Journal of Cellular Biochemistry 70:587–595 (1998)

Regulation of Heregulinβ1-Induced Differentiation in a Human Breast Carcinoma Cell Line by the Extracellular-Regulated Kinase (ERK) Pathway

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Abstract The AU565 breast carcinoma cell line was used to determine the role of the extracellular-regulated kinase (ERK) pathway in mediating Heregulinβ1 (HRGβ1)-induced mammary cell differentiation. ERK activation remained elevated for 2 h following high doses of HRG which induce differentiation. In contrast, a transient 5 min peak of ERK activation in response to doses of HRG which induce proliferation was observed. A MEK specific inhibitor, PD98059, which inhibited activation of ERK in response to HRG, completely blocked HRG-induced differentiation and reversed cell growth arrest. To further assess the importance of sustained ERK activity in cellular differentiation, we transiently transfected a mutant constitutively active MEK1 construct into AU565 cells. Differentiation was induced in the absence of HRG and treatment with HRG potentiated this response. These data indicate that sustained activation of the MEK/ERK pathway is both essential and sufficient for HRG-induced differentiation of AU565 cells. J. Cell. Biochem. 70:587–595, 1998.

Key words: breast carcinoma; ERK pathway

Type 1 receptor tyrosine kinases, which include the epidermal growth factor receptor (EGFR), erbB2, erbB3, and erbB4, are expressed in a variety of tissues, including those of epithelial, neuronal, and mesenchymal origin. In addition to their important roles in a variety of normal physiological processes [Ullrich, 1984; Kraus et al., 1989; Bargmann et al., 1986; Yamamoto et al., 1986; Plowman et al., 1990, 1993a], these receptors have been implicated in the development and progression of several types of human cancers including mammary carcinomas [Slamon et al., 1987, 1989; Gullick, 1991; Sainsbury et al., 1987; Elledge, 1992; Lemoin et al., 1992]. Heregulin (HRG)/neu differentiation factor (NDF) has recently been identified as a ligand for the erbB3 and erbB4 receptors [Tzahar et al., 1994; Wen et al., 1994; Plowman et al., 1993b; Carraway et al., 1994] and has been shown to induce both cell proliferation and differentiation in some mammary carcinoma cell lines [Peles et al., 1992; Bacus et al., 1992, 1993; Marte et al., 1995]. In vitro high concentrations of HRG induce differentiation of, or inhibit, the growth of mammary carcinoma cells overexpressing erbB2, such as the AU565 and MDA-MB-435 cell lines. High concentrations of HRG result in a flattened morphology, increased nuclear size, and large cytoplasmic vesicles. Milk protein is synthesized and fat droplets appear in the cytoplasm, characteristics typical of differentiated mammary cells [Bacus et al., 1992]. There is also an upregulation of ICAM-1 [Bacus et al., 1993], which promotes calcium-independent cell-cell interactions necessary for differentiation. Low concentrations of HRG added to these cell lines

Contract grant sponsors: Interactive Research Grant from the Greenebaum Cancer Center at the University of Maryland and Graduate Program Enhancement Award.

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Received 15 January 1998; Accepted 31 March 1998

induce proliferation. In other mammary cell lines which express low levels of erbB2, HRG is mitogenic [Holmes et al., 1992; Lewis et al., 1996]. In vivo, HRG appears to induce cellular differentiation and tissue remodeling. In organ culture, addition of HRG to the mammary gland induces secretory lobuloalveoli development and terminal differentiation of the mammary epithelium [Yang et al., 1995]. It has also been demonstrated that treatment of prepubescent female mice with HRG induces ductal branching in the absence of steroid hormones, and lobuloalveolar development when steroids are present [Jones et al., 1996]. Taken together, these data demonstrate an important role for heregulin during mammary gland development.

To understand the mechanism of HRG-induced differentiation, it is important to delineate the specific cellular signaling pathways involved. The mitogen-activated protein kinase (MAPK) family is a group of protein kinases that are key to cell signaling pathways activated by a variety of external stimuli [Seger and Krebs, 1995; Marshall, 1994]. The sequential activation of p21Ras, Raf-1, ERK kinase (MEK), and MAPK 1 and 2, occur through a series of phosphorylation events. Upon activation, $p44^{MAPK}$ and $p42^{MAPK}$ (ERK 1 and 2, respectively) translocate to the nucleus and phosphorylate various proteins including transcription factors and cell cycle regulatory proteins [Gille et al., 1992; Pulverer et al., 1991; Seth et al., 1991; Sturgill et al., 1988; Gotoh et al., 1991; Haycock et al., 1992; Lin et al., 1993]. This pathway appears to regulate a number of cellular responses, including cell proliferation and differentiation [Seger and Krebs, 1995; Marshall, 1994].

We hypothesized that cell signals regulating heregulin-induced differentiation of mammary epithelial cells are transmitted by the ERK signaling pathway. We report that sustained activation of the ERK pathway is required for HRG-induced differentiation of AU565 breast carcinoma cells. These findings have important implications for the understanding of the signalling pathways mediating the differentiated phenotype in mammary carcinomas.

MATERIALS AND METHODS

Reagents

Heregulin-β1 was a kind gift from Genentech (San Francisco, CA). The MEK inhibitor, PD98059, was obtained from Calbiochem (San Diego, CA). All chemicals unless otherwise specified were purchased from Sigma (St. Louis, MO).

Cell Culture

AU565 cells were maintained in complete RPMI 1640 media (Biofluids, Rockville, MD) containing 15% fetal bovine serum (Sigma).

Cell Transfection

AU565 cells were cultured in complete media to 50% confluence in six-well plates (Corning, Corning, NY). Cells were washed once with Opti-MEM media (Lifetech, Gaithersburg, MD) prior to transfection. The pcDNA3.1-activated MEK1 vector was constructed as follows. A 2 kb KpnI-XhoI fragment of pMCL-HA tagged constitutively active MEK1 (ΔS222D; a kind gift of Dr. Natalie Ahn, Boulder, CO) was directionally subcloned into the KpnI-XhoI sites in the polylinker region of pcDNA3.1. Cells transiently transfected with this plasmid expressed an HA tagged MEK1 protein. Plasmid DNA (5 μg) was incubated with Lipofectin Reagent (Lifetech) in Optimem medium for 15 min and added to cells for 5 h at 37°C. Cells were washed once with Optimem and propagated in complete media with 15% serum. Cells received HRGβ1 16 h after replacement of fresh media. Cells were harvested 4 days after transfection and cell differentiation assessed by Oil Red O staining as described below. Transfection efficiencies ranged from 17–23% as determined in parallel transfections with pcDNA3lacZ. Cells were stained for β-galactosidase activity as previously described [Aicher et al., 1997].

Growth Analysis

2.5×10^4 cells were seeded in triplicate in 12-well tissue culture plates (Corning) in the presence or absence of different concentrations of PD98059 and HRGβ1 as indicated. After 7 days, the cells were trypsinized and stained with 0.4% Trypan Blue (Gibco BRL, Grand Island, NY). Viable cells were counted using a hemacytometer.

Oil Red O Staining

Oil Red O staining was performed as a measure of cell differentiation as described previously [Bacus et al., 1992]. Differentiated mammary epithelial cells show red, fat droplets while undifferentiated cells do not. Briefly, 5×10^3 cells were seeded in triplicate in the wells of

Lab-Tek 8 chamber slides (Nunc, Inc., Naperville, IL) and incubated in the presence or absence of different concentrations of PD98059 with or without 10 ng/ml HRGβ1. After 7 days, the cells were fixed in a solution of 10% phosphate buffered formalin for 20 min and stained with Oil Red O working solution. Cells were counterstained with Harris Hematoxylin solution.

Western Blotting

Cells were plated in complete media containing 15% serum and grown to 60–70% confluence. Prior to stimulation, cells were serum starved for 16 h in media containing 0.1% serum. Cells were then incubated with either 10 μM PD98059 or vehicle alone (0.1% DMSO) for 60 min followed by stimulation with 10 ng/ml HRGβ1 for 10 min at 37°C. Cells were solubilized in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 1% (v/v) Triton-X-100, 0.1% SDS, 50 mM β-glycerophosphate, 50 mM sodium flouride, 5 mM $MgCl_2$, 1 mM sodium orthovanadate, 1 mM DTT, 10 mM EGTA, 2.5 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Cell lysates were clarified by centrifugation at 16,000*g*, and protein concentrations were quantitated using the Bradford Assay Reagent (Pierce, Rockford, IL). Equal amounts of protein were resuspended and boiled in 3× Laemmli buffer. Proteins were resolved by electrophoresis on 12% SDS polyacrylamide gels and electrotransferred to nitrocellulose. Blots were probed with primary antibodies against activated ERK 1/2 (Promega, Madison, WI) or pan-ERK (clone 16, Transduction Laboratories, Lexington, KY) and the appropriate secondary peroxidase-conjugated antibodies prior to detection using the Enhanced Chemiluminescence (ECL) system (Amersham, Arlington Heights, IL). Protein bands were quantitated by densitometry using a Molecular Dynamics Image Quant Densitometer.

ERK Activity

ERK activity was assayed as previously described [Marte et al., 1995]. Briefly, after HRGβ1 treatment, cells were lysed in lysis buffer as described above and 200 μg of total cell lysates were immunoprecipitated with a mouse monoclonal anti-pan ERK antibody for 16 h at 4°C. Immunocomplexes were recovered by Protein G-plus/Protein A-Agarose (Oncogene Science, Uniondale, NY) for 1 h, centrifuged, and washed three times with lysis buffer and once in kinase reaction buffer containing 80 mM β-glycerophosphate, 20 mM EGTA, 50 mM $MgCl_2$, 20 mM HEPES (pH 7.2). Immune complex-kinase reactions were then performed by resuspending immune complexes in 30 μl kinase buffer containing 15 μg myelin basic protein, 10 μM cold ATP and 2 μCi [γ-^{32}P]ATP (1,000 Ci mmol$^{-1;}$ Amersham). The reactions were performed at 30°C for 30 min and terminated by the addition of 10 μl 3X SDS Laemmli buffer. After boiling, proteins were separated by 12.5% SDS-PAGE. Gels were fixed in a solution containing 10% acetic acid, 25% methanol, dried, and exposed to Kodak XAR film. Autoradiograms were quantitated with a Phosphoimager (Molecular Dynamics).

Statistical Analysis

The two-tailed Student's *t*-test was used to compare control to experimental groups. Data are expressed as mean ± S.E. Statistical significance was established at the 5% level.

RESULTS

Kinetics of ERK Activation Upon Treatment With HRG in AU565 Cells

In the human breast carcinoma cell line, AU565, HRG elicits a biphasic, dose-dependent, response [Bacus et al., 1992]. At low concentrations, HRG stimulates cell proliferation, whereas high concentrations induce differentiation. To determine if treatment with doses of HRG which induce differentiation results in a sustained increase in ERK activities, the kinetics of ERK activation were determined at different concentrations of HRGβ1 (Fig. 1). AU565 cells were treated for up to 60 min with 0.01 and 10 ng/ml HRGβ1. Treatment with 0.01 ng/ml HRGβ1 increased ERK activity approximately seven-fold after 5 min of treatment. Activity then dropped to 60% of control levels by 30 min with a subsequent return to control levels by 90 min. Treatment of cells with 10 ng/ml HRGβ1 increased ERK activity approximately four-fold after 10 min. Although this initial increase dropped to control levels by 15 min, a second sustained increase in activity was observed for the following 105 min. These data were also confirmed with the use of a nonradioactive ERK assay (NEBiolabs, Beverly, MA; data not shown).

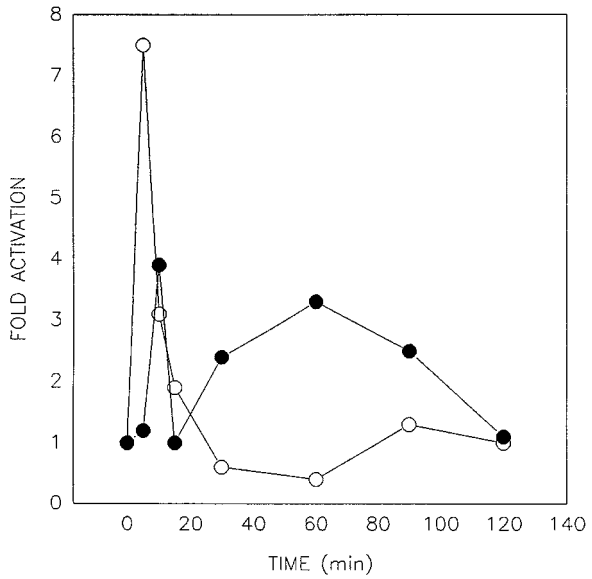

Fig. 1. ERK activation in response to HRGβ1 treatment. Serum-starved AU565 cells were treated with 0.01 ng/ml (open circles) or 10 ng/ml (closed circles) HRGβ1 for 0–120 min. Cells were assayed for ERK activity as described in Experimental Procedures. Fold activation was quantified via densitometry. Data are representative of four independent experiments.

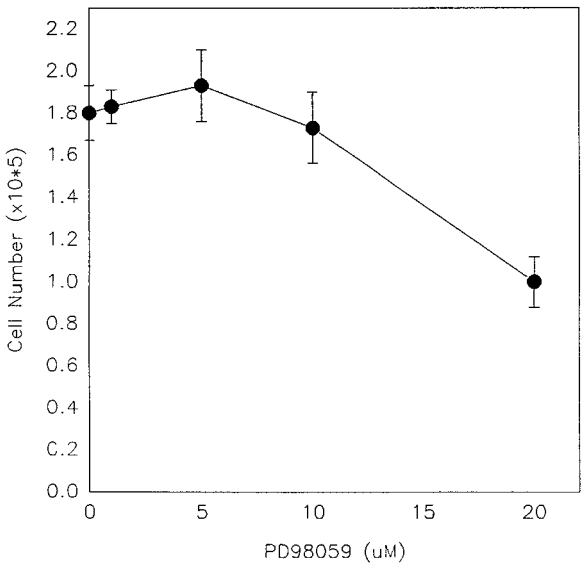

Fig. 2. Effects of PD98059 on growth of AU565 cells. Cells were grown in complete media in the presence of 0–20 μM PD98059. Cell growth was assessed at 7 days as described. Each point represents the mean ± S.D. of four wells.

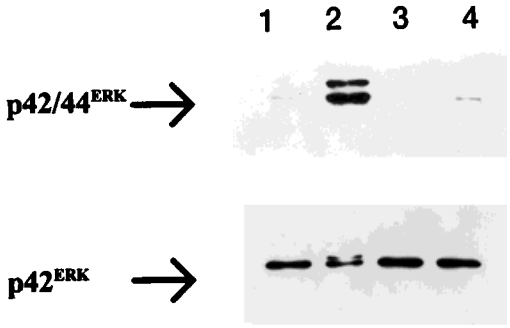

Fig. 3. PD98059 blocks activation of ERK. AU565 cells were treated with or without 10 μM PD98059 for 60 min, followed by a 10 min stimulation with 10 ng/ml HRGβ1. Western blotting was performed with an antibody to activated ERK 1 and 2 (top). Blots were then stripped and reprobed with a pan-ERK antibody (bottom). **Lane 1:** Vehicle control [dimethyl sulfoxide (DMSO), 0.1%]; **Lane 2:** vehicle and HRG; **Lane 3:** PD98059; **Lane 4:** PD98059 and HRG.

Effects of MEK-Specific Inhibitor, PD98059, on AU565 Cells

To study the role of the ERK pathway in HRGβ1-mediated cell growth arrest and differentiation, the specific MEK inhibitor, PD98059, was used to block activation of ERK by MEK. This compound is a selective inhibitor of MEK1 [Alessi et al., 1995], and blocks subsequent activation of ERK [Pumilglia and Decker, 1997; Pang et al., 1995]. To determine the toxicity of PD98059 in AU565 cells, cells were treated with 0–20 μM PD98059 for 7 days and the number of viable cells determined (Fig. 2). No significant decrease in cell growth or increase in cell death was observed at concentrations of 0.1–10 μM.

To confirm that PD98059 inhibits ERK activation, we performed Western blot analysis using an antibody against activated, phosphorylated ERK 1 and 2 (Fig. 3, top panel). Cells were treated with 10 μM PD98059 or vehicle (0.1% DMSO) for 60 min, followed by a 10 min exposure to 10 ng/ml HRGβ1 at 37°C. Cell lysates were Western blotted with the antibody to activated ERK. PD98059 completely blocked phosphorylation of $p44^{ERK}$ and inhibited phosphorylation of $p42^{ERK}$ by 77%. The blot was stripped and reblotted with a pan-ERK antibody which verified that levels of total $p42^{ERK}$ were equal as determined by densitometry (Fig. 3, bottom panel).

Suppression of HRG-Induced AU565 Cell Differentiation by PD98059

The ability of PD98059 to block cellular differentiation induced by a high dose of HRGβ1 was examined. AU565 cells were treated with 10 ng/ml HRGβ1 and 0–10 μM PD98059 for 7 days. As shown in Figure 4, HRGβ1 treatment decreased cell number by 56% as compared to

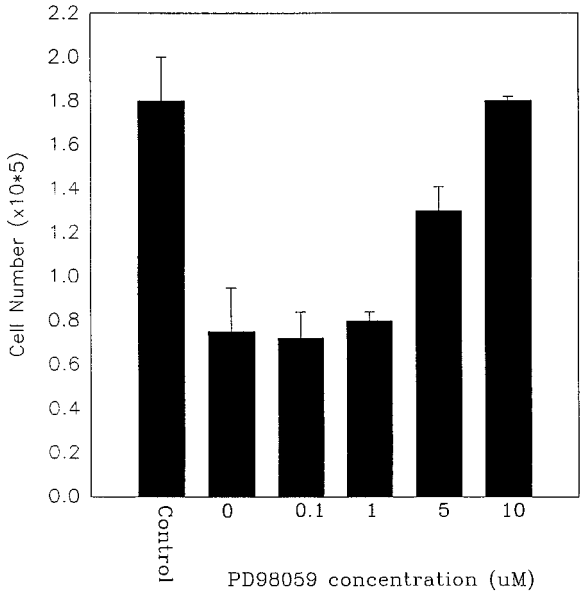

Fig. 4. Effect of the MEK inhibitor PD98059 on HRGβ1-mediated cell growth arrest. 2×10^5 AU565 cells were seeded in triplicate in 12-well plates and treated with vehicle control (0.1% DMSO) or 10 μM PD98059 for 60 min prior to addition of 10 ng/ml HRGβ1 for 7 days. Cells were trypsinized and stained with 0.4% Trypan Blue and viable cells counted as described. Each point represents the mean ± S.E and are representative of two separate experiments.

the nontreated control. Pretreatment of cells with 10 μM PD98059 resulted in a dose-dependent reversal of HRGβ1-induced cell growth arrest. HRGβ1-induced differentiation was also inhibited with PD98059 as determined by Oil Red O Staining for neutral lipids (Fig. 5). Treatment of cells with 10 ng/ml HRGβ1 increased the number of Oil Red O positive cells by 40% (31.3% vs. 72.6%). Pretreatment with PD98059 inhibited differentiation in a dose-dependent manner, with 10 μM PD98059 completely blocking HRG-induced differentiation (35.2%). These data suggest that activated MEK or ERK is required for HRG-induced differentiation.

Effects of Constitutively Active MEK1 on AU565 Cell Differentiation

In order to test the effect of MEK activity on AU565 cell differentiation, a constitutively activated MEK1 mutant was transiently expressed in these cells [Mansour et al., 1994]. Effects of mutant MEK1 expression on basal and HRG-stimulated cell differentiation was assessed by Oil Red O staining (Table 1). As expected, 20.8% of cells transfected with the control pcDNA3.1 vector were Oil Red O positive in the absence of HRGβ1. Treatment with HRG (10 ng/ml) increased the number of differentiated cells to 67% of total cells. In contrast, 53% of cells transfected with the constitutively activated MEK1 vector were Oil Red O positive. Incubation with HRG further increased the number of Oil Red O positive cells to 81% of the total. To verify that constitutively active MEK1 resulted in enhanced ERK activity in AU565 cells, ERK activities were assessed following transient transfection with mutant MEK1. Expression of constitutively active MEK1 led to a significant enhancement of ERK activity as determined by use of an antibody to activated ERK (Fig. 6A,B). These data demonstrate that constitutive activation of MEK/ERK in AU565 cells is sufficient to mediate differentiation.[8]

DISCUSSION

The importance of the MAPK pathways in mediating cellular differentiation and growth arrest has recently gained great attention. In the present study, we demonstrate for the first time that a sustained MEK/MAPK signal is responsible for HRG-induced differentiation of AU565 mammary carcinoma cells, and that sustained ERK activation is sufficient for maintaining the differentiated phenotype in the absence of HRG. This is the first report demonstrating the importance of the ERK signaling pathway in cellular differentiation with a human epithelial cell system in which the same growth factor, HRG, mediates both cell proliferation and differentiation, depending upon dosage.

Our data demonstrate that in the AU565 breast carcinoma cell line, HRG-induced cell differentiation was associated with sustained ERK activity when compared with HRG-induced proliferation, which is more transient in nature. This is consistent with the results of Traverse et al. [1992] that showed a more sustained ERK activation in NGF-induced differentiation of PC12 cells. Two peaks of ERK activation were observed after treatment with a high concentration of HRG. The mechanism of this complex regulation of ERK activation is presently unknown. MAPK phosphatase (MKP)-1 and -2 are two thr/tyr phosphatases which specifically dephosphorylate ERK [Sun et al., 1993; Misra-Press et al., 1995]. It has been suggested that growth factors which activate ERK also rapidly increase MKP-1 and MKP-2 mRNA levels, as demonstrated by treatment of PC12 cells

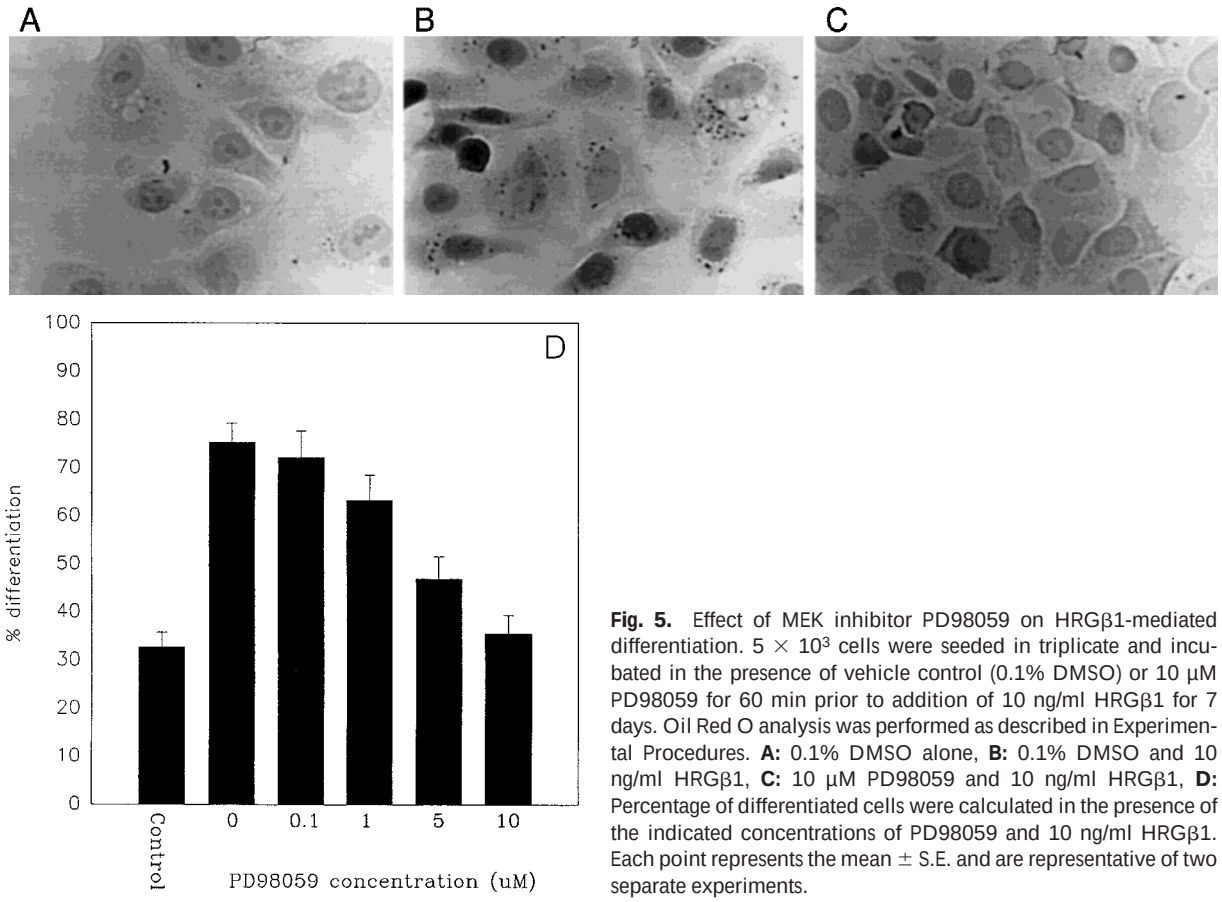

Fig. 5. Effect of MEK inhibitor PD98059 on HRGβ1-mediated differentiation. 5×10^3 cells were seeded in triplicate and incubated in the presence of vehicle control (0.1% DMSO) or 10 μM PD98059 for 60 min prior to addition of 10 ng/ml HRGβ1 for 7 days. Oil Red O analysis was performed as described in Experimental Procedures. **A:** 0.1% DMSO alone, **B:** 0.1% DMSO and 10 ng/ml HRGβ1, **C:** 10 μM PD98059 and 10 ng/ml HRGβ1, **D:** Percentage of differentiated cells were calculated in the presence of the indicated concentrations of PD98059 and 10 ng/ml HRGβ1. Each point represents the mean ± S.E. and are representative of two separate experiments.

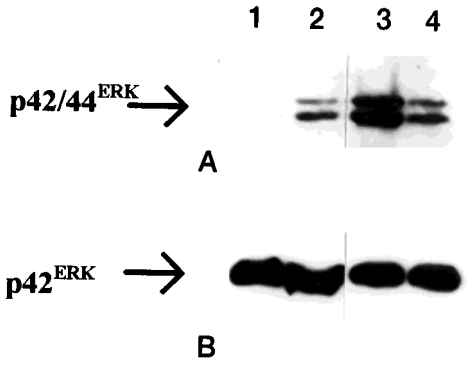

Fig. 6. Effect of transfection of constitutively active MEK on ERK activity. AU565 cells were transiently transfected with constitutively active mutant HA-MEK1, treated with 10 ng/ml HRG for 10 min, and Western blotting performed with and antibody to activated ERK (**A**), or pan-ERK (**B**). **Lane 1:** Control (pcDNA3.1 vector alone), **Lane 2:** control vector and HRG, **Lane 3:** HA-MEK1 and HRG, **Lane 4:** HA-MEK1 alone.

TABLE I. Effect of Constitutively Active MEK1 on AU565 Cell Differentiation

	Percent oil red O positive cells	
Plasmid	−HRG	+HRG
pcDNA3.1	20.8 ± 9.3[a]	67.0 ± 5.6
pcDNA3.1-MEK1	53.6 ± 8.6	81.8 ± 6.5

[a]Mean ± S.D. (four wells).

with EGF and NGF [Sun et al., 1993]. However, the role these phosphatases play in the kinetics of ERK inactivation in our system remains unclear. Another phosphatase which may determine the kinetics of ERK inactivation is PP2A. PP2A is a general ser/thr phosphatase which also dephosphorylates ERK and has been shown to colocalize to microtubules with ERK [Waskiewicz and Cooper, 1995; Fiore et al., 1993; Sontag et al., 1995]. Upon growth factor stimulation, PP2A becomes inactivated after tyrosine phosphorylation, rendering it unable to dephosphorylate ERK (and other substrates) [Chen et al., 1992]. Studies are underway to examine the role of phosphatase activation in the AU565 mammary carcinoma cell line.

In addition to internal control of ERK activity, it has been suggested that the number of receptors activated at the cell surface determines the nature of the ERK signal (sustained

or transient) thereby determining downstream cellular events [Marshall, 1995; Traverse et al., 1994]. In AU565 cells, low concentrations of HRG activate a small number of high affinity erbB2/erbB3 heterodimers, whereas high concentrations of HRG activate a large number of both high (erbB2/erbB3 heterodimers) and low (erbB3/erbB3 homodimers) affinity erbB receptors [Sliwkowski et al., 1994], suggesting that this may, at least in part, regulate differential ERK activity.

PD98059 is a compound which has been previously shown to specifically block the activation of MEK, and subsequently ERK [Alessi et al., 1995; Pumiglia and Decker, 1997; Pang et al., 1995]. In AU565 cells, 10 μM PD98059 blocked HRG-induced differentiation and reversed HRG-mediated cell growth arrest without significant effect on cell viability. This finding demonstrates that MEK (and/or ERK) activation is essential for HRG-induced differentiation. Furthermore, a constitutively activated mutant of MEK1, which results in activation of MEK1 [Mansour et al., 1994], and ERK in the absence of HRG, induced a differentiated phenotype. In the presence of ligand, there was a further increase in differentiation, as determined by Oil Red O staining, accompanied by a further increase in activated ERK suggesting that this signaling pathway is potentiated by the presence of constitutively active MEK. It is currently unknown which ERK substrates are required for AU565 cell differentiation. However, upon translocation to the nucleus, ERK is known to phosphorylate a number of general transcription factors resulting in immediate early gene expression [Gille et al., 1992; Pulverer et al., 1991; Seth et al., 1991; Sturgill et al., 1988; Gotoh et al., 1991; Haycock et al., 1992; Lin et al., 1993; Davis, 1993; Kortenjann et al., 1994; O'Neill et al., 1994; Towatari et al., 1995]. Our data demonstrate that sustained activation of the MEK/ERK pathway is both necessary and sufficient for the differentiation of a human breast carcinoma cell line. Current efforts are directed towards understanding how ERK signaling regulates transcription of genes important in mammary cell differentiation. These findings will have important implications into our understanding of signaling pathways involved in HRG-induced mammary cell differentiation, in both normal mammary gland development and breast carcinomas.

ACKNOWLEDGMENTS

We thank Dr. Arun Rishi and Weidong Yang for technical assistance, and Dr. Craig Webb for critical review of the manuscript. This work was supported in part by an Interactive Research Grant from the Greenebaum Cancer Center at the University of Maryland (to A.W.H.) and a Graduate Program Enhancement Award (to T.L).

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