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Advances in Brief

Heregulin Induces Expression, DNA Binding Activity, and Transactivating Functions of Basic Leucine Zipper Activating Transcription Factor 4[1]

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Abstract

Heregulin β1 (HRG), a combinatorial ligand for human epidermal growth factor receptor 3 and human epidermal growth factor receptor 4 receptors, is a regulatory secretory polypeptide with distinct biological effects such as growth stimulation, differentiation, invasiveness, and migration in breast cancer cells. The mechanism underlying the diverse functions of HRG is not well established, but it is believed to be dependent on the induced changes in expression of specific cellular gene products, their modification, or both. The binding of basic leucine zipper transcription factors to the cAMP response element is known to activate a variety of gene products with a role or roles in growth regulation. In the studies presented here, we identified basic leucine zipper activating transcription factor (ATF) 4 as one of the HRG-inducible gene product. We demonstrated that HRG stimulation of human cancer cells induces expression of ATF4 mRNA and protein, ATF4 DNA binding activity, and ATF4 transactivating function. Consistent with its role as a transcriptional activator, HRG-stimulated ATF4 protein stimulated the transcription from an artificial promoter with three tandem ATF sites or from a naturally occurring promoter with ATF4 sites such as E-selectin. We also demonstrated a preferential role of the HRG-stimulated mitogen-activated protein kinase pathway, but not the phosphatidylinositol 3′-kinase pathway, in supporting the observed increase in ATF4 DNA binding activity and transcription from E-selectin promoter in HRG-stimulated cells. Because ATF4 binding sites are present in a variety of growth-regulating cellular genes, these findings suggest that the stimulation of ATF4 expression and its transactivating functions may constitute an important mechanism of HRG-mediated regulation of putative genes with diversified functions. The present study is the first demonstration of regulation of expression and transactivation ability of ATF4 by any polypeptide growth factor.

Introduction

Growth factors and their receptors play an essential role in the regulation of epithelial cell proliferation. It has been demonstrated that abnormalities in growth factor expression and action contribute to the progression and maintenance of the malignant phenotype. For example, c-*erb*B2 encodes HER2,[3] the overexpression of which is frequently associated with an aggressive clinical course and increased metastasis in human breast cancer (1). Recently, additional members, HER3 and HER4, have been added to the HER2 family because these receptors also share sequence homology with the tyrosine kinase domain of HER1 (2). The family receptors can be transactivated by receptor-receptor interaction in a ligand-dependent manner and thus can use more than one pathway to execute their biological functions (2–4). In addition, accumulating evidence suggests that the progression of human breast cancer cells may be regulated by heregulin, a combinatorial ligand for HER3 and HER4 receptors. Recently, we and others (5–9) have demonstrated that HRG activation of breast cancer cells (in the absence of HER2 overexpression) also promotes the development of more aggressive phenotypes in breast cancer cells. Although these observations suggest that ligand-driven activation of HER receptors may play an important biological role or roles in the progression of breast cancer cells to a malignant phenotype, the nature of the pathways by which HRG signals are relayed to the nucleus in breast cancer cells remains poorly understood.

It is widely believed that the induction of a set of early growth-responsive (also known as immediately early) genes, in the absence of *de novo* protein synthesis, may constitute the first step in the cellular molecular response to extracellular signals. Transcription of protein-coding genes is one of the major regulatory steps in gene expression. Among the major regulatory elements that contribute to transcriptional regulation of extracellular signals are the CRE and activator protein 1 sequence motifs. The CRE element (TGACGTCA) is defined as the ATF binding site. The process of transcription involves physical interactions of sequence-specific DNA binding transcriptional activations with general transcription factor directly, indirectly, or both (10). It is increasingly accepted that the CRE site is recognized by a family of the bZIP-containing proteins known as CREBs or ATFs. The bZIP DNA-binding proteins need to dimerize for productive DNA binding (11). The dimeric bZIP factors are characterized by a bipartite α-helical structure: the basic NH_2-terminal half of the motif interacts with sequence-specific DNA; whereas the COOH-terminal half of the motif dimerizes to form a leucine zipper (11, 12). The bZIP factors not only homodimerize but also heterodimerize with specific bZIP proteins by the leucine zipper (12). Because binding sites for ATF factors are present in several growth-regulating cellular promoters (13), they are believed to be involved in different regulatory circuits, allowing cells to integrate signals from distinct pathways. In addition, the activity of ATF is also regulated by coactivators such as CREB-binding protein/p300 family members (14). In the present study, we have investigated the possible involvement of ATF proteins in the action of HRG in breast cancer cells. We report that recombinant HRG up-regulates the expression of bZIP transcription factor ATF4, its DNA binding activity, and the ability of ATF4 to transactivate the target genes in HRG-responsive cancer cells.

Materials and Methods

Cell Cultures and Reagents. Human breast cancer MCF-7 cells (8) and colon cancer cell lines LS174T, CaC02, and FET (15) were maintained in DMEM-Ham's F-12 (1:1) supplemented with 10% FCS. Recombinant HRG was purchased from Neomarkers, Inc., and anti-ATF4 Ab was purchased from Santa Cruz, Inc.

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[3] The abbreviations used are: HER, human epidermal growth factor receptor; HRG, heregulin β1; ATF, activating transcription factor; bZIP, basic leucine zipper; CRE, cAMP response element; CREB, CRE-binding protein; MAPK, mitogen-activated protein kinase; PI-3K, phosphatidylinositol 3′-kinase; Ab, antibody; RT-PCR, reverse transcription-PCR; CAT, chloramphenicol acetyltransferase; HUVEC, human umbilical vein endothelial cell; MEKK, MAPK kinase kinase.

Cell Extracts and Immunoprecipitation. Cell lysates containing equal amounts of protein were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with the appropriate Abs. An equal number of cells were metabolically labeled for 12 h with 100 μCi/ml [^{35}S]methionine in methionine-free medium containing 2% dialyzed fetal bovine serum in the absence or presence of HRG. Cell extracts (equal perceptible trichloroacetic acid counts) were immunoprecipitated with the desired or control Ab, resolved on a SDS-PAGE gel, and analyzed by autoradiography (16).

RT-PCR and Northern Hybridization. The sequences of the following primers were obtained from the Clontech: (*a*) Tax, Tax-responsive enhancer element-binding protein 107; (*b*) PUF, c-Myc purine-binding transcription factor PUF; and (*c*) Mac, Merkes-related protein. The RT-PCR for ATFD and others genes was done according to the manufacturer's instructions using access RT-PCR systems (Promega). The forward primer sequence for ATF4 was 5′-AATGGCTGGC′rGTGGATGGG′I″I′GGTCA-3′, and the reverse primer sequence was 5′-GATCATGGCAACGTAAGCAGTGTAGTCTG-3′. Human E-selectin-specific primers were purchased from R&D Systems. Total cytoplasmic RNA was analyzed by Northern hybridization using an ATF4 cDNA probe (17) or a RT-PCR-generated 436-bp fragment. Ribosomal 28S RNA and 18S RNA were used to assess the integrity of the RNA.

Nuclear Extract Preparation and Electrophoresis Mobility Shift Assay. Nuclear extracts were prepared as described previously (18). The synthetic oligonucleotides corresponding to the consensus sequence of human CRE (5′-AGAGATTGCCtGACGTCAGAGAGCTAG-3′), activator protein 1 (5′-CGCTTGATGACTCAGCCGGAA-3′), and nuclear factor κB (5′-AGTT-GAGGGGACTTTCCCAGG-3′) were purchased from Santa Cruz, Inc. Oligonucleotides were end-labeled with ^{32}P by using the end-labeling kit (Boehringer Mannheim). To confirm the ATF4 protein in the DNA-protein complex, 5-μg extracts were incubated for 10 min with or without antisera recognizing different nuclear factors.

Promoter Assays. Dr. T. Hai (Ohio State University, Columbus, OH) generously provided pEC $(ATF)_3$-CAT (19) and E-selection-CAT (20). Cells were serum starved in low serum medium (0.1% serum) for 48 h before transfection. Serum-starved cells were transiently cotransfected with ATF4 plasmid and a control vector using Lipofectamine (Life Technologies, Inc.). CAT activity was measured 48 h after transfection using a CAT assay kit (Promega).

Results

Identification of ATF4 as a HRG-inducible Gene. To better understand the mechanism of HRG action in breast cancer cells, we screened MCF-7 cells for HRG-inducible genes using the Atlas cDNA Gene Array (Clontech). Total RNA was isolated from control and HRG-treated cells, and cDNAs were generated by reverse transcriptase in the presence of [α-^{32}P]dCTP and hybridized to gene array filters. This screening resulted in the identification of bZIP transcription factor ATF4 as a HRG-inducible gene in breast cancer cells (Fig. 1*A*). To validate the results of RT-PCR screening, Northern blot analysis was performed using a 406-bp PCR probe specific for ATF4. Data in Fig. 1*B* demonstrate that HRG increased the steady-state levels of the 1.6-kb mRNA of ATF4 by 2–4-fold in MCF-7 cells, with maximal induction between 10 and 24 h after HRG treatment. Similar results were obtained when the Northern hybridization was performed using a human ATF4 cDNA (data not shown). Because there was no precedent of growth factor-inducible up-regulation of ATF4, the experiment was independently repeated five times, and similar results were obtained.

HRG Regulates ATF4 at the Pretranslational Level. The observed HRG-mediated increase in ATF4 mRNA could be due to increased synthesis of newly transcribed mRNA, enhanced stability of ATF4 mRNA, or both. To delineate this possibility, we examined the effect of actinomycin D, an inhibitor of transcription. Pretreatment of cells with actinomycin D abolished the HRG-mediated induction of ATF4 mRNA, suggesting the need for continuous RNA synthesis in the observed increased expression of ATF4 mRNA in HRG-treated cells (Fig. 1*C*). To address the issue of translational regulation, we used cycloheximide, a translational inhibitor. Treatment of cells with cycloheximide by itself induced the expression of ATF mRNA (2–3-fold). However, HRG treatment further induced the expression of ATF4 mRNA compared with the level in cycloheximide-treated culture (compare *Lane 3* with *Lane 4*). These results suggest that HRG regulates ATF4 expression at a pretranslational level.

HRG Induces the Expression of Newly Synthesized ATF4 Protein and Its DNA Binding Activity. To determine whether the observed increase in the level of ATF4 mRNA in HRG-treated MCF-7 breast cancer cells was associated with an increase in the expression of ATF4 protein, Western blot analysis was performed. The results seen in Fig. 2*A* demonstrate that treatment of MCF-7 cells with HRG was accompanied by a significant increase in the steady-

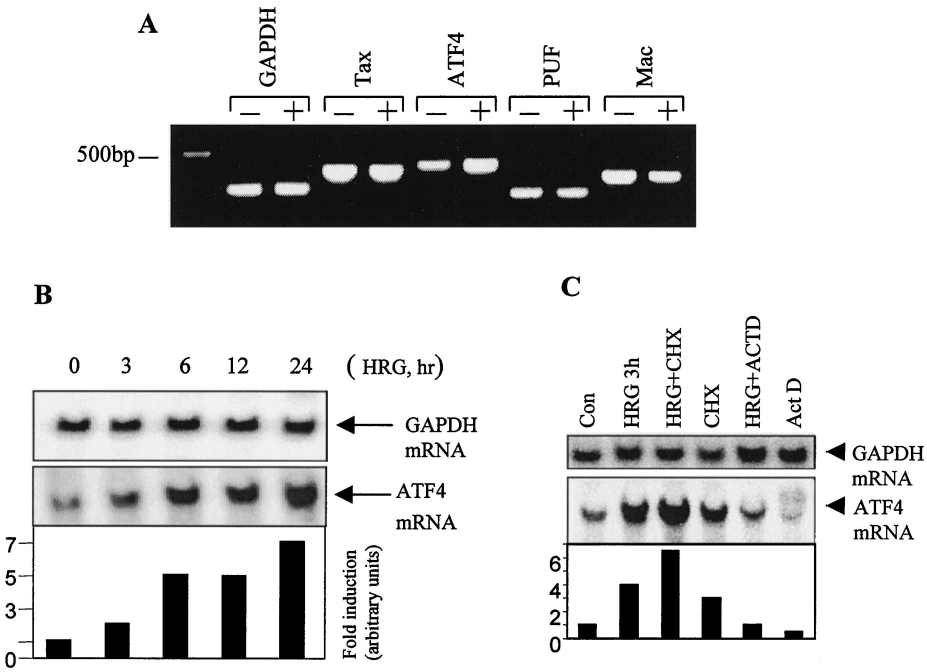

Fig. 1. HRG regulation of ATF4 mRNA expression. *A*, identification of ATF4 as a HRG-inducible gene. *Tax*, Tax-responsive enhancer element binding protein 107; *PUF*, c-Myc purine-binding transcription factor PUF; *Mac*, Merkes-related protein; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *B*, MCF-7 cells were treated with HRG for the indicated times. Total RNA (20 μg) was analyzed by Northern blotting using a PCR-generated 436-bp human ATF4 fragment as a probe. The blot was reprobed with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe. Quantitation of ATF4 mRNA is shown in the *bottom panel*. The results shown are representative of five independent experiments. *C*, HRG induces ATF4 mRNA expression. MCF-7 cells were treated with cycloheximide (50 μg/ml) or actinomycin D (10 μg/ml) in the presence or absence of HRG (10 ng/ml) for 3 h. Total RNA was isolated, and the levels of ATF4 mRNA were detected by Northern blotting. Quantitation of ATF4 mRNA is shown in the *bottom panel*. Results shown are representative of two independent experiments.

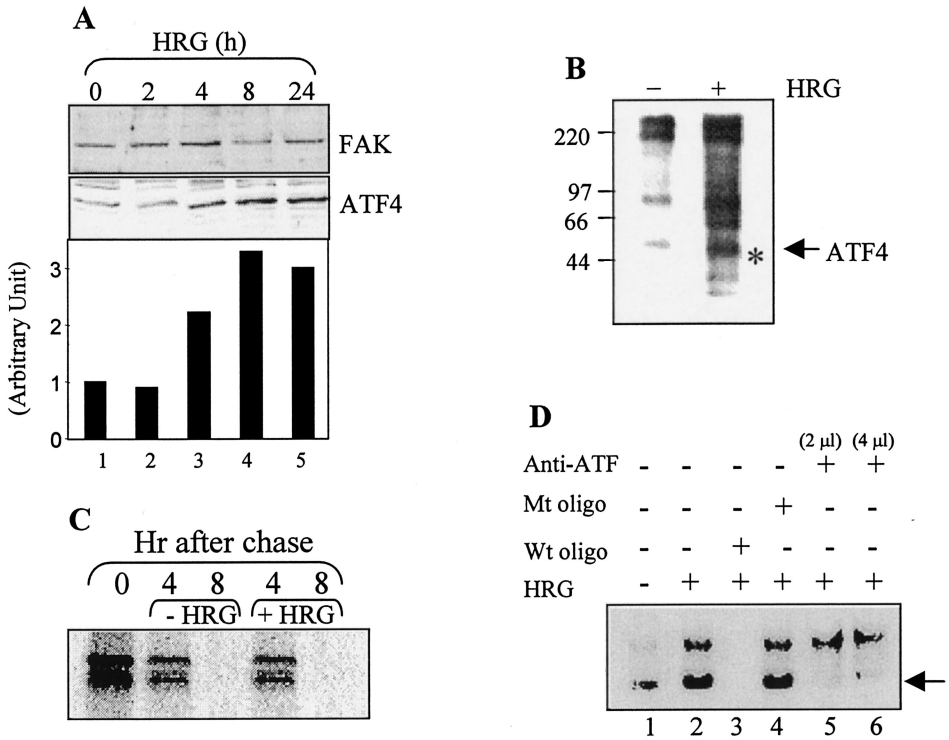

Fig. 2. HRG up-regulates the level of ATF4 protein. *A*, MCF-7 cells were treated with HRG for the indicated times. Total lysates were subjected to SDS-PAGE and blotted with anti-ATF4 Ab (*middle panel*). As an internal control, the upper portion of same blot was probed with anti-FAK Ab (*top panel*). Quantitation of ATF4 mRNA is shown in the *bottom panel*. *B*, MCF-7 cells were stimulated with HRG for 12 h and metabolically labeled with [^{35}S]methionine during the last 4 h before harvesting. Cell lysates were immunoprecipitated with an anti-ATF4 Ab and analyzed by SDS-PAGE, followed by fluorography. *C*, MCF-7 cells were metabolically labeled with [^{35}S]methionine for 2 h and cultured further in the presence or absence of HRG for the indicated times. Cell lysates were immunoprecipitated with an anti-ATF4 Ab and analyzed by SDS-PAGE, followed by fluorography. Results shown are representative of three independent experiments. *D*, induction of ATF4 DNA binding activity. Nuclear extracts were incubated with a ^{32}P-labeled oligonucleotide containing the consensus CRE element for 30 min at 37°C. When indicated, nuclear extracts were preincubated with a 50-fold excess of unlabeled wild-type or mutated oligonucleotide and also by ATF4 Ab (2 or 4 μl) for 15 min. An *arrow* shows the specific DNA-protein complex with which ATF4 Ab competed. Results shown are representative of three independent experiments.

state level of −50 kDa ATF4 protein. The observed HRG-mediated increase in ATF4 expression was due to increased expression of newly synthesized ATF4 in HRG-treated cells, as confirmed by metabolic labeling studies (Fig. 2*B*). There was no effect of HRG on the half-life (<4 h) of ATF4 protein as assessed by pulse-chase experiments (Fig. 2*C*).

To understand the physiological significance of ATF4 in HRG activity in breast cancer cells, we investigated whether HRG signaling could enhance the DNA binding of ATF4 to the CRE motif by using a gel-shift assay. Data in Fig. 2*D* show that HRG treatment for 30 min was associated with a significant enhancement of DNA binding activity of ATF4 because this activity could be competed by wild-type (Fig. 2*D, Wt oligo*) unlabeled CRE oligonucleotide but not by mutated (Fig. 2D, *Mt oligo*) CRE oligonucleotide; the activity was blocked by pretreatment of the nuclear extracts with a well-characterized anti-ATF4 Ab (Ref. 17; Fig. 2*D*, the lower band is indicated by an *arrow*).

HRG Regulates ATF4 Expression and Its DNA Binding Activity in Diversified Cell Types. To determine whether the observed induction of ATF4 expression is an effect restricted to HRG in MCF-7 cells or whether it could be demonstrated in other HRG-responsive cells, we examined the effect of HRG on ATF4 expression in HRG-responsive human colorectal LS174T, CaCO2, and FET cells (15). As illustrated in Fig. 3, HRG treatment was associated with a significant up-regulation of ATF4 mRNA expression in a number of HRG-responsive cells (Fig. 3*A*) and also of ATF4 protein levels (Fig. 3*B*). Although HRG could induce ATF4 expression and its DNA binding activity in a number of human cell lines, we have chosen human breast cancer MCF-7 cells as a model system to further ascertain the potential significance of ATF4 pathway in the regulation of HRG action.

Regulation of ATF4 Transactivation Functions by HRG. We next determined whether HRG could activate CRE site-driven transcription using a CAT reporter system. MCF-7 cells were transiently transfected with a CAT reporter driven by artificial ATF sites [p(ATF4)$_3$ CAT] and stimulated with or without HRG. As illustrated in Fig. 4*A*, HRG treatment stimulated the CAT reporter up to 3–5-fold more than untreated control cells in a time-dependent manner. In addition to activating the CAT reporter system driven by an artificial promoter, HRG also stimulated the transcription from naturally occurring promoters with ATF4 sites such as E-selection (19), although to a lesser degree. In general, E-selectin promoter was about 2.6 times less active, as compared to (ATF4)$_3$ promoter. This may be related to the fact that MCF-7 cells do not express E-selectin, probably due to the presence of the appropriate transcription factors in the appropriate places to drive full expression of the promoter. Results in Fig. 4*B* demonstrate that HRG activated the E-selectin promoter, although to a lesser extent than did the synthetic ATF4 promoter. It is possible that HRG-induced ATF4 protein may not interact efficiently with the natural promoter due to its interactions with other factors. Alternatively, the observed activation may be sufficient to regulate the expression of E-selectin mRNA. The putative role of HRG-induced ATF4 in the stimulation of E-selectin promoter was confirmed by cotransfection studies using a ATF4 plasmid or control vector. As shown in Fig. 4*C*, cotransfection of MCF-7 cells with E-selectin CAT with human pATF4-sense (*Lanes 2* and *3*), and not with pATF4-antisense (*Lane 4*), resulted in a modest but significant stimulation of promoter activity (*Lane 3*). Because MCF-7 cells do not express E-selectin, we next examined whether HRG-mediated activation of the E-selectin promoter could lead to up-regulation of E-selectin mRNA in HUVECs. Results in Fig. 4*D* demonstrate that HRG treatment of HUVECs was accompanied by stimulation of E-selectin expression as determined by RT-PCR.

A Role of the MAPK Pathway in HRG-mediated ATF4 Transactivation. To understand the nature of signaling pathways leading to ATF4 regulation, we next examined the potential involvement of MAPK and PI-3K, two prototype signaling pathways that are widely activated by growth factors. To demonstrate the functionality of these pathways, MCF-7 cells were treated with or without HRG, and stimulation of PI-3K and MAPK pathways was determined by thin-layer chromatography and immunoblotting using phospho-p42/44 Ab, respectively (Fig. 5*A*). In these studies, the activation of the MAPK and PI-3K pathways was blocked by using well-characterized dominant-negative mutants of MEKK (Ref. 21; upstream regulator of

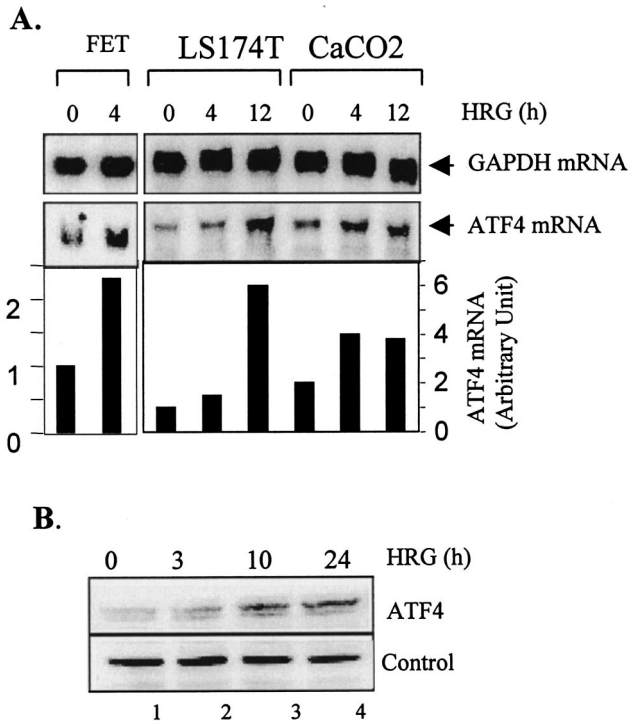

Fig. 3. HRG stimulates ATF4 expression in multiple cell lines. *A*, FET, CaC02, and LS174T colon cancer cell lines were treated with or without HRG for the indicated times, and ATF4 mRNA expression was determined by Northern blotting. The blot was sequentially reprobed with a glyceraldehyde-3-phosphate dehydrogenase probe (*GAPDH*). *B*, lysates from control and HRG-treated FET cells were analyzed by Western blotting using ATF4 mAb. Results shown are representative of two independent experiments.

MAPK) and the p85 subunit of PI-3K (8), respectively. As illustrated in Fig. 5, *B* and *C*, there was no stimulatory effect of HRG on ATF4 DNA binding activity (Fig. 5*B*) and transactivation function (Fig. 5*C*) in cells transfected with dominant-negative MEKK compared to control cells. In contrast, HRG stimulated both DNA binding activity (Fig. 5*B*, *Lanes 5* and *6*) and transactivation function (Fig. 5*C*, *Lanes 5* and *6*) of ATF4 in cells expressing dominant-negative p85. In brief, these results suggest a potential role of MAPK in regulation of ATF4 transactivation in HRG-treated breast cancer cells.

Discussion

HRG has been shown to modulate the growth, differentiation, and motility of breast cancer cells. The multifunctional nature of HRG is thought to be dependent on the induced changes in expression, modification, or both of specific cellular gene products. Because we identified the bZIP transcription factor ATF4 as one of the HRG-inducible genes and because the binding sites for ATF4, the CRE element, are commonly present in genes that regulate cell growth, we explored the possible involvement of ATF protein in the action of HRG using human breast cancer MCF-7 cells as a model system.

The results presented here indicate that treatment of human cells with HRG induced significantly induced higher levels of ATF4 protein expression. The expression and induction of ATF4 protein by HRG open a whole new area of investigation regarding the role of bZIP transcription factor in HRG activity. These observations, together with the fact that ATF4 promoter itself contains CRE sites (22), raise the possibility of an autoregulatory role of increased ATF4 DNA binding activity in the induction of ATF4 expression in HRG-stimulated cells.

Our conclusion that HRG is a very potent inducer of ATF4 expression is supported by the following lines of evidence: (*a*) HRG stimulated the expression of ATF4 mRNA as early as 3 h after treatment; (*b*) elevated expression of ATF4 mRNA was sensitive to the transcriptional inhibitor; (*c*) HRG-mediated increased expression of ATF4 mRNA was accompanied by the enhancement of ATF4 protein; and (*d*) HRG stimulated the expression of newly synthesized ^{35}S-labeled ATF4 protein. These findings establish that ATF4 is a *bona fide* HRG-inducible gene product. The induction of ATF4 expression by HRG was not a phenomenon restricted to MCF-7 cells because HRG also induces the expression of ATF4 mRNA and protein in other HRG-responsive human tumor cell lines, such as colon carcinoma, FET, and CaCO2 cells (15).

Another notable finding of this study is the potential role of HRG-initiated signals in transcriptional activation of genes containing CRE sites including ATF4 and E-selectin (19). The evidence that cotrans-

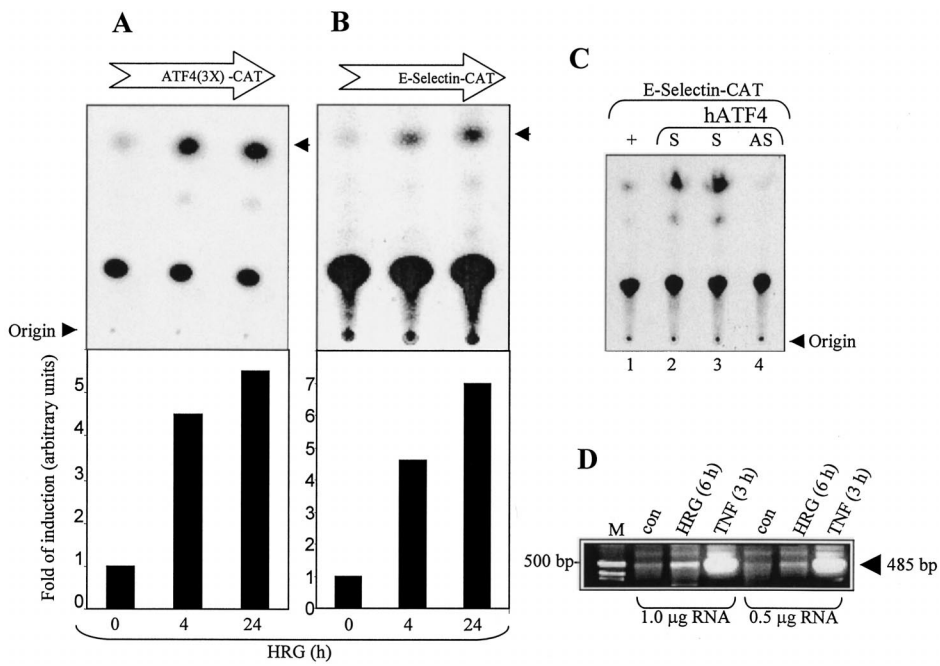

Fig. 4. Regulation of ATF4 transactivation functions by HRG. *A* and *B*, MCF-7 cells were transiently transfected with p(ATF)$_3$-CAT (*A*) or E-selectin CAT (*B*) constructs, and CAT activity was measured after 36 h of transfection. Some cultures were treated with HRG for 4 or 24 h before lysis. These studies were independently repeated four times with similar results. Quantitative means of four experiments are shown in the *bottom panel*. *C*, MCF-7 cells were transfected with E-selectin-CAT in the absence (*Lane 1*) or presence of human ATF4-sense (*Lanes 2* and *3*) or human ATF4-antisense (*Lane 4*). Results shown are representative of three separate experiments. *D*, HUVECs were treated with HRG or tumor necrosis factor α (as a positive control), and total RNA was analyzed by RT-PCR for E-selectin mRNA expression (485-bp product).

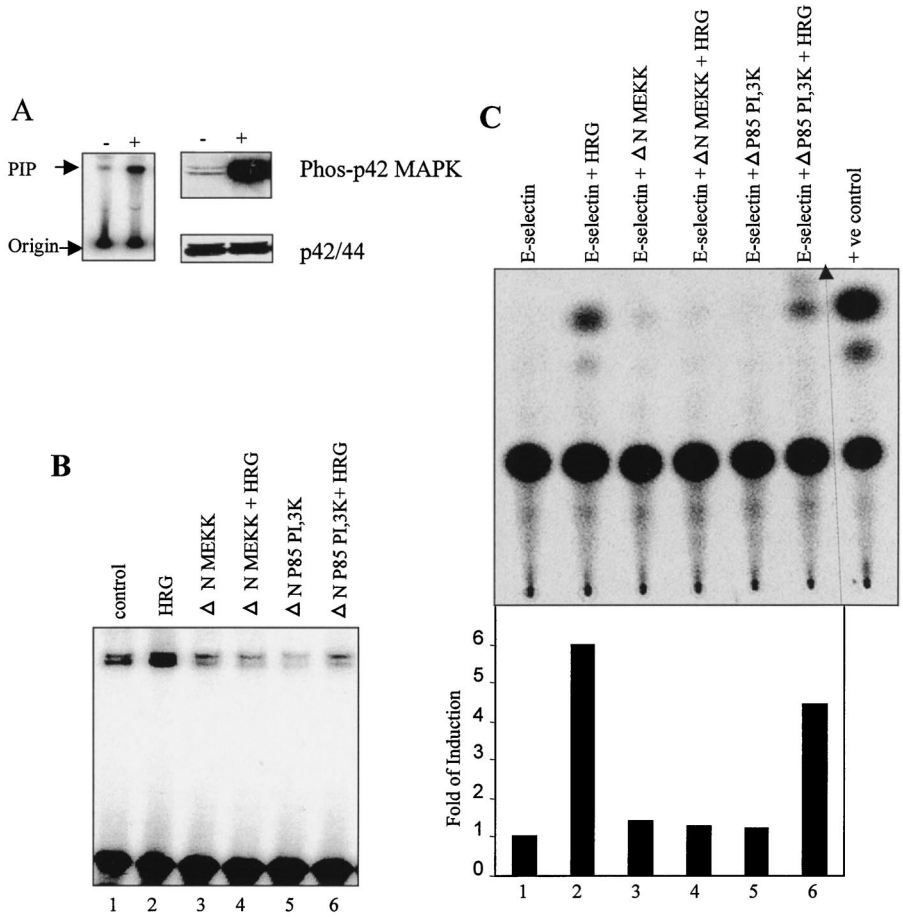

Fig. 5. Role of MAPK in regulation of ATF4 transactivation functions by HRG. *A*, MCF-7 cells were treated for 30 min with (+) or without (−) HRG, and cell lysates were assessed for the activation of PI-3K (using thin-layer chromatography) or MAPK (using phospho-MAPK Ab immunoblotting). *B*, cells were transiently transfected with E-selectin CAT construct in the absence or presence of dominant-negative mutants of MEKK and p85. Some cultures were treated with HRG for 16 h before harvesting for electrophoresis mobility shift assay. *C*, cells were transiently transfected with E-selectin CAT construct in the absence or presence of dominant-negative mutants of MEKK and p85. Some cultures were treated with HRG for 16 h before lysis. CAT activity was measured after a total of 36 h of transfection. These studies were repeated two times with similar results. Quantitative means of four experiments are shown in the *bottom panel*.

fection of human ATF4 has the capacity to activate E-selectin promoter-driven transcription is of special interest because it strongly suggests that HRG-induced ATF4 expression may contribute in the modulation of expression of genes containing ATF4 binding sites either directly, indirectly, or both by influencing the physical interactions between ATF4 and its binding factors. Data from the literature suggest that up-regulation of E-selectin expression in endothelial cells may be closely associated with increased metastases and invasion of human breast cancer cells (23). Because HRG is a paracrine growth factor, our preliminary finding of HRG-mediated stimulation of E-selectin expression in HUVECs implies that HRG may use the ATF4 transcription factor to regulate the expression of gene products with a role in breast cancer progression to a more invasive phenotype.

Data from the literature suggest that in addition to transactivation, ATF4 may also act as a transcriptional repressor, presumably due to sequestration of factors and cofactors from the proximity of the target promoter (10, 11). In view of these earlier findings, it seems reasonable to speculate that HRG may also use inducible modification of ATF4 to potentially repress the expression of unidentified cellular genes that negatively regulate the invasiveness of breast cancer cells. Although we demonstrated the stimulation of E-selectin as an example of transcription activation of a cellular gene by bZIP transcription factor ATF4 in HRG-treated cells, our findings may have a general implication for regulation of cellular genes with a CRE motif by HRG-inducible signals. In the present study, we have not identified the nature of ATF4 target genes in HRG-stimulated cells. Another emerging concept from our present investigation is the possibility that HRG regulation of ATF4 may provide an explanation for multiple cellular functions of HRG because binding sites for the ATF4 factor are present in several growth-regulating cellular promoters and because ATF4-interacting factors, particularly CREB-binding protein/p300, are widely believed to be involved in integrating regulatory signals from distinct pathways (13). Together, these events may lead to differential regulation of genes that may participate in the mediation of HRG in breast cancer cells.

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