cells by the rapid, protein synthesis-independent activation of both mitogen-activated protein (MAP) kinase kinase and p42/p44 MAP kinase and by phosphorylation of the resident p74^{raf-1} protein as demonstrated by decreased electrophoretic mobility. The phosphorylation of p74^{raf-1} had no effect on the kinase activity of the protein, indicating that mobility shift is an unreliable indicator of p74^{raf-1} enzymatic activity. Removal of estradiol from the growth media led to a rapid inactivation of the MAP kinase cascade. These results demonstrate that Raf-1 can activate the MAP kinase cascade in vivo, independent of other "upstream" signaling components. Parallel experiments performed with rat1a cells conditionally transformed by hrafER demonstrated activation of MAP kinase kinase in response to estradiol but no subsequent activation of p42/p44 MAP kinases or phosphorylation of p74^{raf-1}. This result suggests that in rat1a cells, p42/p44 MAP kinase activation is not required for Raf-1-mediated oncogenic transformation. Estradiol-dependent activation of p42/p44 MAP kinases and phosphorylation of p74^{raf-1} was, however, observed in rat1a cells expressing hrafER when the cells were pretreated with okadaic acid. This result suggests that the level of protein phosphatase activity may play a crucial role in the regulation of the MAP kinase cascade. Our results provide the first example of a cytosolic signal transducer being harnessed by fusion to the hormone-binding domain of the estrogen receptor. This conditional system not only will aid the elucidation of the function of Raf-1 but also may be more broadly useful for the construction of conditional forms of other kinases and signaling molecules.

The Raf serine/threonine-specific protein kinases are believed to be key components in the machinery of eukaryotic signal transduction (33, 42, 47). Raf homologs have been found in both *Drosophila melanogaster* (37) and *Caenorhabditis elegans* (23). In both of these organisms there is strong evidence that the *raf* gene plays a pivotal role in both cell proliferation and the specification of cell fate, such that loss-of-function mutations in the *raf* genes have severe developmental consequences for the organism (2, 17, 23, 43). In addition recent work has identified a role for the *Xenopus laevis raf-1* homolog in fibroblast growth factor-mediated mesoderm induction (35).

Both humans and mice have three *raf* genes, A-*raf*, B-*raf*, and *raf-1* (42, 49). In mice the three *raf* genes vary in their level and tissue distribution of expression (50). All three proteins are apparently activated in response to a wide variety of extracellular mitogens and differentiation agents. In addition, similar amino-terminal truncations of all three *raf* genes gives rise to potent oncogenes (5, 27, 46, 48).

The p42/p44 mitogen-activated protein (MAP) kinases are serine/threonine-specific protein kinases whose activity is also modulated in cells by treatment with mitogens and differentiation agents (6, 13, 14, 53). The p42/p44 MAP kinases are believed to be important regulators of a diverse group of proteins that include phospholipase A_2 (34), p85^{RSK} (11, 51), and the transcription factor Elk-1 (25, 36). The p42/p44 forms of MAP kinase are activated by tyrosine and threonine phosphorylation, which is mediated by a protein kinase known as MAP kinase kinase (MKK) (1, 4, 15, 29, 38, 44, 60).

Evidence from genetic studies with *D. melanogaster* suggests that the Raf protein kinase acts upstream of MKK (55). Furthermore, in Raf-1-transformed 3T3 cells MAP kinases are constitutively activated (26). However, in these cells numerous events secondary to the expression of Raf-1 could account for the activation of the MAP kinases. Immunoprecipitates and partially purified preparations of the Raf-1 protein have been reported to activate MKK in vitro, but it is uncertain whether this reflects a normal in vivo function of p74^{raf-1}, a cross-talk activity between parallel pathways or an adventitious phosphorylation (16, 31). Two additional points of uncertainty concerning the interaction between p74^{raf-1} and MAP kinases come from reports that expression of an oncogenic allele of Raf-1 fails to activate the MAP kinase pathway in rat1a cells (19, 22) and PC12 cells (59) and reports that the p74^{raf-1} molecule is a substrate for MAP kinases both in vitro and in vivo (3, 32). For these reasons we thought it was important to develop a system in which Raf-1 protein kinase activity could be rapidly and condition-

* Corresponding author.

ally activated in mammalian cells. Here we describe the development of such a system and its use to document the control of the MAP kinase cascade by Raf-1.

MATERIALS AND METHODS

Cell culture and virus production. Cell lines were maintained in Dulbecco's modified Eagle's minimal medium supplemented with 10% (vol/vol) fetal bovine serum (FBS). Replication-defective recombinant retroviruses were produced as previously described (39). Estradiol (β-estradiol; Sigma) was made up as a 1 mM stock in ethanol and stored at −20°C. 4-Hydroxy-tamoxifen and ICI 164,384, molecules that bind and inhibit the functions of native estrogen receptors, were gifts of Sabine Schirm (University of California, San Francisco) and Alan Wakeling (ICI Pharmaceuticals), respectively, and were prepared and stored as described for estradiol. Okadaic acid (UBI) was dissolved in dimethyl sulfoxide at a concentration of 200 μM and stored at −20°C. Sodium orthovanadate was dissolved in H_2O at a concentration of 100 mM, the pH was adjusted to 10, and it was stored at −80°C. Cells were treated with 500 nM okadaic acid and 50 μM sodium orthovanadate for 30 min prior to subsequent treatment with estradiol.

Construction of *raf* alleles and vectors for their expression in cells. Polymerase chain reaction primers (PCR1, GGCT CGAGCCATGGAGTACTCACAGCCGAAAACCCCCGT GCC; and PCR2, CCATCGATTATGAATTCCCGAAGAC AGGCAGCCTCGGGGACT) were used to amplify, in 10 cycles, a portion of the human $p74^{raf-1}$ cDNA encoding amino acids 305 to 648 (hraf) that has previously been shown to be highly transforming in C7 3T3 cells (48). This allele encodes all of the kinase domain contained in conserved region 3 (CR3) but none of conserved regions 1 or 2 (CR1 and CR2). This polymerase chain reaction product was blunt-end cloned into the *Eco*RV site of pBluescript II (Promega) to generate pBShraf. An *Eco*RI-*Cla*I fragment encoding the hormone-binding domain of the HE14 allele (30) of the estrogen receptor (hbER) was subcloned from a tyrosine kinase-ER fusion protein (40) and ligated into pBShraf to generate pBShrafER.

The $p74^{raf-1}$, hraf, and hrafER alleles were each subcloned into two replication-defective murine retrovirus vectors: pLNCX (41) and pMLVneo (39). In these vectors, expression of the raf alleles is promoted by the cytomegalovirus promoter and the murine leukemia virus long terminal repeat, respectively. Both vectors encode resistance to G418, allowing selection of infected cells in the absence of a biological effect of the oncogene. Expression constructs were transfected into Ψ2 cells and virus stocks were derived as has been previously described (39).

Virus infection and measurement of transforming activity. Virus stocks were tested for their transforming potential by infecting 2×10^5 C7 3T3 cells with a series of virus dilutions as described previously (39). At 48 h after infection, the cells were trypsinized; 25% of the cells were placed in medium with no additions, and 25% were placed in medium containing 1 μM estradiol to measure the number of transformed foci per ml of virus in the absence or presence of estradiol. A further 25% of the cells were placed in medium containing 700 μg of G418 per ml, and the remaining 25% were placed in medium containing 700 μg of G418 per ml and 1 μM estradiol to measure the number of G418-resistant colonies obtained by virus infection. This number was unaffected by the presence of estradiol in the growth media. Cells were cultured for 14 to 21 days in the appropriate media, at which time foci and $G418^r$ colonies were counted. $G418^r$ colonies were inspected under the microscope to assess their morphology. Several hundred colonies were trypsinized and pooled to give rise to the populations of cells that were used in subsequent experiments. Cells were photographed with a Nikon TMS photomicroscope at ×120 magnification.

Measurement of cloning efficiency in agarose. G418-resistant populations of C7 3T3 (3T3:hrafER) and rat1a (rat1:hrafER) cells expressing hrafER were trypsinized and counted in a hemocytometer. We placed 10^3 cells in medium containing 0.35% (wt/vol) low-melting-temperature agarose in either the absence or presence of 1 μM estradiol. After 21 days the cells were stained with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). The total number of colonies was counted, and the stained plates were photographed with 200ASA Kodak color print film as described previously (39).

Preparation of cell extracts and analysis by Western blotting. Cells were washed with cold phosphate-buffered saline (PBS) and lysed on ice in Gold lysis buffer (GLB) containing 20 mM Tris (pH 7.9), 137 mM sodium chloride, 5 mM Na_2EDTA, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 1 mM leupeptin, 1 μM pepstatin A, 1 mM sodium orthovanadate, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 mM sodium fluoride, 1 mM tetrasodium PP_i, and 100 μM β-glycerophosphate. Insoluble material was removed by centrifugation at 12,000 × *g*. The protein concentration of the soluble material was estimated by using the BCA protein assay kit (Pierce).

Cellular proteins were analyzed by electrophoresis through polyacrylamide gels followed by Western immunoblotting onto polyvinylidene difluoride membranes (Immobilon P; Millipore). Western blots were probed with the following antibodies: an anti-hbER polyclonal antiserum (gift of S. Robbins [University of California, San Francisco]), an anti-MAP kinase monoclonal antibody that recognizes both p42 and p44 MAP kinase (Zymed), an anti-$p74^{raf-1}$ polyclonal antiserum (gift of R. C. Schatzman [Syntex Research]), an anti-phosphotyrosine monoclonal antibody (4G10), and an anti-MKK polyclonal antiserum (UBI). Western blots were incubated with the appropriate primary antibody at a dilution of 1:1,000 to 1:2,000 and then washed in Tris-buffered saline containing 0.5% (vol/vol) Nonidet P-40. Antigen-antibody complexes were visualized by using 1:10,000-diluted goat anti-rabbit antiserum, sheep anti-mouse antiserum, or protein A coupled to horseradish peroxidase as appropriate and the Enhanced Chemiluminescence detection system (Amersham). The blots were exposed to Kodak XAR5 X-ray film.

Western blots were "stripped" for reprobing with other primary antibodies by incubation for 2 h at 68°C in a buffer containing 0.2 M glycine (pH 2.5), and 1% (wt/vol) sodium dodecyl sulfate (SDS).

Assay for $p74^{raf-1}$ kinase activity. Immune complexes of hrafER or $p74^{raf-1}$ were prepared by incubating 100 μg of cell lysate with either the anti-hbER or anti-$p74^{raf-1}$ antisera. Immune complexes were collected by using protein A-Sepharose 4B (Pharmacia) and washed twice in 1 ml of GLB. The in vitro kinase activities of $p74^{raf-1}$ and hrafER were assessed by incubation of immune complexes for 30 min at 30°C in a reaction mix containing 25 mM *N*′-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol (DTT), 1 μM ATP, and 30 μCi of [γ-^{32}P]ATP (Amersham) with 50 ng of purified recombinant MKK (MKK1; gift of P.

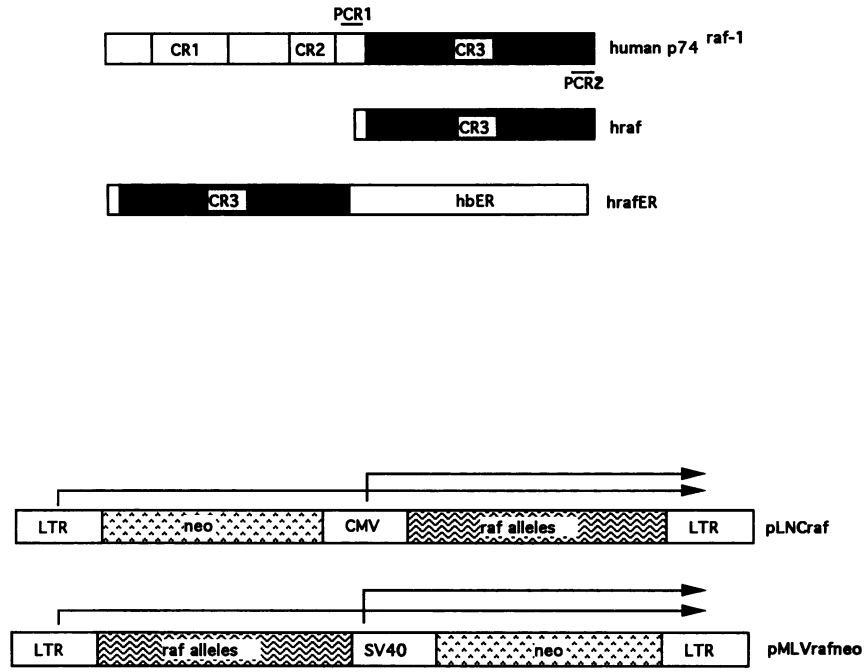

FIG. 1. Construction of Raf alleles and vectors for their expression in cells. Standard cloning techniques were used to construct retrovirus vectors encoding either Neo alone; full-length p74$^{raf\text{-}1}$; a truncated, oncogenic form of p74$^{raf\text{-}1}$ (hraf); and a fusion between hraf and the hormone-binding domain of the HE14 allele of the human estrogen receptor (hrafER). Retrovirus vectors were introduced into Ψ2 cells and retrovirus stocks were generated as described in Materials and Methods. Abbreviations: LTR, long terminal repeat; CMV, cytomegalovirus immediate-early promoter; SV40, simian virus 40 early region promoter.

Dent and T. Sturgill) as a substrate. The reactions were denatured by boiling in SDS-DTT buffer and electrophoresed through a polyacrylamide gel. After electrophoresis, proteins were Western blotted. Western blots were exposed first to X-ray film to quantitate Raf-1 kinase activity and subsequently probed sequentially with the anti-p74$^{raf\text{-}1}$ and the anti-hbER antisera to quantitate the amounts of Raf proteins in the immunoprecipitates.

Assay for MKK activity. Aliquots (30 μg) of protein from unfractionated cell lysates were incubated for 30 min at 30°C in a reaction mix containing 40 mM HEPES (pH 7.8), 10 mM $MgCl_2$, 1 mM DTT, 40 μM ATP, and 10 μCi of [γ-^{32}P]ATP with 2 μg of bacterially expressed rp42, which is an enzymatically inactive form of human p42 MAP kinase that serves as a substrate of MKK in this assay. The reaction was stopped by the addition of SDS-DTT sample buffer. The reaction mixtures were electrophoresed through a 10% polyacrylamide gel, which was Western blotted and exposed to X-ray film. Tyrosine phosphorylation of the rp42 substrate was quantitated by probing the Western blot with an anti-phosphotyrosine monoclonal antibody as described above. In all experiments, both methods of quantitation yielded identical results.

Assay for p42 MAP kinase activity. Aliquots (100 μg) of protein were incubated for 2 h at 4°C with an anti-MAP kinase polyclonal antiserum (TR2) that specifically immunoprecipitates p42 MAP kinase. Immune complexes were collected by using protein A-Sepharose 4B and washed twice with GLB and once with a buffer containing 25 mM Tris (pH 7.5), 137 mM NaCl, 40 mM $MgCl_2$, and 10% (vol/vol) glycerol. The immune complexes were incubated for 30 min at 30°C in a reaction mix containing 40 mM HEPES (pH 7.4), 40 mM $MgCl_2$, 200 μM ATP, and 5 μCi of [γ-^{32}P]ATP with 20 μg of myelin basic protein (MBP) as a substrate. The reactions were analyzed on polyacrylamide gels as described above.

In-gel MBP kinase assay. The in-gel MBP kinase assay was carried out essentially as described previously (21). Briefly, 50-μg aliquots of cell extracts were electrophoresed through a 12.5% polyacrylamide gel containing 500 μg of MBP per ml, which had been copolymerized into the gel. After electrophoresis the gel was washed once each in 20% (vol/vol) isopropanol in 50 mM Tris (pH 8), buffer A (50 mM Tris [pH 8], 5 mM β-mercaptoethanol), and 6 M guanidine HCl in buffer A. After the guanidine HCl wash, the proteins in the gel were allowed to renature at 4°C by extensive washing in buffer A containing 0.04% (vol/vol) Tween 40. Renatured MBP kinase activity was detected by incubating the gel for 60 min at room temperature in a reaction buffer containing 40 mM HEPES (pH 7.4), 2 mM DTT, 15 mM $MgCl_2$, 300 μM sodium orthovandate, 100 μM EGTA, 25 μM ATP, and 100 μCi of [γ-^{32}P]ATP. Unincorporated radioactivity was removed by extensive washing in 5% (vol/vol) trichloroacetic acid containing 1% (wt/vol) tetrasodium PP_i. The gel was dried and exposed to X-ray film for 24 to 48 h.

RESULTS

Transformation of C7 3T3 and rat1a cells by an estradiol-dependent Raf-1 protein kinase. Replication-defective retrovirus vectors encoding either Neo^r alone or the Raf alleles diagrammed in Fig. 1 were constructed by standard techniques, and viruses were produced by transfection of Ψ2 cells (Fig. 1) (39). Infection of C7 3T3 cells with viruses encoding Neo alone or p74$^{raf\text{-}1}$ gave rise to few transformed foci (Table 1). G418-resistant cells derived from these infec-

TABLE 1. Hormone-dependent transformation of C7 3T3 cells

Virus stock[a]	No. of G418[r] cells/ml	No. of transformed FFU/ml	
		Estradiol absent	Estradiol present
LNCX (Neo alone)	1×10^5	<10	<10
LNC:raf1	1×10^5	<10	<10
LNC:hraf	1×10^5	1×10^5	1×10^5
MLV:hraf	5×10^4	5×10^4	5×10^4
LNC:hrafER	1×10^5	<10	1×10^5
MLV:hrafER	5×10^4	<10	5×10^4

[a] Abbreviations: MLV, murine leukemia virus; LNC, LTR, Neo, CMV; LNCX, LNC plus insert.

tions had a flat, nontransformed morphology identical to that of uninfected cells (Fig. 2A). Infection of C7 3T3 cells with viruses encoding hraf gave rise to large numbers of foci (Table 1) and G418-resistant cells that were highly refractile, displaying a characteristic transformed morphology (Fig. 2C). Addition of 1 μM estradiol, 4-hydroxy-tamoxifen, or ICI 164,384 had no effect on the focus-forming ability of any of these viruses or on the morphology of virus-infected cells (Table 1, Fig. 2B, and Fig. 2D, respectively; data not shown). Infection of C7 3T3 cells with viruses encoding hrafER gave rise to large numbers of foci only in the presence of estradiol (Table 1). G418-resistant cells derived in the absence of estradiol had a flat, nontransformed morphology (Fig. 2E), whereas those derived in the presence of estradiol were highly refractile and transformed (Fig. 2F). Addition of 1 μM estradiol, 4-hydroxy-tamoxifen, or ICI 164,384 to previously untreated C7 3T3 cells expressing hrafER led to morphological transformation that was visible after 6 h and complete after 16 h of exposure (data not shown). Addition of 1 μM estradiol to rat1a cells expressing hrafER led to morphological transformation that, although more subtle than in C7 3T3 cells, was clearly visible after 16 h of treatment (Fig. 2G and H). Pools of cells expressing hrafER were derived by infection of C7 3T3 (3T3:hrafER) and rat1a (rat1:hrafER) cells. These cells were used for all subsequent experiments.

Both 3T3:hrafER and rat1:hrafER cells were capable of anchorage-independent growth in low-melting-temperature agarose. rat1:hrafER cells displayed a strong estradiol dependence for anchorage-independent growth in agarose. Cells grown in the presence of estradiol gave rise to large colonies, whereas cells grown in the absence of estradiol showed little or no growth (Fig. 3d and c, respectively). By contrast, 3T3:hrafER cells gave rise to smaller colonies and showed less hormone stimulation of anchorage-independent growth than the rat1:hrafER cells did (Fig. 3a and d). C7 3T3 and rat1a cells expressing the *neo*[r] gene did not form colonies in agarose and were unaffected by the addition of exogenous estradiol (data not shown).

Rapid, protein synthesis-independent phosphorylation of p42/p44 MAP kinases and p74$^{raf\text{-}1}$ upon activation of hrafER by estradiol in C7 3T3 cells. To investigate the mechanism of conditional transformation by hrafER, we isolated Triton X-100-soluble proteins from C7 3T3 cells expressing *neo* and from 3T3:hrafER cells treated for 16 h with 0.1% (vol/vol) ethanol (Fig. 4, lanes 1 and 4, respectively), 1 μM estradiol (lanes 2 and 5, respectively), or 1 μM 4-hydroxy-tamoxifen (lanes 3 and 6, respectively) and analyzed them by probing Western blots with a panel of antibodies. Anti-hbER antisera detected the hrafER fusion protein at a predicted molecular mass of 66 kDa in 3T3:hrafER cells. This protein was not detected in control cells. The abundance of the fusion protein increased approximately 10-fold after 16 h of treatment with estradiol or 4-hydroxy-tamoxifen (Fig. 4a), at which time the cells were fully morphologically transformed.

Antibodies against p74$^{raf\text{-}1}$ and p42/p44 MAP kinases revealed that estradiol and 4-hydroxy-tamoxifen treatment of 3T3:hrafER cells had no effect on the overall abundance of these proteins, but a significant proportion of the proteins became phosphorylated as demonstrated by reduced electrophoretic mobility (Fig. 4b and c, respectively). Treatment of cell lysates with potato acid phosphatase abrogated the observed electrophoretic mobility shifts of p74$^{raf\text{-}1}$ and p42/p44 MAP kinases, indicating that the shifts were the result of phosphorylation (data not shown). Western blotting with an anti-phosphotyrosine antibody revealed estradiol-inducible tyrosine phosphorylation of two proteins of 42 and 40 kDa in 3T3:hrafER cells (Fig. 4d). Immunoprecipitation with an anti-p42 MAP kinase antiserum followed by Western blotting with an anti-phosphotyrosine antibody revealed that the 42-kDa protein was tyrosine phosphorylated p42 MAP kinase (data not shown). The identity of the 40-kDa protein remains unknown.

Estradiol-stimulated phosphorylation of p42/p44 MAP kinases and p74$^{raf\text{-}1}$ was specific to 3T3:hrafER cells, since 3T3 cells conditionally transformed by two tyrosine kinase: hbER fusion proteins (v-SrcER and v-ErbBER) did not show similar phosphorylations in response to estradiol (data not shown).

We wished to assess the rapidity of activation of hrafER and to determine whether de novo protein synthesis was required for the phosphorylation of p42/p44 MAP kinases and p74$^{raf\text{-}1}$. 3T3:hrafER cells were treated with 25 μg of cycloheximide per ml to inhibit de novo protein synthesis and then stimulated with 1 μM estradiol in the continued presence of cycloheximide for 15, 30, and 60 min. The level of expression of the hrafER fusion protein was unchanged throughout the experiment (Fig. 5a). Phosphorylation of p42/p44 MAP kinases (Fig. 5c) and p74$^{raf\text{-}1}$ (Fig. 5b) was clearly detected after 15 min of estradiol treatment and was maintained over the time course analyzed. Tyrosine phosphorylation of p42 MAP kinase (Fig. 5d) was detected 15 min after addition of estradiol and correlated with the mobility shift of p42 MAP kinase.

Time course of activation of the MAP kinase cascade after addition of estradiol. To further analyze the kinetics of activation of the MAP kinase cascade, we treated 3T3:hrafER cells with 25 nM estradiol for different lengths of time from 1 to 60 min and assessed the phosphorylation and enzymatic activity of a number of cellular proteins. Over this time course the level of the hrafER protein did not change significantly (Fig. 6a). Mobility shifts of p42 MAP kinase and of p74$^{raf\text{-}1}$ were detected after 10 and 15 min of estradiol treatment, respectively (Fig. 6b and c). Tyrosine phosphorylation of p42 MAP kinase was clearly detected after 15 min of estradiol treatment (Fig. 6d). To assess the enzymatic activity of p42 MAP kinase, the protein was immunoprecipitated and incubated with [γ-^{32}P]ATP and MBP as a substrate. Analysis of the reactions revealed that p42 MAP kinase was enzymatically activated after 10 min of estradiol treatment (Fig. 6f). To measure MKK activity, unfractionated cell lysates were incubated with [γ-^{32}P]ATP and recombinant, enzymatically inactive p42 MAP kinase (rp42) as a substrate. Activation of an MKK activity was detected after 10 min of estradiol treatment (Fig. 6e).

In separate experiments we have shown that prolonged

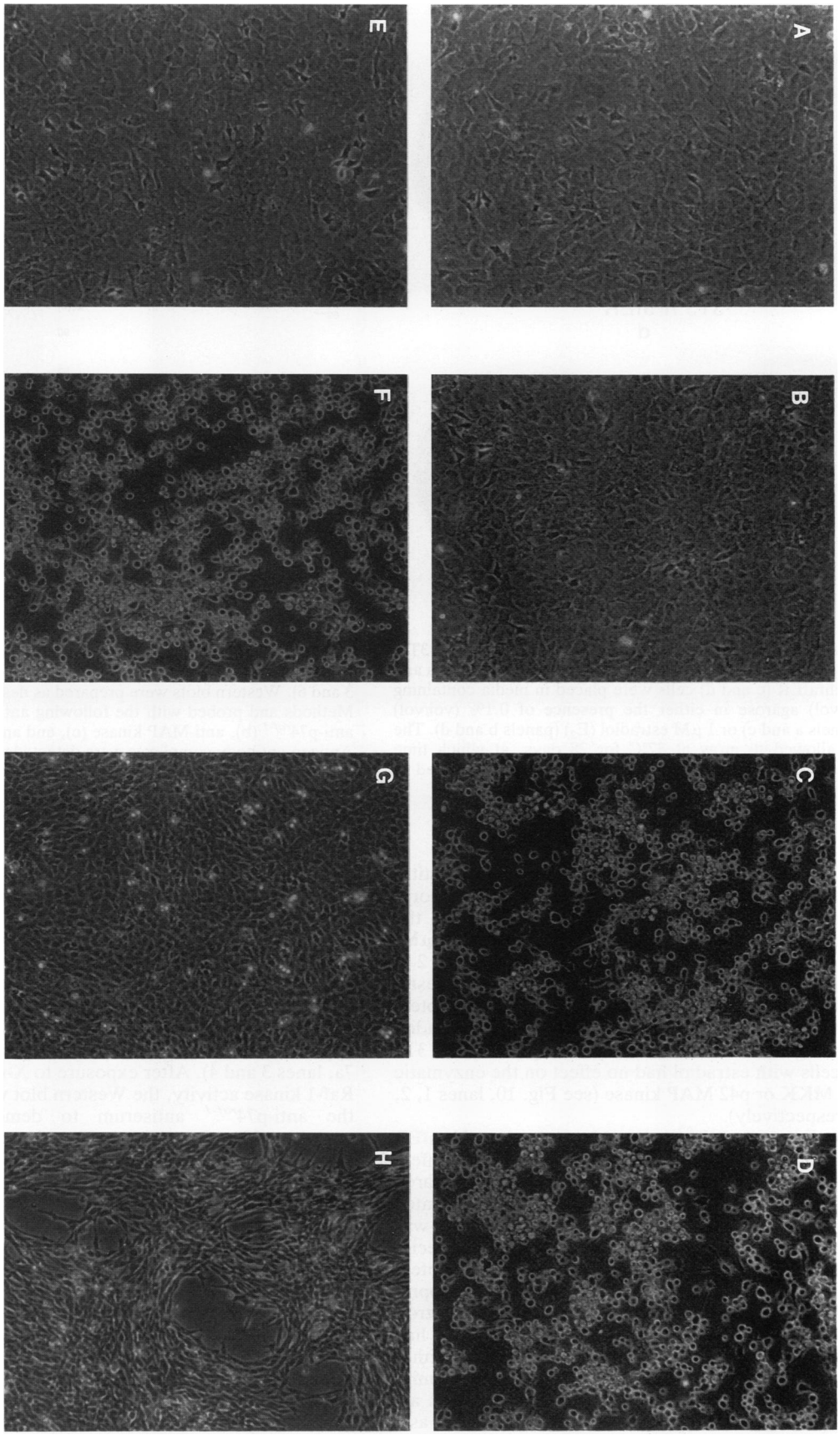

FIG. 2. Morphological transformation of C7 3T3 and rat1a cells expressing hrafER is dependent on the addition of estradiol to the growth media. Pools of G418-resistant C7 3T3 cells expressing the *neo* gene (A and B), hraf (C and D), or hrafER (E and F) were cultured in the presence of 0.1% (vol/vol) ethanol (A, C, and E), or 1 μM estradiol (B, D, and F). rat1a cells expressing the hrafER gene were cultured in the presence of 0.1% (vol/vol) ethanol (G) or 1 μM estradiol (H). Morphological transformation was assessed 16 to 48 h after the addition of the various agents.

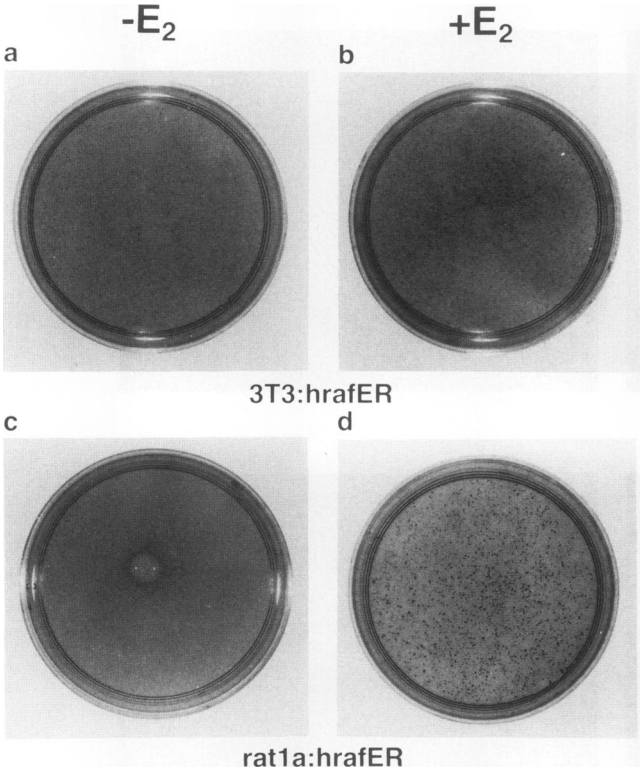

FIG. 3. Anchorage-independent growth in agarose of 3T3:hrafER and rat1:hrafER cells. Approximately 10^3 3T3:hrafER (a and b) and rat1:hrafER (c and d) cells were placed in media containing 0.35% (wt/vol) agarose in either the presence of 0.1% (vol/vol) ethanol (panels a and c) or 1 μM estradiol (E_2) (panels b and d). The cells were allowed to grow at 37°C for 28 days, at which time colonies were stained with MTT. The plates were photographed as described in Materials and Methods.

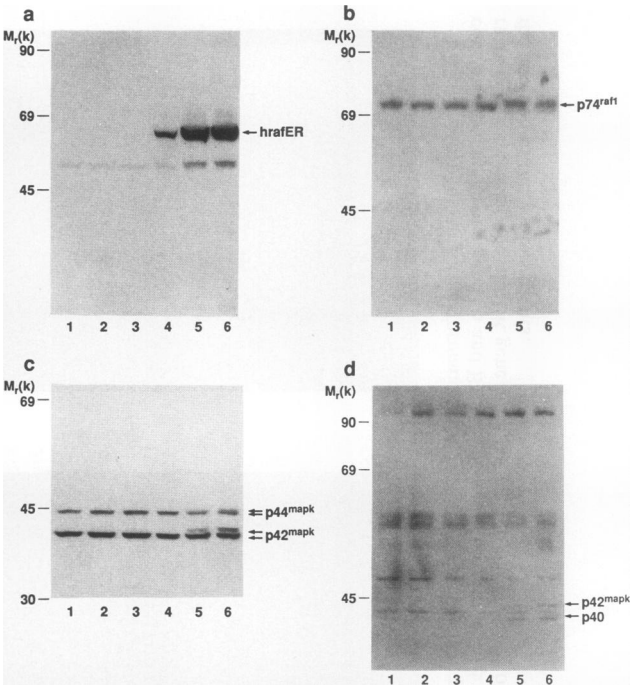

FIG. 4. Treatment of 3T3:hrafER cells with estradiol or 4-hydroxy-tamoxifen leads to elevated expression of the hrafER protein and phosphorylation of p42 MAP kinase and $p74^{raf\text{-}1}$. 3T3 cells expressing the *neo* gene (lanes 1 to 3) or the hrafER gene (lanes 4 to 6) were treated for 16 h with 0.1% (vol/vol) ethanol (lanes 1 and 4), 1 μM estradiol (lanes 2 and 5), or 1 μM 4-hydroxy-tamoxifen (lanes 3 and 6). Western blots were prepared as described in Materials and Methods and probed with the following antibodies: anti-hbER (a), anti-$p74^{raf\text{-}1}$ (b), anti-MAP kinase (c), and anti-phosphotyrosine (d). Antigen-antibody complexes were detected with horseradish peroxidase-coupled secondary reagents and the Enhanced Chemiluminescence system (Amersham). The positions of proteins of known molecular weight and the proteins of interest are indicated.

treatment of 3T3:hrafER cells with estradiol led to constitutive activation of the MAP kinase cascade and phosphorylation of $p74^{raf\text{-}1}$ (data not shown). In addition, when the dose of estradiol on 3T3:hrafER cells was increased to 1 μM, activation of the MAP kinase cascade could be detected 2 to 3 min after hormone addition (data not shown). This result suggests that the rapidity of activation of the hrafER protein can be modulated in cells by the concentration of estradiol added to the growth media. Treatment of parental C7 3T3 and rat1a cells with estradiol had no effect on the enzymatic activity of MKK or p42 MAP kinase (see Fig. 10, lanes 1, 2, 8, and 9, respectively).

Phosphorylation of $p74^{raf\text{-}1}$ induced by activation of hrafER had no effect on $p74^{raf\text{-}1}$ kinase activity. To measure the effect of phosphorylation on the activity of $p74^{raf\text{-}1}$, we prepared extracts from 3T3:hrafER cells either untreated or treated with 1 μM estradiol for 60 min. All of the hrafER protein was removed from these extracts by quantitative immunoprecipitation with the anti-hbER antiserum and protein A-Sepharose 4B. The $p74^{raf\text{-}1}$ protein was then immunoprecipitated with the anti-$p74^{raf\text{-}1}$ antiserum. As a control, $p74^{raf\text{-}1}$ was immunoprecipitated from C7 3T3 cells that had been maintained in 0.5% (vol/vol) FBS and then either untreated or stimulated with 20% (vol/vol) FBS for 20 min. Raf-1 kinase activity in these immune complexes was assayed with [γ-^{32}P]ATP and purified recombinant MKK1 as a substrate. The kinase activity of hrafER toward the MKK1 substrate was significantly stimulated by the addition of estradiol to cells (Fig. 7a, lanes 3 and 4). Furthermore, $p74^{raf\text{-}1}$ protein immunoprecipitated from a serum-stimulated cell also had increased kinase activity (Fig. 7a, lanes 1 and 2). The phosphorylation of $p74^{raf\text{-}1}$ that occurred in 3T3:hrafER cells in response to estradiol, however, had no apparent effect on the kinase activity of the molecule (Fig. 7a, lanes 3 and 4). After exposure to X-ray film to quantitate Raf-1 kinase activity, the Western blot was probed first with the anti-$p74^{raf\text{-}1}$ antiserum to demonstrate equivalent amounts of Raf-1 proteins in lanes 1 and 2 and lanes 5 and 6 (Fig. 7b). The blot was then "stripped" and reprobed with the anti-hbER antiserum (Fig. 7c) to demonstrate equivalent amounts of hrafER protein in lanes 3 and 4 and to further demonstrate that there was no residual hrafER protein in the immunoprecipitates of $p74^{raf\text{-}1}$ in lanes 5 and 6.

Time course of inactivation of the MAP kinase cascade after removal of estradiol. Removal of estradiol from transformed 3T3:hrafER cells caused phenotypic reversion of the cells to a nontransformed morphology over the course of 10 to 12 h. We wished to assess the rapidity of inactivation of the MAP kinase cascade after inactivating hrafER by removing estradiol from the growth medium. 3T3:hrafER cells, grown for 16 h in the presence of estradiol, were washed with PBS to

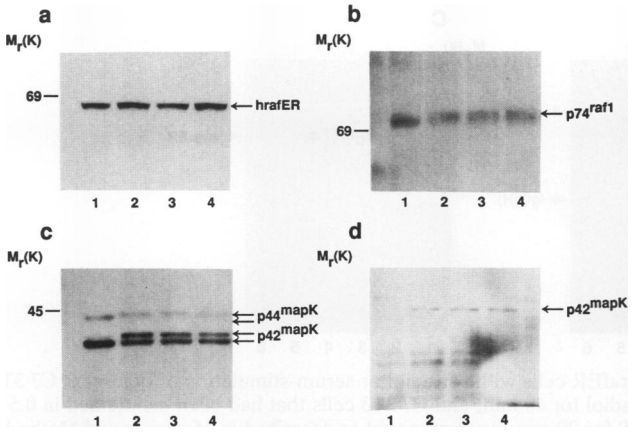

FIG. 5. Phosphorylation of p42/p44 MAP kinases and p74^{raf-1} in response to hrafER activation occurs rapidly and in the absence of de novo protein synthesis. 3T3:hrafER cells were treated with 25 μg of cycloheximide per ml for 60 min to inhibit protein synthesis and were either untreated (lane 1) or treated with 1 μM estradiol for 15, 30, or 60 min (lanes 2 to 4, respectively) in the continuous presence of cycloheximide. The cells were lysed, and Western blots were prepared and probed with the following antibodies: anti-hbER (a), anti-p74^{raf-1} (b), anti-MAP kinase (c), and anti-phosphotyrosine (d). The positions of proteins of known molecular weight and the proteins of interest are indicated.

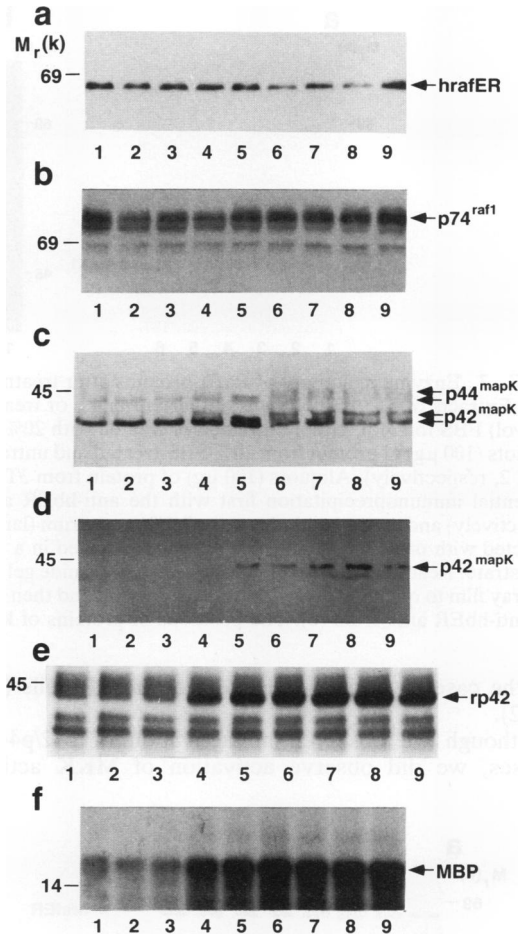

FIG. 6. Time course of events following stimulation of 3T3:hrafER cells with estradiol. 3T3:hrafER cells either untreated (lane 1) or treated with 25 nM estradiol for 1, 5, 10, 15, 20, 25, 30, or 60 min (lanes 2 to 9, respectively) were lysed and Western blots were prepared as described previously. Western blots were probed with anti-hbER antiserum (a), anti-p74^{raf-1} antiserum (b), anti-MAP kinase antibody (c), and anti-phosphotyrosine antibody (d). The Western blot in panel d was prepared after stripping the Western blot in panel a. The blot was first incubated with sheep anti-mouse horseradish peroxidase secondary antibody to ensure that all of the anti-hbER antibody had been removed prior to the incubation with the anti-phosphotyrosine antibody. MKK activity (e) was assessed by incubation of 30 μg of unfractionated cell lysate with [γ-^{32}P]ATP and 2 μg of rp42 MAP kinase substrate. p42 MAP kinase activity was assessed by immunoprecipitating p42 MAP kinase from 100 μg of cell lysate. Immune complexes were collected and incubated with [γ-^{32}P]ATP and MBP as a substrate. The MKK and p42 MAP kinase assays were analyzed by electrophoresis on a polyacrylamide gel and exposure of the dried gel to X-ray film (f). The positions of proteins of known molecular weight and the proteins of interest are indicated.

remove the hormone and refed with normal growth medium. Cells were harvested at different times after the removal of estradiol, and the activity and/or phosphorylation of MKK, p42 MAP kinase, and p74^{raf-1} was determined as described above. The enzymatic activity of both MKK and p42 MAP kinase rapidly decreased, declining to basal levels 60 min after the removal of estradiol (Fig. 8b and c, respectively).

Dephosphorylation of p74^{raf-1} occurred with slightly slower kinetics, returning to the level of untreated cells 2 h after the removal of estradiol (Fig. 8d). The expression of the hrafER fusion protein, which is 10-fold higher than in the untreated cell, was unchanged throughout the experiment (Fig. 8a). These results demonstrated that the elevated levels of hrafER found in an estradiol-treated 3T3:hrafER cell can be rapidly enzymatically repressed by the removal of estradiol from the growth medium.

p42/p44 MAP kinases are not activated in hrafER-transformed rat1a cells. Previous work (19, 22) had raised the possibility that the regulation of the MAP kinase cascade in rat1a cells was fundamentally different from that in 3T3 cells; hence, we wished to address the regulation of the MAP kinase cascade in rat1a cells by hrafER. rat1:hrafER cells were treated for 16 h with 0.1% (vol/vol) ethanol, 1 μM estradiol, or 1 μM 4-hydroxy-tamoxifen. Cell lysates were analyzed by Western blotting as shown in Fig. 9. Low but detectable levels of hrafER were expressed in these cells in the absence of estradiol. As was seen in similarly treated 3T3:hrafER cells, the level of expression of the fusion protein was increased 10-fold in the presence of estradiol or 4-hydroxy-tamoxifen (Fig. 9a). Activation of hrafER in rat1a cells, however, had no effect on the phosphorylation of p42/p44 MAP kinases or p74^{raf-1} (Fig. 9b and c, respectively). The inability of hrafER to activate the p42/p44 MAP kinases in these cells was not a consequence of the lack of expression of MKK, which was clearly detected by Western blotting (Fig. 9d).

As further confirmation that p42/p44 MAP kinases were not activated in rat1:hrafER cells, the enzymatic activity of p42/p44 MAP kinases was determined by both an immune-complex kinase assay of p42 MAP kinase (Fig. 9e) and by an in-gel MBP kinase assay (Fig. 9f). In these experiments, lysates were prepared from 3T3:hrafER cells grown in the absence and presence of estradiol for 16 h as controls. The results clearly demonstrated that treatment of 3T3:hrafER cells with estradiol led to the activation of both p42 and p44 MAP kinases (lanes 3 and 4, respectively) but that this was

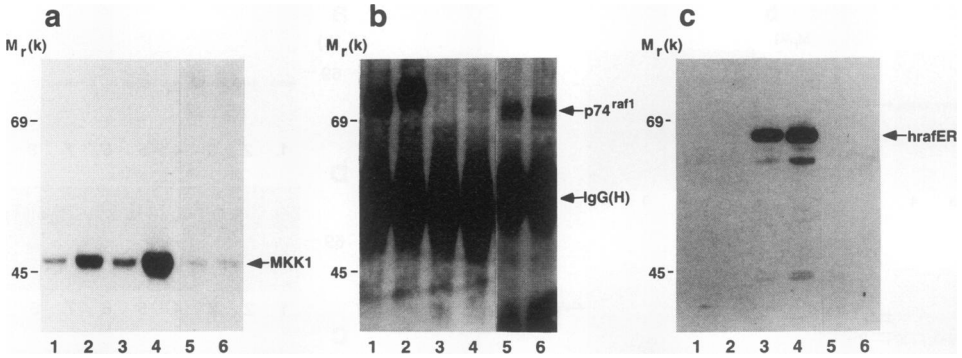

FIG. 7. Enzymatic activity of Raf-1 proteins after treatment of 3T3:hrafER cells with estradiol or serum-stimulation of quiescent C7 3T3 cells. Extracts of 3T3:hrafER cells, either untreated or treated with estradiol for 60 min, and C7 3T3 cells that had been maintained in 0.5% (vol/vol) FBS for 24 h, either untreated or treated with 20% (vol/vol) FBS for 20 min, were prepared as described in Materials and Methods. Aliquots (100 μg) of protein from 20% FBS-treated and untreated C7 3T3 cells were immunoprecipitated with the anti-p74^{raf-1} antiserum (lanes 1 and 2, respectively). Aliquots (100 μg) of protein from 3T3:hrafER cells cultured in the absence or presence of estradiol were subjected to sequential immunoprecipitation first with the anti-hbER antisera to remove all of the hrafER protein from the extracts (lanes 3 and 4, respectively) and second with the anti-p74^{raf-1} antiserum (lanes 5 and 6, respectively). Immune complexes from all immunoprecipitations were collected with protein A-Sepharose 4B and incubated in a reaction mix containing [γ-^{32}P]ATP and 50 ng of purified recombinant MKK1 as a substrate. Reactions were analyzed by polyacrylamide gel electrophoresis followed by Western blotting. The Western blot was first exposed to X-ray film to quantitate Raf-1 kinase activity (a) and then probed with the anti-p74^{raf-1} antiserum (b). It was then stripped and reprobed with the anti-hbER antiserum (c). The positions of proteins of known molecular weight and the proteins of interest are indicated.

not the case in similarly treated rat1:hrafER cells (lanes 1 and 2).

Although we observed no activation of p42/p44 MAP kinases, we did observe activation of MKK activity in rat1:hrafER cells in response to estradiol, as demonstrated by its ability to tyrosine phosphorylate rp42 as a substrate (Fig. 9g, lanes 1 and 2). The extent of activation of MKK by estradiol in rat1:hrafER cells was less than that observed in similarly treated 3T3:hrafER cells (Fig. 9g, lanes 3 and 4).

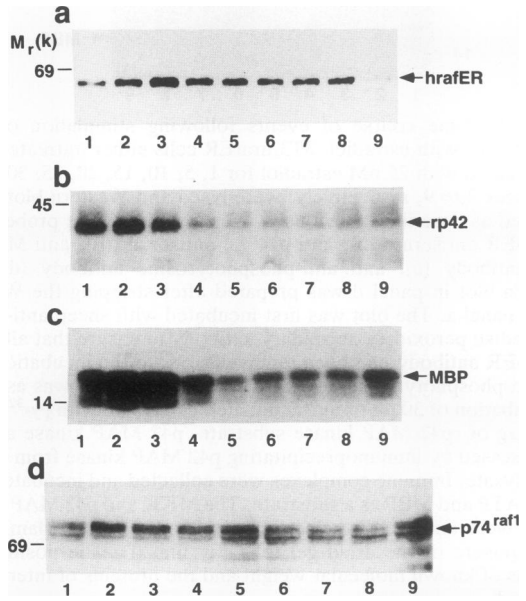

FIG. 8. Time course of events following removal of 3T3:hrafER cells with estradiol. 3T3:hrafER cells that had been treated with estradiol for 16 h were washed in PBS and refed with fresh medium to remove the hormone. Cell extracts were prepared prior to removal of the hormone and again 15 min, 30 min, and 1, 2, 3, 4, and 5 h (lanes 2 to 8, respectively) after the removal of estradiol. In addition, an extract was prepared from cells that had not been treated with estradiol (lane 9). The levels of expression of hrafER (a), the enzymatic activity of MKK (b) and p42 MAP kinase (c), and the extent of phosphorylation of p74^{raf-1} (d) in these extracts were assessed as described in Materials and Methods. The positions of proteins of known molecular weight and the proteins of interest are indicated.

Activation of the MAP kinase cascade in 3T3:hrafER and rat1:hrafER cells in response to estradiol, PMA, and serum stimulation. We wished to compare the extent of activation of the MAP kinase cascade in 3T3:hrafER and rat1:hrafER cells in response to estradiol treatment or treatment with two different mitogens, phorbol-12-myristate-13-acetate (PMA; Calbiochem) and 20% (vol/vol) FBS. Estradiol treatment of 3T3:hrafER cells for 15 min led to activation of both MKK and p42 MAP kinase activity (Fig. 10, lanes 3 and 4, respectively). Treatment of 3T3:hrafER cells, which had been cultured for 48 h in medium containing 0.5% FBS, with either 50 nM PMA or 20% FBS for 15 min gave rise to activation of MKK activity that was lower than that observed in response to hrafER activation (Fig. 10a, lanes 5 to 7). Perhaps surprisingly, the activation of p42 MAP kinase activity in response to PMA or serum was greater than that observed in response to hrafER activation, even though there was apparently less activation of MKK activity in these cells (Fig. 10b, lanes 5 to 7). No activation of p42 MAP kinase activity was observed in rat1:hrafER cells in response to estradiol (Fig. 10b, lanes 3 and 4), even though MKK was activated to an extent similar to that observed in 3T3:hrafER cells treated with PMA or 20% FBS (Fig. 10a and b, compare lanes 6, 7, and 11). Curiously, treatment of serum-deprived rat1:hrafER cells with PMA or 20% FBS had no effect on the activity of MKK or p42 MAP kinase (Fig. 10, lanes 12 to 14).

Okadaic acid treatment of hrafER-expressing rat1a cells overcomes the block to MAP kinase activation. We wished to address the potential role of protein phosphatases in the regulation of the MAP kinase cascade in 3T3:hrafER and rat1:hrafER cells. Okadaic acid and sodium orthovanadate have previously been shown to inhibit the activity of protein phosphatase 2A (24) and protein tyrosine phosphatases (8), respectively. Treatment of 3T3:hrafER cells with okadaic acid, prior to the addition of estradiol, potentiated the

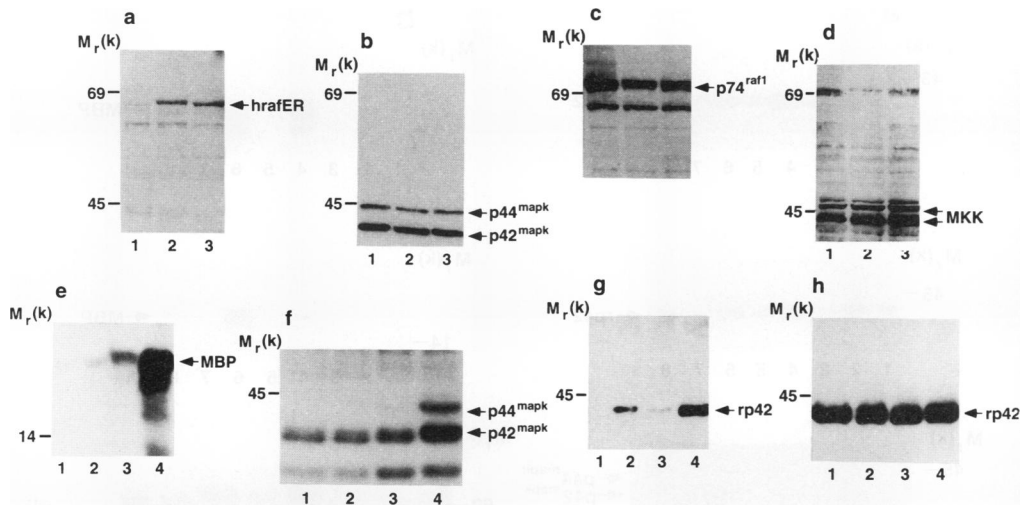

FIG. 9. Analysis of MAP kinase cascade activation in rat1a cells expressing hrafER. rat1:hrafER cells were treated with 0.1% (vol/vol) ethanol (lane 1), 1 μM estradiol (lane 2), or 1 μM 4-hydroxy-tamoxifen (lane 3) for 16 h, and cell extracts were prepared for analysis by Western blotting. Western blots were probed with an anti-hbER antiserum (a), an anti-MAP kinase monoclonal antibody (b), an anti-p74^{raf-1} antiserum (c), and an anti-MKK antibody (d). Cell extracts for the experiments presented in panels e to h were prepared from rat1:hrafER cells treated with 0.1% (vol/vol) ethanol (lanes 1) or 1 μM estradiol (lanes 2) for 16 h and from similarly treated 3T3:hrafER cells (lanes 3 and 4, respectively). These extracts were assayed for p42 MAP kinase activity by an immune-complex kinase assay with MBP as a substrate (e) and for the activity of both p42 and p44 MAP kinases by an in-gel MBP kinase assay (f) as described in Materials and Methods. MKK activity was assayed with rp42 as a substrate (g) as described in Materials and Methods. The MKK assay mixtures were electrophoresed through a polyacrylamide gel and Western blotted. MKK activity was quantitated by probing the blot with an anti-phosphotyrosine antibody (g). Equal loading of the added rp42 substrate was confirmed by stripping this blot and reprobing with an anti-MAP kinase monoclonal antibody (h) as described in Materials and Methods. The positions of proteins of known molecular weight and the proteins of interest are indicated.

activation of both MKK and p42 MAP kinase activity (Fig. 11a and b, lanes 3 and 4). Treatment of these cells with sodium orthovanadate prior to addition of estradiol led to potentiation of p42 MAP kinase activity with little or no effect on MKK activity (Fig. 11a and b, lanes 7 and 8). Treatment of cells with a combination of the two inhibitors led to the greatest potentiation of MKK and p42 MAP kinase activity (Fig. 11a and b, lanes 5 and 6).

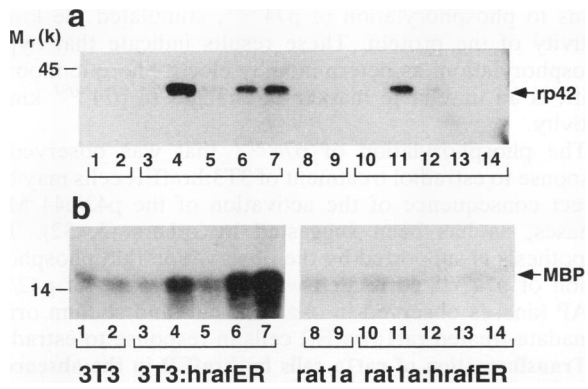

FIG. 10. Activation of the MAP kinase cascade in 3T3:hrafER and rat1:hrafER cells in response to treatment with estradiol, PMA, and serum. Cell extracts were prepared from parental C7 3T3 and rat1a cells either untreated (lanes 1 and 8, respectively) or treated with 1 μM estradiol for 16 h (lanes 2 and 9, respectively). Further cell extracts were prepared from 3T3:hrafER and rat1:hrafER cells either untreated (lanes 3 and 10, respectively) or treated with estradiol for 15 min (lanes 4 and 11, respectively) and from 3T3:hrafER and rat1:hrafER cells grown in 0.5% (vol/vol) FBS for 24 h and then either untreated (lanes 5 and 12, respectively) or treated for 15 min with either 50 nM PMA (lanes 6 and 13, respectively) or 20% (vol/vol) FBS (lanes 7 and 14, respectively). MKK (a) and p42 MAP kinase (b) assays were carried out as described in Materials and Methods.

Treatment of rat1:hrafER cells with okadaic acid prior to the addition of estradiol potentiated the activation of MKK activity (Fig. 11c, lanes 3 and 4). Under these conditions, p42/p44 MAP kinase activity was activated in response to treatment of the cells with estradiol as measured by both immune-complex kinase assay and in-gel MBP kinase assay (Fig. 11d and e, lanes 3 and 4). Treatment of rat1:hrafER cells with a combination of okadaic acid and sodium orthovanadate permitted the greatest activation of p42/p44 MAP kinase activity in response to estradiol treatment (Fig. 11d and e, lanes 5 and 6). This latter induction of p42/p44 MAP kinase activity occurred without further potentiation of the MKK activity observed with okadaic acid alone (Fig. 11c, compare lanes 4 and 6). Sodium orthovanadate alone had little or no effect on the ability of hrafER to activate p42/p44 MAP kinase activity in rat1:hrafER cells (Fig. 11d and e, lanes 7 and 8). In addition, we observed a correlation between the activation of p42/p44 MAP kinases in rat1:hrafER cells and the phosphorylation of p74^{raf-1} as detected by electrophoretic mobility shift (Fig. 11f, lanes 4 and 6).

DISCUSSION

Estradiol-dependent transformation of C7 3T3 and rat1a cells by hrafER. It has previously been demonstrated that fusion of the hormone-binding domain of steroid receptors to a variety of transcription factors renders the activity of those proteins dependent on the addition of exogenous steroids to the growth media (7, 9, 12, 18, 20, 45, 52, 56). This report

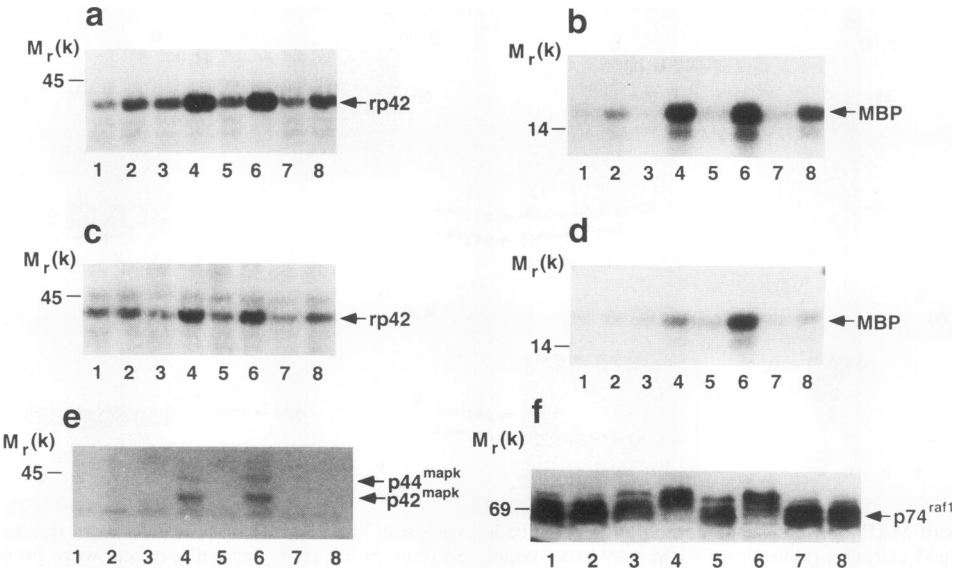

FIG. 11. Okadaic acid and sodium orthovanadate treatment potentiate the activation of the MAP kinase cascade by hrafER in both 3T3:hrafER and rat1:hrafER cells. Cell extracts were prepared from 3T3:hrafER (a and b) and rat1:hrafER (c to f) cells either untreated or treated with estradiol for 20 min (lanes 1 and 2, respectively). Cell extracts were prepared from similarly treated cells that had been pretreated for 30 min prior to the addition of estradiol with either 500 nM okadaic acid (lanes 3 and 4, respectively), 50 μM sodium orthovanadate (lanes 7 and 8, respectively) or a combination of the two (lanes 5 and 6, respectively). Assays for MKK (panels a and c), p42 MAP kinase (panels b and d), in-gel MBP kinase (panel e), and anti-p74^{raf-1} Western blotting were carried out as described in Materials and Methods.

documents the first example of the harnessing of an enzymatic activity by a similar strategy.

The results presented here demonstrate that fusion of the hormone-binding domain of the human estrogen receptor to an activated form of the human p74^{raf-1} protein kinase renders transformation of C7 3T3 and rat1a cells by this oncogene dependent on the addition of exogenous estradiol to the growth media. Transformation of cells is accompanied by a 10-fold increase in the level of expression of the fusion protein, which is due, at least in part, to increased stability of the fusion protein (data not shown). However, even when expressed at high level, the removal of estradiol from the growth media leads to the rapid inactivation of the enzymatic activity of the protein.

4-Hydroxy-tamoxifen and ICI 164,384, anti-estrogens which bind and inhibit the functions of native estrogen receptors (54, 57, 58), are potent activators of the hrafER fusion protein. The ability of these molecules to induce and maintain the transformed state of cells expressing hrafER rules out the possibility that endogenous estrogen receptors play a role in the transformation process (18).

Activation of the MAP kinase cascade in C7 3T3 cells by hrafER. These results demonstrate that activation of Raf-1 kinase activity in C7 3T3 cells, independent of other "upstream" factors, leads to the rapid activation of MKK and MAP kinase activity. These results demonstrate that the activation of MAP kinases observed in Raf-1-transformed C7 3T3 cells is an immediate consequence of the activation of the Raf-1 protein kinase.

Inhibition of cellular protein synthesis had no effect on the ability of estradiol to stimulate phosphorylation of p42/p44 MAP kinases and p74^{raf-1}. It would seem that the fusion protein is maintained in a latent form that can be rapidly activated, in a dose-dependent manner, by the addition of estradiol.

In addition, we demonstrated that activation of hrafER by estradiol led to the phosphorylation of endogenous p74^{raf-1}. Hyperphosphorylation of p74^{raf-1} is frequently used as an indicator of the enzymatic activity of the protein. With this in mind, we measured the enzymatic activity of the endogenous p74^{raf-1} protein with purified recombinant MKK1 as a substrate. We found that p74^{raf-1} protein kinase activity was unaffected by this phosphorylation. By contrast, serum stimulation of quiescent C7 3T3 cells, a treatment that also leads to phosphorylation of p74^{raf-1}, stimulated the kinase activity of the protein. These results indicate that hyperphosphorylation, as determined by electrophoretic mobility shift, is an unreliable marker of changes in p74^{raf-1} kinase activity.

The phosphorylation of p74^{raf-1} that was observed in response to estradiol treatment of 3T3:hrafER cells may be a direct consequence of the activation of the p42/p44 MAP kinases, as has been suggested by others (3, 32). This hypothesis is supported by the observation that phosphorylation of p74^{raf-1} correlated with the activation of p42/p44 MAP kinases observed in okadaic acid- and sodium orthovanadate-treated rat1:hrafER cells in response to estradiol.

Transformation of rat1a cells by hrafER in the absence of p42/p44 MAP kinase activation. In rat1a cells, activation of hrafER led to activation of MKK activity but no subsequent activation of p42/p44 MAP kinase activity. Since hrafER transforms rat1a cells very efficiently, there must be a p42/p44 MAP kinase-independent pathway leading to oncogenic transformation. The extent of MKK activation in rat1:hrafER cells was lower than that observed in estradiol-treated 3T3:hrafER cells but similar to that observed in 3T3:hrafER cells in response to serum stimulation or PMA treatment. Both of these last treatments led to increased p42/p44 MAP kinase activity in 3T3:hrafER cells.

Although in-gel MBP kinase assays failed to detect activation of any MBP kinases in rat1:hrafER cells, it is possible

that MAP kinases with different substrate specificities are being activated by hrafER in rat1a cells.

The inability of hrafER to activate p42/p44 MAP kinases in rat1a cells is not due to the absence of an intermediate or to an intrinsic difference in the regulation of the MAP kinase cascade in these cells. This was demonstrated by the fact that pretreatment of rat1:hrafER cells with okadaic acid permitted the activation of p42/p44 MAP kinases in an estradiol-dependent manner. It is possible that the threshold of MKK activity required for activation of p42/p44 MAP kinase activation is higher in rat1a cells than in C7 3T3 cells. This would explain the observation that equivalent amounts of MKK activity in 3T3:hrafER and rat1:hrafER cells have different consequences for the activation of p42/p44 MAP kinases.

On the basis of these results, we would like to propose a model for the activation of the MAP kinase cascade in a Raf-1-transformed C7 3T3 cell. We propose that the activation of Raf-1 kinase activity leads to the phosphorylation and activation of MKK. Activated MKK phosphorylates p42/p44 MAP kinases on threonine and tyrosine residues and activates their kinase activity toward a number of substrates. The simplest, but by no means the only, interpretation of the phosphatase inhibitor studies is that MKK activity is regulated predominantly by protein phosphatase 2A, whereas the p42/p44 MAP kinases are regulated predominantly by a vanadate-sensitive protein phosphatase such as the recently described 3CH134 protein-tyrosine-phosphatase (10).

The rapid activation of a kinase cascade in C7 3T3 cells by the hrafER fusion protein clearly demonstrates the utility of this approach and will be useful in the identification of new substrates of the Raf-1 kinase and aid the mapping of specific sites of in vivo phosphorylation of those substrates. In addition, we believe that control by estradiol will be generally applicable to a variety of oncogenic protein kinases. Indeed, others have demonstrated hormone-dependent transformation by an Abl:ER fusion protein (28), and we have demonstrated hormone-dependent transformation with two tyrosine kinase:hbER fusion proteins, v-SrcER and v-ErbBER (40). This approach may be broadly applicable to a number of enzymes and thus may be generally useful in helping to elucidate the contributions of individual proteins to complex signal transduction pathways.

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