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Regulation of Elongation Factor-1α Expression by Growth Factors and Anti-receptor Blocking Antibodies*

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The epidermal growth factor (EGF) family and its receptors regulate normal and cancerous epithelial cell proliferation, a process that could be suppressed by anti-receptor blocking antibodies. Polypeptide elongation factor-1α (EF-1α) is a multifunctional protein whose levels are positively correlated with the proliferative state of cells. To identify genes, whose expression may be modulated by anti-receptor blocking antibodies, we performed a differential display screening and isolated differentially expressed cDNAs. Isolates from one clone were 100% identical to human *EF-1α*. Both EGF and heregulin-β1 (HRG) induced *EF-1α* promoter activity and mRNA and protein expression. Growth factor-mediated EF-1α expression was effectively blocked by pretreatment with humanized anti-EGF receptor antibody C225 or anti-human epidermal growth factor receptor-2 (HER2) antibody herceptin. Mutants and pharmacological inhibitors of p38^{MAPK} and MEK, but not phosphatidylinositol 3-kinase, suppressed both constitutive and HRG-induced stimulation of *EF-1α* promoter activity in MCF-7 cells. Deletion analysis of the promoter suggested the requirement of the −393 to −204 region for growth factor-mediated transcription of *EF-1α*. Fine mapping and point mutation studies revealed a role of the SP1 site in the observed HRG-mediated regulation of the *EF-1α* promoter. In addition, we also provide new evidence to suggest that HRG stimulation of the *EF-1α* promoter involves increased physical interactions with acetylated histone H3 and histone H4. These results suggest that regulation of EF-1α expression by extracellular signals that function through human EGF receptor family members that are widely deregulated in human cancers and that growth factor regulation of EF-1α expression involve histone acetylation.

Growth factors and their receptors play an important role in the regulation of epithelial cell growth. Abnormalities in the expression, structure, or activity of proto-oncogene products contribute to the development and the pathogenesis of cancer. For example, the human epidermal growth factor receptor (HER1)[1] is overexpressed in a number of epithelial tumor cells (1). HER2, the second member of the HER family, shares extensive sequence homology with the tyrosine kinase domain of HER1 (1, 2) and is overexpressed and/or amplified in a number of human malignancies, including breast, ovarian, colon, lung, prostate, and cervical cancers. Recently, HER3 and HER4 have been added to the family, as they share sequence homology with the tyrosine kinase domain of HER1 (2). Regulation of these receptor family members is complex, and they can be transactivated in a ligand-dependent manner. For example, binding of heregulin-β1 (HRG) to HER3 or HER4 can activate HER2 receptor as a result of HER2/HER3 or HER4/HER2 heterodimeric interactions (3, 4). HER1 and HER2 have been shown to induce transformation in recipient cell, possibly because of excessive activation of signal transduction pathways. In contrast, transformation by HER3 or HER4 requires the presence of HER1 or HER2 (3, 4).

Since growth factors regulate the proliferation of cancer cells by activating receptors on the surface of the cells, one approach to controlling cell proliferation is to use anti-receptor blocking monoclonal antibodies that interfere with growth factor receptor-mediated autocrine/paracrine growth stimulation. The humanized antibody C225 against the EGF receptor (EGFR) blocks binding of ligand and prevents ligand-induced activation of receptor tyrosine kinase (5, 6). C225 is currently being used in phase IIA multicenter clinical trials alone and in combination with chemotherapy or radiation to treat to patients with head and neck, lung, or prostate carcinomas (7–9). Similarly, the humanized form of anti-HER2 monoclonal antibody HCT (herceptin) inhibits the growth of breast cancer cells overexpressing HER2 (10, 11) and is currently being used as an effective drug against some forms of breast cancer (12). Anti-receptor antibodies are known to inhibit many processes, including mitogenesis, cell cycle progression, invasion and metastasis, angiogenesis, and DNA repair.

Mitogenic growth factors stimulate protein synthesis in eukaryotic cells. Polypeptide elongation factor-1α (EF-1α) is a ubiquitously expressed protein that plays a key role in the elongation cycle during translation. EF-1α forms a complex

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[1] The abbreviations used are: HER, human epidermal growth factor receptor; HRG, heregulin-β1; EGF, epidermal growth factor; EGFR, EGF receptor; HCT, herceptin; C225, anti-EGF receptor antibody; EF-1α, elongation factor-1α; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; CAT, chloramphenicol acetyltransferase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; TGF-α, transforming growth factor-α.

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with aminoacyl-tRNA and GTP that transfers the aminoacyl-tRNA group to the 80 S ribosome and hydrolyzes GTP (13). EF-1α is also involved in cytoskeleton reorganization (14, 15) and proliferation (16). It can regulate embryogenesis (17), actin bundling, and microtubule severing and is associated with the centrosome and mitotic machinery (14, 18, 19). EF-1α is also one of the actin-associated activators of phosphatidylinositol 4-kinase, which regulates the levels of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate (20). These phospholipids regulate the capping of actin filaments by actin-binding protein (18). In brief, EF-1α regulates cellular functions that are both dependent on and independent of translational controls.

Next only to actin, EF-1α is the most abundant protein in normal cells, accounting for 1–2% of total protein. Regulation of its levels may be important for normal cell function; rapidly growing cells usually exhibit a large increase in their EF-1α mRNA levels (21); overexpression of EF-1α correlates with metastasis (22); and EF-1α mRNA levels decrease during murine erythroleukemic cell differentiation (23). EF-1α expression can be regulated at both the transcriptional and post-transcriptional levels (24, 25). EF-1α mRNA levels have been shown to be up-regulated by oncogenes and induced by phytohemagglutinin in human blood lymphocytes (22, 26). In addition, overexpression of EF-1α in fibroblasts leads to increased susceptibility to oncogenic transformation (27). In addition to its predominant cytoplasmic presence, EF-1α has been reported in the nucleus (28) where it binds to RNA polymerase (29). Recently, EF-1α has been shown to be physically associated with the novel zinc finger protein ZPR1 in A-431 cells and to be translocated to the nucleus in an EGF-dependent manner (30). Furthermore, insulin can regulate translational elongation activity of EF-1α by regulating its phosphorylation by multipotential S6 kinase (31–33). Yet, despite knowledge of these cellular functions, the possible regulation of EF-1α by the EGF family of growth factors and by therapeutic anti-receptor antibodies remains unexplored.

To identify genes whose expression may be down-regulated by anti-receptor blocking antibodies, presumably owing to ligand-induced activation of receptor tyrosine kinase and/or interference of receptor-associated functions, we used differential display approach to isolate differentially expressed cDNAs. We report that one of these clones had 100% identity with human elongation factor-1α (EF-1α). Both EGF and HRG induced *EF-1α* promoter activity and mRNA and protein expression that could be effectively blocked by pretreatment with anti-receptor monoclonal antibodies. Our results also suggest involvement of specific signaling pathways in the base-line regulation of EF-1α transcription. In addition, we also provide new evidence to suggest that HRG stimulation of *EF-1α* promoter requires the SP1 site and that the *EF-1α* promoter undergoes histone acetylation in response to HRG.

EXPERIMENTAL PROCEDURES

Cell Cultures and Reagents—Human breast cancer cells MCF-7, MDA-MB468, BT-474, SK-BR-3, MDA-MB231, and MDA-MB435 (34), mouse NIH3T3 cells transfected with human EGFR (HER14 cells, Fan *et al.* (35)), and vulvar carcinoma A-431 cells (6) were maintained in Dulbecco's modified Eagle's medium/F-12 (1:1) supplemented with 10% fetal calf serum. Recombinant HRG-β1 was purchased from Neomarkers Inc. Anti-vinculin antibody and recombinant EGF were purchased from Sigma.

Differential Display and Cloning of EF-1α cDNA—Differential display was performed according to the method described in Ref. 36. In brief, MDA-MB435 and MDA-MB231 cells were treated with or without C225 or herceptin. Total RNA was isolated using Trizol reagent (Life Technologies, Inc.). Total RNA was digested with RNase-free DNase (Promega) and purified by phenol/chloroform extraction. First strand cDNA was synthesized by reverse transcriptase reaction containing 200 ng of total RNA using four different degenerate anchored oligo(dT) primer set (dT12VA, dT12VG, dT12VC, dT12VT; Operon Technologies Inc.). Reactions were performed in a 20-μl volume using Moloney murine leukemia virus reverse transcriptase (Promega). Amplification of cDNA fragments was performed using 2 μl of the cDNA in reaction buffer containing 2 μl of 10× PCR buffer, 10 μCi of α-^{35}S-dATP, dNTPs (2 μM), 1 unit of *Taq* polymerase (Roche Molecular Biochemicals), the same 3′-degenerate oligo(dT) primer, and 1 of the 10 5′-orbitary decamers (OP-26–01 to OP-26–10, Operon Technology, Inc.). PCR cycles were as follows: denaturation 95 °C for 5 min, 40 cycles at 94 °C for 30 s, 40 °C for 2 min, 72 °C for 30 s with a final extension of 72 °C for 10 min. PCR products were separated on a 6% polyacrylamide gel and developed by autoradiography. Bands of interest were excised, extracted, and reamplified with same set of primers. Amplified DNA was separated on an agarose gel, and bands were purified and cloned into PCR2.1 vector using Topocloning kit (Invitrogen). Five to ten independent clones were isolated, miniprepared, and sequenced at the M. D. Anderson Cancer Core facility. Sequences were compared with GenBank™ sequences using BLAST search.

Construction of EF-1α Promoter Deletion Constructs—Construction of deletion constructs pEF (construct numbers 1–7) was described (37). Sub-fragmentation of the insert of construct 6 was done using restriction sites *Hin*dIII/*Sph*I, *Hin*dIII/*Sty*I, and *Ava*II/*Sph*I. Cloning of bluntended fragments into the *Xba*I site of pBLCAT5 (38) yielded constructs 8, 9, and 12, respectively. The mutations in construct 9 were made using the QuickChange kit (Stratagene) according to the instructions.

Cell Extracts, Immunoblotting, and Immunoprecipitation—To prepare cell extracts, cells were washed three times with phosphate-buffered saline and lysed in buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 mM $NaVO_5$, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) for 15 min on ice. The lysates were centrifuged in an Eppendorf centrifuge at 4 °C for 15 min. Cell lysates containing equal amounts of protein were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with the appropriate antibodies. An equal number of cells were metabolically labeled for 4–8 h with 100 μCi/ml [^{35}S]methionine in methionine-free medium containing 2% dialyzed fetal bovine serum in the absence or presence of indicated treatments. Cell extracts containing equal trichloroacetic acid-precipitable counts were immunoprecipitated with the desired antibody, resolved on SDS-PAGE, and analyzed (34).

Northern Hybridization—Total cytoplasmic RNA was isolated using the Trizol reagent (Life Technologies, Inc.), and 20 μg of RNA was analyzed by Northern hybridization using a 1.8-kilobase pair cDNA fragment of human EF1-α. rRNA (28 S and 18 S) was used to assess the integrity of the RNA, and for RNA loading and transfer control, the blots were routinely reprobed with glyceraldehyde-3-phosphate dehydrogenase cDNA.

Transfection and Promoter Assays—Cells were split in 100-mm tissue culture dishes (Falcon) 24 h before transfection. Subconfluent cells were transiently transfected with pEFΔ1090CATSp1 or with other constructs as needed or control pSVb-Gal vector using LipofectAMINE method (Life Technologies, Inc.). After 5 h of transfection, medium was changed to Dulbecco's modified Eagle's medium containing 10% serum. After 24 h, cultures were shifted to 0% serum (for growth factor treatment) or 2% serum (for antibody treatment) for 12 h before harvesting. CAT activity was measured 48 h after transfection using a CAT assay kit (Promaga) (39). When indicated, cells were treated with HRG or EGF (30-ng/ml medium) or herceptin or C225 (50 nM final concentration). In some experiments, cells were pretreated with 20 μM PD098059 (a MEK inhibitor), 20 μM SB203580 (a p38^{MAPK} inhibitor), and 20 μM LY294002 (PI3K inhibitor) for 1 h before HRG treatment. Each experiment was repeated two to five times and transfection efficiency varied between 30 and 50%.

Chromatin Immunoprecipitation Assays—MCF-7 cells were split in 100-mm tissue culture dishes (Falcon). About 70% confluent dishes were serum-starved for 24 h followed by overnight treatment with HRG (30 ng/ml). Quantitative chromatin immunoprecipitation assay was done as described previously (40–42) with some modifications. Approximately 10^6 cells were treated with formaldehyde (1% final concentration) for 10 min at 37 °C to cross-link histones to DNA. The cells were washed twice with phosphate-buffered saline, pH 7.4, containing protease inhibitor mixture (Roche Molecular Biochemicals). Cells were lysed and sonicated as described (40). Sonicated lysate was centrifuged for 10 min at 12,000 rpm at 4 °C. Supernatant was diluted 10-fold by dilution buffer containing 0.01% SDS, 1.1% Triton X-100, and protease inhibitor mixture (Roche Molecular Biochemicals). A portion (1%) of the chromatin solution was kept to check the amount of input DNA in different samples before immunoprecipitation. Chromatin solutions

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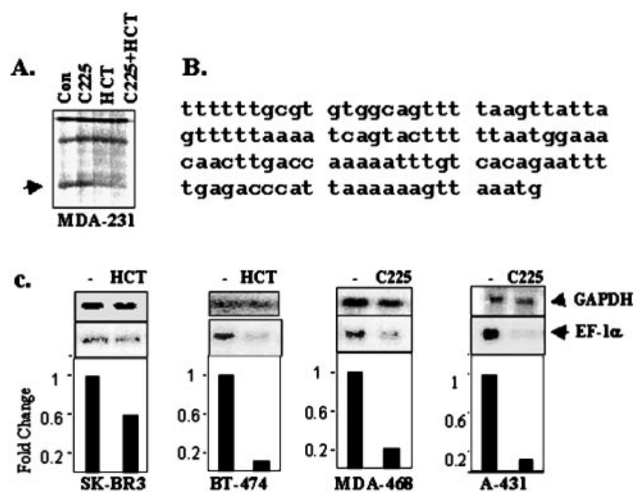

FIG. 1. **Identification of *EF-1α* as a differentially expressed gene.** *A*, representative differential display band patterns of control, C225, or HCT-treated MDA-MB231 cells. *Arrow* indicates the band of interest, which is down-regulated by herceptin treatment. *B*, sequence of the purified bands that matches (100%) those with human EF-1α. *C*, Northern blot analysis showing down-regulation of EF-1α mRNA in herceptin- or C225-treated breast cancer cell lines. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

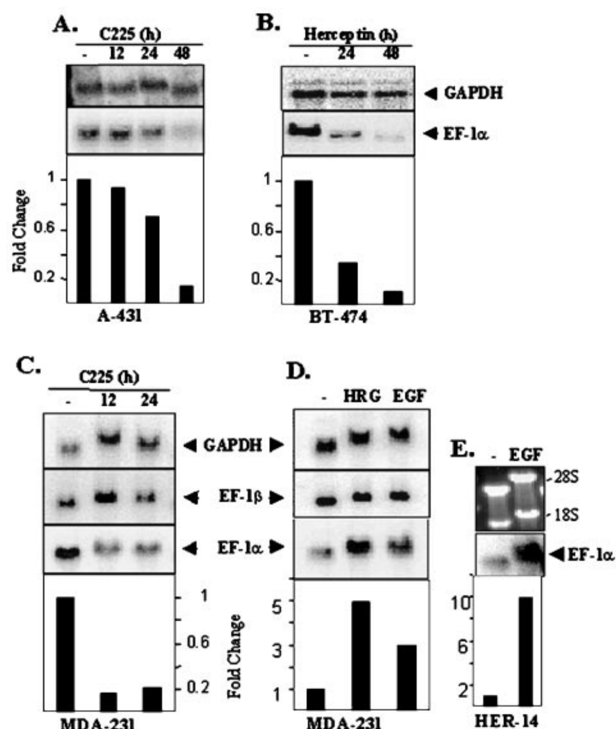

FIG. 2. **Regulation of EF-1α mRNA expression by anti-receptor antibodies and growth factors.** *A–C*, tumor cell lines were treated with or without C225 or (50 nM) for the indicated times. *D* and *E*, MDA-MB231 or HER14 cells were treated with EGF or HRG (30 nM) for 16 h. Total RNA was isolated, and EF-1α mRNA levels were detected by Northern blot analysis. Subsequently, the blot was reprobed with a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA probe. Quantitation of mRNA is shown in the *bottom panel.* Results are representative of three experiments.

were precleared with 80 μl of protein A-Sepharose beads (60 mg/ml) saturated with salmon sperm DNA and bovine serum albumin for 30 min at 4 °C before immunoprecipitating with either anti-acetylated histone H3 or anti-acetylated H4 antibody (Upstate Biotechnology, Inc.) at 4 °C overnight. Immunocomplexes were recovered with 60 μl of protein A- Sepharose beads at 4 °C for 1 h. Beads were washed as described (40) on a rotating platform before eluting the immunocomplexes by incubation with 400 μl of 1% SDS containing 0.1 M $NaHCO_3$. The elution was heated to 65 °C for 6 h to reverse the formaldehyde cross-links. Phenol/chloroform extraction was performed, and the supernatant was ethanol-precipitated (using 20 μg of glycogen as an inert carrier). DNA was resuspended in 50 μl of 10 mM Tris, 1 mM EDTA, pH 8.0. Quantitative PCR was done with 10 μl of DNA sample restricted to 25 cycles. The EF-1α DNA sequence of the 5′ primer was 5′ GATTT-GTCCCGGACTAGCGAG and of the 3′ primer was 5′ TCTTCTCCAC-CTCAGTGATGACG 3′. The PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide.

RESULTS AND DISCUSSION

Identification of Human Polypeptide Elongation Factor-1α as an Anti-receptor Antibody-regulated Gene—In an attempt to identify genes whose expression may be modulated in human cancer cells by anti-receptor blocking antibodies, total RNA was isolated from two highly invasive human breast cancer cell lines, MDA-MB435 and MDA-MB231, and treated with or without C225 and herceptin for 10 h. Although MDA-MB435 and MDA-MB231 cells have normal levels of EGFR and HER2, the *in vitro* invasive properties of these cells were inhibited by herceptin and C225 (43). A total of 160 reactions was performed using four 3′-degenerate oligo(dT) primers and 10 5′-random primers for each antibody treatment. Analysis of gels showed amplification of a number of bands ranging from 100 to 600 base pairs, and the majorities of the bands were of equal intensity. By using these bands as internal control, we analyzed for the bands with differences in intensity in C225- or herceptin-treated lanes. This analysis resulted in the identification of five differentially expressed gene products ranging in size from 120 to 350 base pairs. A representative portion of the gel is shown in Fig. 1*A*.

Each differentially expressed gene product was amplified, cloned, and sequenced. The resulting sequence was compared with sequences deposited in GenBank™. For each gene product five clones were sequenced, and all five sequences from one band were 100% identical to human elongation factor-1α from 1580 to 1694 (EF1α, GenBank™ accession number X16869) (Fig. 1*B*). To determine whether EF-1α expression can be modulated by anti-receptor blocking antibodies C225 and herceptin on EF-1α mRNA levels in tumor cells, we did Northern blot hybridization using the 1.8-kilobase pair EF-1α cDNA as a probe. Treatment of human breast carcinoma cells (SK-BR-3, BT-474, and MDA-MB468) and vulvar carcinoma cells (A-431) with C225 or herceptin was accompanied by a significant decrease in EF-1α mRNA levels (Fig. 1*C*).

Regulation of EF-1α mRNA Expression by Anti-receptor Antibodies and Growth Factors—A-431 cells, which overexpress EGFR, are growth-stimulated by autocrine transforming growth factor-α (TGF-α) and growth-inhibited by C225 (6). To determine whether TGF-α is involved in the regulation of EF-1α, we asked whether C225, which blocks TGF-α from binding to EGFR, could down-regulate the steady-state level of EF-1α mRNA. Treatment of A-431 cells with C225 was accompanied by a gradual decrease in EF-1α mRNA expression (Fig. 2*A*). Similarly, herceptin treatment of BT-474 cells, which overexpress HER2, was associated with reduced expression of EF-1α mRNA (Fig. 2*B*). The observed suppression was not related to high levels of EGFR, as C225 was effective in selectively reducing EF-1α levels (but not EF-1β) in breast cancer MDA-MB231 cells, which have normal levels of EGFR and HER2 (Fig. 2*C*).

Since MDA-MB231 cells are known to constitutively secrete HRG, a combinatorial ligand for HER3 and HER4 that can transactivate EGFR and HER2, the above results raised the possibility that EF-1α mRNA expression could be induced by HRG. Indeed, both HRG and EGF were potent inducers of EF-1α mRNA (but not EF-1β) in MDA-MB231 cells (Fig. 2*D*). EGF regulation of EF-1α mRNA expression was confirmed using a mouse NIH3T3 cell line (HER14) that stably expressed

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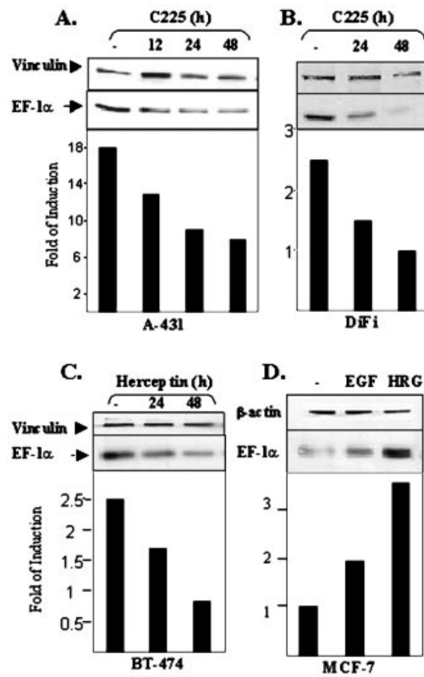

FIG. 3. **Anti-receptor antibodies decrease and growth factors increase EF-1α protein level.** *A–C*, cells were treated with C225 or HCT for the indicated times; *D*, cells were treated with EGF or HRG for 16 h. Total lysates were run on SDS-PAGE and blotted with anti-EF-1α monoclonal antibody. Anti-vinculin antibody or anti-β-actin antibody was used as an internal control. Quantitation of the ratio of EF-1α to β-actin is shown in the *bottom panel*. Results are representative of three to five separate experiments.

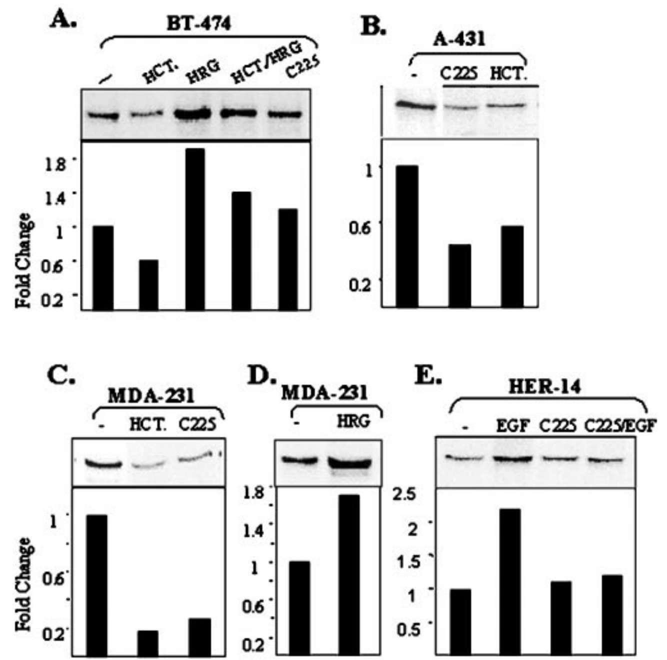

FIG. 4. **Regulation of newly synthesized EF-1α protein by anti-receptor antibodies and growth factors.** *A–E*, cells were treated with C225 or HCT in the presence or absence of EGF or HRG for 16 h and metabolically labeled with [^{35}S]methionine for 10 h before harvesting. Cell lysates were immunoprecipitated with an anti-EF-1α monoclonal antibody and analyzed by SDS-PAGE and fluorography. Results are representative of three independent experiments.

human EGFR and responded to exogenous recombinant EGF by growth stimulation (35). EGF treatment of HER14 cells for 8 h was accompanied by a significant increase in EF-1α mRNA levels (Fig. 2*E*). Taken together, these results suggest that EF-1α mRNA levels in a number of cell types are modulated by EGF, HRG, and anti-receptor monoclonal antibodies that interfere with EGFR and HER2.

Regulation of EF-1α Protein Expression by Anti-receptor Antibodies and Growth Factors—Western blot analysis was performed to determine whether the modulation of EF-1α mRNA levels by growth factors and anti-receptor monoclonal antibodies was associated with a corresponding modulation in the expression of EF-1α protein. Results demonstrated that A-431 cells and human colon carcinoma DiFi cells, which have a functional TGF-α autocrine loop (44), expressed a lower level of the 51-kDa EF-1α protein, after C225 treatment (Fig. 3, *A* and *B*). Similarly, herceptin inhibited EF-1α protein levels in BT-474 cells (Fig. 3*C*). In contrast, treatment of MCF-7 cells with EGF or HRG significantly increased the level of EF-1α protein (Fig. 3*D*).

To validate further the modulation of EF-1α protein expression by growth factors, we examined the effects of growth factors and anti-receptor monoclonal antibodies on the level of newly synthesized EF-1α protein in cells metabolically labeled with [^{35}S]methionine. Similar to our results from Western analysis, treatment with C225 or herceptin resulted in a reduction of ^{35}S-labeled EF-1α protein in A-431, BT-474, and MDA-MB231 cells (Fig. 4, *A–C*). In contrast, HRG treatment caused an increase in ^{35}S-labeled EF-1α protein in BT-474 (Fig. 4*A*) and MDA-MB231 (Fig. 4*D*) cells and EGF in HER14 cells (Fig. 4*E*). The observed induction of ^{35}S-labeled EF-1α protein by HRG and EGF was mediated through HER2 and EGFR, as it was effectively suppressed by herceptin and C225 (Fig. 4, *A* and *E*). These results indicate the ability of growth factors to induce expression of EF-1α and of growth factor receptor antibodies to block it.

Regulation of the EF-1α Promoter by Anti-receptor Antibodies and Growth Factors—The regulatory elements in the human *EF-1α* are not completely understood. Recently, Clark and colleagues (37) have shown the significance of specific elements (44), located in the first intron and also close to the TATA box, in the regulation of EF-1α transcription. To examine the effect of growth factor-blocking monoclonal antibodies on *EF-1α* promoter activity, tumor cells were transfected with the *EF-1α* promoter construct pEFΔ1090CATSp1, which contains all regulatory elements upstream of the human EF-1α TATA box fused to the thymidine kinase promoter (37). Treatment of A-431 and MDA-MB231 cells with C225 (Fig. 5, *A* and *B*) and of MDA-MB231 and BT-474 cells with herceptin (Fig. 5, *B* and *C*) resulted in a significant inhibition of *EF-1α* promoter-driven reporter transcription. Conversely, exposure of HER14 cells to EGF was accompanied by 2–4-fold stimulation of *EF-1α* promoter-driven transcription, and EGF receptor antagonist C225 blocked the EGF-mediated stimulation of *EF-1α* promoter activity (Fig. 4*D*). Similarly, stimulation of *EF-1α* promoter activity by HRG was also suppressed by pretreatment of MCF-7 cells with herceptin (Fig. 4*E*).

Growth Factor Signaling and Regulation of EF-1α Promoter Activity—Distinct signaling pathways regulate different functions of growth factors. For example, HRG utilizes $p38^{MAPK}$ and PI3K pathways to regulate the spreading and formation of lamellipodia (39, 45). A careful analysis of *EF-1α* promoter (GenBank™ accession number E02627) revealed several important motifs, including AP1, SP1, CREB, CRE-BP, and NF-κB that are activated by multiple growth factor signaling pathways. To understand the nature of growth factor signaling pathways leading to stimulation of the *EF-1α* promoter, we employed cotransfection of *EF1α* promoter pEFΔ1090CATSp1 reporter with dominant-negative tagged mutants that specifically inhibit $p38^{MAPK}$ and PI3K activation or dominant-nega-

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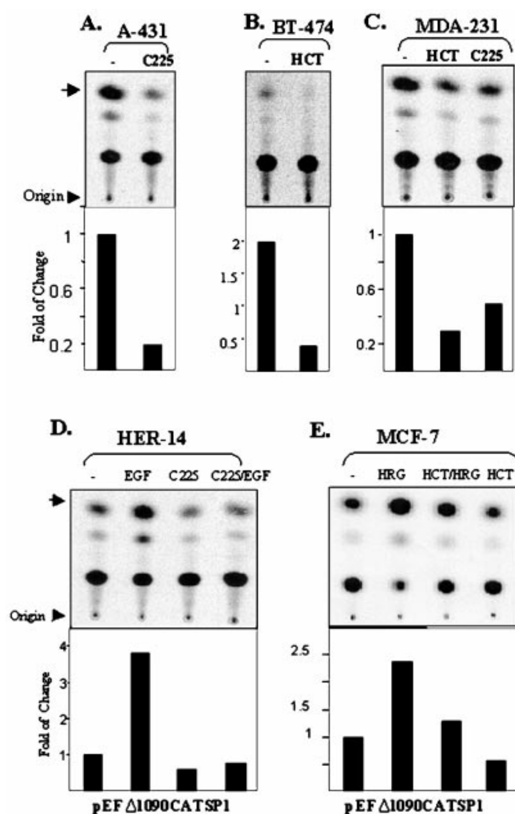

FIG. 5. **Modulation of *EF-1α* promoter activity by C225 and herceptin and growth factors.** Tumor cells were transiently transfected with an *EF-1α* promoter (*pEFΔ1090CATSp1*), and CAT activity was measured 36 h after transfection. *A–C*, cultures were treated with C225 and HCT (50 nM) for 16 h before lysis. Results are representative of two experiments. *D* and *E*, after transfection with *EF-1α* promoter (pEFΔ1090CATSp1), HER14 and MCF-7 cells were treated with EGF or HRG (30 nM) in the presence or absence of C225 or herceptin (50 nM) for 16 h, and CAT activity was measured. These studies were independently repeated four times. Relative CAT activities are shown in the *bottom panels.*

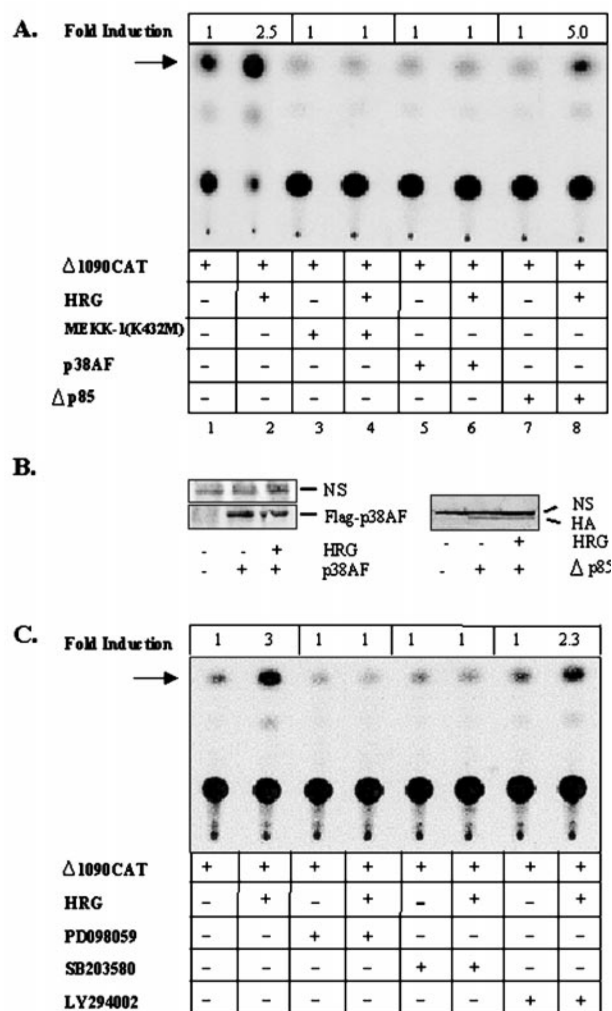

FIG. 6. **Delineation of signaling pathways involved in ligand-induced stimulation of *EF-1α* promoter activity.** *A,* MCF-7 cells were transiently transfected with a full-length *EF-1α* promoter (pEFΔ1090CATSp1) in the absence or presence of dominant-negative mutants of MEK, $p38^{MAPK}$, and PI3K. One set of cultures was treated with or without HRG for 16 h before assaying for CAT activity. Quantitation of CAT activity is shown as fold induction of CAT reporter activity by HRG treatment over the control culture for each mutant group. *B,* Western blot analysis of expression of FLAG-tagged $p38^{MAPK}$ and HA-tagged PI3K mutants in lysates from the above panel, using antibodies against FLAG-tagged and HA epitopes, respectively. *NS* indicates nonspecific band in the same blot. Results shown are representative of two experiments. *C,* cells were pretreated with specific inhibitors PD098059, SB203580, and LY294002 (20 μM each) for 1 h before HRG treatment for 16 h. Quantitation of CAT activity is shown as fold induction of CAT reporter activity by HRG treatment over the control culture in each inhibitor.

tive mutant of MEK (39, 45, 46). In these studies, we used HRG as a model growth factor. Mutants of $p38^{MAPK}$ and MEK, but not PI3K, suppressed both constitutive and the extent of HRG-induced stimulation of *EF-1α* promoter activity in MCF-7 cells (Fig. 6*A*). The observed inhibitory effects of mutants were not due to variability in the expression levels of the transfected genes in cells treated with or without HRG, as shown by the expression levels of FLAG-tagged p38AF and HA-tagged Δp85 by antibodies against FLAG and HA moieties, respectively (Fig. 6*B*). To confirm these findings further, we next used pharmacological inhibitors, such as PD098059 (for MEK), SB203580 (for $p38^{MAPK}$), and LY294002 (for PI3K), and similar results were obtained (Fig. 6*C*). In brief, these results suggested a preferential involvement of MEK and $p38^{MAPK}$, but not PI3K, in the base-line and HRG-inducible regulation of *EF-1α* promoter activity.

Stimulation of cytoplasmic kinases by growth factors leads to phosphorylation and activation of multiple transcription factors, including c-Jun, ATF-2, NF-κB, CREB, and SP1. Since the binding motifs for these transcription factors are present in the *EF-1α* promoter (GenBank™ accession number E02627), it is possible that a combination of these factors may be responsible for optimal *EF-1α* promoter regulation. Recent studies have shown transcriptional regulation of the human *EF-1α* gene by upstream sequences, including a novel C8-stretch element.[2]

[2] H. F. Jørgensen and B. F. C. Clark, unpublished data.

A Role of SP1 in HRG Regulation of EF-1α Promoter—To understand the mechanism by which HRG mediates its effect on *EF-1α* promoter, we employed a series of deletion mutants to map the HRG-responsive regulatory region in the *EF-1α* promoter. All mutants were fussed to a CAT reporter system. Initially, we used constructs 1–6, and we examined the effect of HRG on the *EF-1α* promoter activity. There was minimal effect of the deletions from −1090 to −393 of the *EF-1α* promoter on the levels of HRG-mediated up-regulation of EF-1α (Fig. 7, *construct numbers 1–6*). To delineate further the minimal region required for HRG stimulation of the *EF-1α* promoter, we next used additional mutants (Fig. 7, *construct numbers 8, 9,* and *12*). Constructs 8 (−393 to −204) and 9 (−313 to −314) responded to HRG well, whereas constructs 12 (−204 to −127) and 7 (−119 to −29) showed no regulation in response to HRG.

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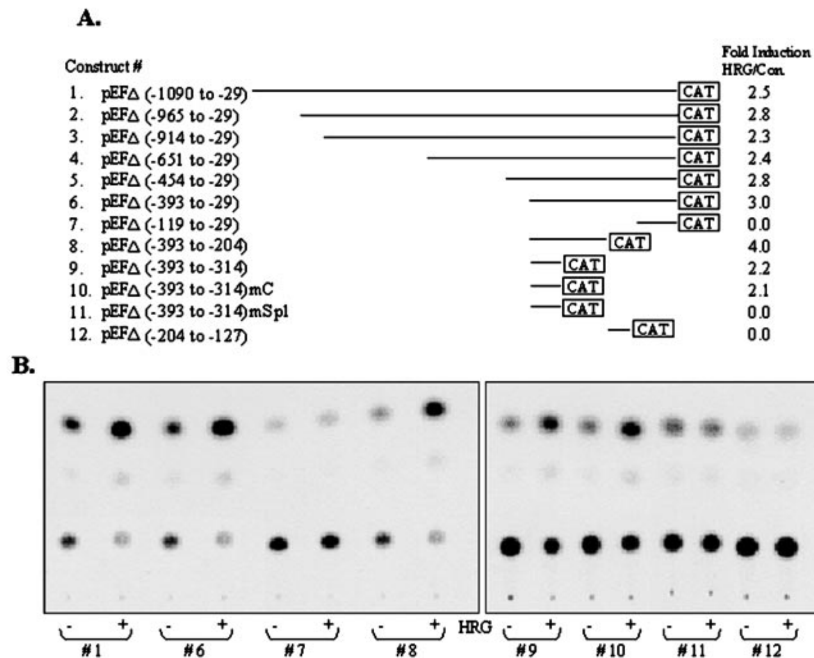

FIG. 7. **A role of SP1 in HRG Regulation of *EF-1α* promoter.** *A,* deletion constructs were made as described under "Experimental Procedures." Fold of induction by HRG over untreated control cells is shown in the *right-hand columns. Construct numbers 10–12* have similar sequence, except that *number 10* is wild type and has one Sp1 site at −379 to −374 position; *construct number 11* was mutated nonspecifically at −387 to −385 where CCC were replaced with AGA, and *construct number 12* was mutated at Sp1 site at −391 to −389 where CGC were replaced with 5′ TTA 3′. *B,* regulation of EF-1α deletion constructs by HRG (16 h). Results shown are representative of three experiments.

The results indicated that −393 to −314 region contained the regulatory elements that may confer the HRG-mediated induction of EF-1α. Analysis of the transcription factor sites present in the −393 to −314 revealed presence of an SP1 site. Recently it was shown that HRG regulate the activity of SP1 transcription factor (47). To verify the potential involvement of the SP1 site in HRG induction of *EF-1α* promoter, we next mutated the SP1 site at −369 to −363 (construct 11). Point mutation of the SP1 site completely abolished the HRG-mediated induction of the construct containing −393 to −314 region. Mutation of another region other than Sp1 site has no effect on HRG-mediated induction (construct 10), suggesting a role of SP1 in the HRG regulation of *EF-1α* promoter. Interestingly, construct 8 (−393 to −204), which contains two Sp1 sites, demonstrated a significantly higher HRG-inducible activity (4-fold) than the construct containing −393 to −314 (construct number 10) with one SP1 site (2.1-fold). Together, these observations suggested a role of SP1 site in the observed HRG-mediated regulation of *EF-1α* promoter.

Involvement of Histone Acetylation in HRG Regulation of EF-1α—The eukaryotic genome is compacted with histone and other proteins to form chromatin, which consists of repeating units of nucleosomes (48, 49). For transcription factors to access DNA, the repressive chromatin structure needs to be remodeled. Dynamic alterations in the chromatin structure can facilitate or suppress the access of the transcription factors to nucleosomal DNA, leading to transcription regulation. One way to achieve this is through alterations in the acetylation state of nucleosomal histones. Hyperacetylated chromatin is generally associated with transcription activation, whereas hypoacetylated chromatin is associated with transcriptional repression (48, 49). To investigate whether the HRG regulation of EF-1α expression involves histone acetylation on the *EF-1α* gene, we next performed chromatin immunoprecipitation (ChIP) assay of the target gene, *i.e.* EF-α around a target sequence in the promoter (−535 to −209) which has multiple SP1 sites by using antibodies specific for acetylated forms of H3 and H4. Representative results from several independent experiments are shown in Fig. 8. HRG treatment of MCF-7 cells was accompanied by a significant enhancement in the association of *EF-1α* promoter region with the acetylated histones H3 and H4 (5.0- and 3.5-fold induction of associated-acetylated H3 and -acetylated H4 by HRG as compared with untreated cells). In addition, there was also easily detectable levels of *EF-1α* promoter association with the acetylated H3 and H4 in control untreated cells (Fig. 8, *lanes 1* and *3*), implying a potential role of histone acetylation in the base-line expression of EF-1α.

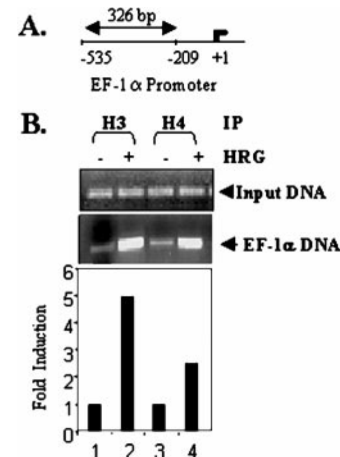

FIG. 8. **Acetylation of histone H3 and histone H4 at the identified multiple SP1 sites by chromatin immunoprecipitation assay.** *A,* schematic representation of *EF-1α* promoter sequence used for *in vivo* chromatin association. *B,* MCF-7 cells were treated with (*lanes 2* and *4*) or without (*lanes 1* and *3*) HRG (30 ng/ml for 16 h), and chromatin lysates were immunoprecipitated with antibodies against acetylated H3 (*lanes 1* and *2*) or acetylated H4 (*lanes 3* and *4*), and samples were processed as described under "Experimental Procedures." The *top panel* shows the PCR analysis of the input DNA. The *middle panel* demonstrates the PCR analysis of 326 base pairs of EF-1α DNA fragment associated with acetylated histone H3 or H4. Quantitation of signals in the *bottom panel* is presented as fold induction over control untreated cells. *IP*, immunoprecipitation. Results are representative of three independent experiments.

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Earlier reports have shown a close correlation of up-regulation of histone H3 and H4 acetylation with an increased transcriptional activity (48, 49). In brief, our findings clearly demonstrated the involvement of histone acetylation in HRG-mediated stimulation of *EF-α* gene expression.

In summary, we have presented new evidence that treatment of tumor cells with growth factors significantly increases *EF-1α* promoter activity and mRNA and protein expression and that HRG increases the acetylation of H3 and H4 on the *EF-1α* promoter. Since constitutive EF-1α expression was not well correlated with EGFR or HER2 overexpression in different cell types, ligand-activated cellular pathways, rather than receptor levels, may be significant in the regulation of EF-1α expression. These views are supported by a recent report showing no effect of EGFR and HER2 overexpression on levels of related EF-1δ in human keratinocytes (50). However, despite the lack of correlation with receptor levels, the ligand-induced up-regulation of EF-1α expression was mediated by a specific ligand-receptor interaction, as the anti-receptor blocking antibodies C225 and herceptin could effectively reduce it. Our study also shows the potential roles of MEK and $p38^{MAPK}$ in the constitutive regulation of *EF-1α* promoter activity.

There are a number of possible functional implications for growth factor-regulated EF-1α expression as follows: 1) promotion of polypeptide elongation and thus potential contribution to increased translation of mRNA encoding growth-related proteins; 2) increased reorganization of the cytoskeleton, since it is one of the earliest phenotypic responses of most cells to growth factors and since EF-1α regulates actin bundling; 3) potential undefined roles in the nucleus, due to the ability of EF-1α to form a complex with ZPR1 (30). In summary, our findings have clearly demonstrated for the first time a potential role of EF-1α in the actions of HER growth factors that are widely deregulated in human cancers and that ligand-dependent EF-1α expression was effectively inhibited with humanized anti-receptor blocking antibodies C225 and herceptin. In addition, we also provide new evidence to suggest that HRG stimulation of *EF-1α* promoter requires the SP1 site and that the *EF-1α* promoter undergoes histone acetylation in response to HRG.

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Regulation of Elongation Factor-1α Expression by Growth Factors and Anti-receptor Blocking Antibodies

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