Cyclooxygenase-2 Is Overexpressed in HER-2/neu-positive Breast Cancer

Kotha Subbaramaiah‡, Larry Norton§, William Gerald¶, and Andrew J. Dannenberg‡

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From the ‡Department of Medicine, New York Presbyterian Hospital, Weill Medical College of Cornell University and the Strang Cancer Prevention Center and the §Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Markedly increased levels of cyclooxygenase-2 (COX-2) mRNA, protein, and prostaglandin E2 synthesis were detected in HER-2/neu-transformed human mammary epithelial cells (184B5/HER) compared with its nontransformed partner cell line (184B5). HER-2/neu stimulated COX-2 transcription via the Ras → Raf → MAPK pathway. The inductive effects of HER-2/neu were mediated, in part, by enhanced binding of AP-1 (c-Jun, c-Fos, and ATF-2) to the cyclic AMP-response element (−59/−53) of the COX-2 promoter. The potential contribution of the transcription factor PEA3 was also investigated. Elevated levels of PEA3 were detected in 184B5/HER cells. A PEA3 site (−75/−72) was identified juxtaposed to the cyclic AMP-response element. HER-2/neu-mediated activation of the COX-2 promoter was blocked by mutagenizing the PEA3 site or overexpressing antisense to PEA3. To determine whether HER-2/neu status was also a determinant of COX-2 expression in vivo, we compared levels of COX-2 protein in HER-2/neu-positive and -negative human breast cancers. Increased amounts of COX-2 were detected in HER-2/neu-positive tumors. Taken together, these results suggest that closely spaced PEA3 and cyclic AMP-response elements are required for HER-2/neu-mediated induction of COX-2 transcription. The clear relationship between HER-2/neu status and COX-2 expression in human breast tumors suggests that this mechanism is likely to be operative in vivo.

The HER-2/neu (erbB-2) gene encodes a 185-kDa transmembrane receptor with tyrosine kinase activity that belongs to the family of receptors for epidermal growth factor (1). Amplification and/or overexpression of HER-2/neu occurs in 20–30% of human breast cancers, and increased expression has been associated with a poor prognosis for the patient (2–4). Overexpression of HER-2/neu causes non-neoplastic mammary epithelial cells to undergo malignant transformation (5). Transgenic mice that express HER-2/neu develop mammary tumors (6, 7). Antibodies directed at HER-2/neu inhibit the growth of tumors that express high levels of this receptor (8, 9). Overexpression of HER-2/neu activates the Ras pathway and increases mitogenic signaling (10). Although the precise mechanism by which HER-2/neu regulates oncogenesis is incompletely understood, the PEA3 subfamily of ets genes appears to be important (11, 12). PEA3 is overexpressed in 93% of HER-2/neu-positive human breast tumors (13). Moreover, expression of dominant negative PEA3 in the mammary gland of MMTV-neu transgenic mice dramatically delayed the onset of mammary tumors and reduced the number and size of tumors in individual mice (12). It is logical to postulate, therefore, that the identification of PEA3 target genes should provide new insights into the mechanism by which overexpression of HER-2/neu causes breast cancer.

Multiple lines of evidence suggest that cyclooxygenase-2 (COX-2),¹ an enzyme that catalyzes the formation of prostaglandins (PGs), is also important in carcinogenesis. COX-2 is overexpressed in transformed cells (14, 15) and in malignant tissues (16–23). Recently, overexpression of COX-2 was found to be sufficient to induce breast cancer in multiparous transgenic mice (24). Mice engineered to be null for COX-2 were protected against the development of both intestinal and skin tumors (25, 26). In addition to the genetic evidence implicating COX-2 in carcinogenesis, selective inhibitors of COX-2 reduce the formation and growth of tumors in experimental animals (27–31) and decrease the number of intestinal tumors in familial adenomatous polyposis patients (32). Several different mechanisms can potentially explain the link between COX-2 and cancer. Enhanced synthesis of COX-2-derived PGs favors tumor growth by stimulating cell proliferation (33), promoting angiogenesis (34, 35), increasing invasiveness (36, 37), and inhibiting apoptosis (38, 39).

A link between HER-2/neu signaling and COX-2 expression has been established (40, 41). Overexpression of HER-2/neu in the biliary epithelium of transgenic mice led to increased levels of COX-2 (40). Additionally, activation of the HER2/HER3 pathway induced COX-2 in colorectal cancer cells (41). Although these studies established a relationship between HER-2/neu signaling and COX-2 expression, the underlying mechanism was not evaluated. The main purpose of the current study was to elucidate the signaling mechanism and cis-acting elements in the COX-2 promoter that mediate the inductive effects of HER-2/neu. We show that HER-2/neu stimulates COX-2 transcription via the Ras pathway in cultured human mammary epithelial cells. Notably, closely spaced PEA3 and AP-1 sites are necessary for HER-2/neu-mediated induction of

¹ The abbreviations used are: COX-2, cyclooxygenase-2; MAPK, mitogen-activated protein kinase; PG, prostaglandin; CRE, cyclic AMP-response element; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.
COX-2. This mechanism is likely to be operative in vivo because COX-2 was also overexpressed in HER-2/neu-positive human breast cancers.

EXPERIMENTAL PROCEDURES

Materials—Minimum Eagle’s medium and LipofectAMINE were from Invitrogen. Keratinocyte basal medium (KBM) was from Clonetics Corp. (San Diego, CA). Sodium arachidonate, epidermal growth factor, hydrocortisone, poly(dI-H9252dC), actin antiserum, and o-nitrophenyl-D-galactopyranoside were from Sigma. 2-Amino-3-methoxyflavone (PD 98059) and 4-[(3-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB 202190) were from Calbiochem. Enzyme immunoassay reagents for PGE2 assays were from Cayman Co. (Ann Arbor, MI). [32P]CTP, [32P]UTP, and [32P]ATP were from PerkinElmer Life Sciences. Random priming kits were from Roche Molecular Biochemicals. Nitrocellulose membranes were from Schleicher & Schuell. Reagents for the luciferase assay were from Analytical Luminescence (San Diego, CA). The 18 S rRNA cDNA was from Ambion, Inc. (Austin, TX). Anti-sera to COX-2, ATF-2, c-Fos, c-Jun, and PEA3 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The PEA3 protein standard (K-562 nuclear extracts) was also from Santa Cruz Biotechnology, Inc. Anti-HER-2/neu antiserum was from Zymed Laboratories Inc. (South San Francisco, CA). MAP kinase activities were measured using kits from New England Biolabs, Inc. (Beverly, MA). Western blotting detection reagents (ECL) were from Amersham Biosciences. Plasmid DNA was prepared using a kit from Promega Corp. (Madison, WI). Oligonucleotides were synthesized by Sigma and Genosys (The Woodlands, TX). Quick change site-directed mutagenesis kits were purchased from Stratagene (La Jolla, CA).

Tissue Culture—The 184B5 and 184B5/HER cell lines have been described previously (5, 42). The 184B5 cell line is an immortalized but

FIG. 1. HER-2/neu-mediated transformation of human mammary epithelial cells is associated with increased rates of COX-2 transcription. A, 184B5 and 184B5/HER cells were grown in culture medium for 6 h. This medium was replaced with fresh medium containing 10 μM sodium arachidonate. 30 min later, the medium was collected to determine amounts of PGE2. Production of PGE2 was determined by enzyme immunoassay. Columns, means; bars, S.D.; n = 6. *, p < 0.001 compared with 184B5 cells. B, cellular lysate protein (25 μg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. The immunoblot was probed with antibody specific for COX-2. Cell lysates were prepared from 184B5 (lane 2) and 184B5/HER (lane 3) cells. Lane 1 represents an ovine Cox-2 standard. C, total cellular RNA was isolated from 184B5 (lane 1) and 184B5/HER (lane 2) cells. 10 μg of RNA was added to each lane. The blot was hybridized with probes that recognized COX-2 mRNA and 18 S rRNA. D, nuclei were isolated from 184B5 (lane 1) and 184B5/HER (lane 2) cells. The COX-2 and 18 S rRNA cDNAs were immobilized onto nitrocellulose membranes and hybridized with labeled nascent RNA transcripts. E, total cellular RNA was isolated from HEK293 cells transfected with empty vector (lane 1) or HER-2/neu (lane 2). 10 μg of RNA was added to each lane. The blot was hybridized with probes that recognized COX-2 mRNA and 18 S rRNA.

FIG. 2. HER-2/neu stimulates COX-2 promoter activity via the Ras pathway. 184B5 cells were transfected with 0.9 μg of a human COX-2 promoter construct (–327/+59) (Control) and 0.2 μg of pSVgal. A, cells were co-transfected with 0.4 μg of expression vectors for HER-2/neu, Ras, and dominant negative Ras. B, cells were co-transfected with 0.4 μg of expression vectors for HER-2/neu, Raf-1, and dominant negative Raf-1. The total amount of DNA in each reaction was kept constant at 2 μg by using corresponding empty expression vectors. Luciferase activity represents data that have been normalized to β-galactosidase activity. Columns, means; bars, S.D.; n = 6.

reagents for PGE2 assays were from Cayman Co. (Ann Arbor, MI). [32P]CTP, [32P]UTP, and [32P]ATP were from PerkinElmer Life Sciences. Random priming kits were from Roche Molecular Biochemicals. Nitrocellulose membranes were from Schleicher & Schuell. Reagents for the luciferase assay were from Analytical Luminescence (San Diego, CA). The 18 S rRNA cDNA was from Ambion, Inc. (Austin, TX). Antibodies to COX-2, ATF-2, c-Fos, c-Jun, and PEA3 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The PEA3 protein standard (K-562 nuclear extracts) was also from Santa Cruz Biotechnology, Inc. Anti-HER-2/neu antiserum was from Zymed Laboratories Inc. (South San Francisco, CA). MAP kinase activities were measured using kits from New England Biolabs, Inc. (Beverly, MA). Western blotting detection reagents (ECL) were from Amersham Biosciences. Plasmid DNA was prepared using a kit from Promega Corp. (Madison, WI). Oligonucleotides were synthesized by Sigma and Genosys (The Woodlands, TX). Quick change site-directed mutagenesis kits were purchased from Stratagene (La Jolla, CA).

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Cox-2 expression is regulated by MAP kinase activity in HER-2/neu transformed mammary epithelial cells. A–C, the activities of ERK1/2 (A), p38 (B), and JNK (C) were measured in 184B5 (lane 1) and 184B5/HER (lane 2) cells. Lane 3 represents a standard for phospho-Elk1 (A), phospho-ATF-2 (B), and phospho-c-Jun (C), respectively. D, 184B5/HER cells were treated with vehicle (lane 1) or 10 μM PD 98059 (lane 2) for 4.5 h. E, 184B5/HER cells were treated with vehicle (lane 1) or SB 202190 (1, 5, 10 μM; lanes 2–4) for 4.5 h. Cell lysate protein was loaded onto a 10% SDS-polyacrylamide gel, electroblotted, and subsequently transferred onto nitrocellulose. Immunoblots in D and E were probed for COX-2.

non-tumorigenic human breast epithelial cell line that was established from a reduction mammaryplasty (42). The 184B5/HER cell line was derived by stably transfecting 184B5 cells with a mutationally activated HER-2/neu oncogene (5). These cells form tumors when injected into athymic nude mice (5). Cells were maintained in minimum Eagle’s medium/KBM mixed in a ratio of 1:1 (basal medium) containing epidermal growth factor (10 ng/ml), hydrocortisone (0.5 μg/ml), transferrin (10 μg/ml), gentamicin (5 μg/ml), and insulin (10 μg/ml) (growth medium). Cells were grown to 60% confluence, trypsinized with 0.05% trypsin, 2 mM EDTA, and plated for experimental use. HEK293 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

FIG. 3. COX-2 expression is regulated by MAP kinase activity in HER-2/neu transformed mammary epithelial cells. A–C, the activities of ERK1/2 (A), p38 (B), and JNK (C) were measured in 184B5 (lane 1) and 184B5/HER (lane 2) cells. Lane 3 represents a standard for phospho-Elk1 (A), phospho-ATF-2 (B), and phospho-c-Jun (C), respectively. D, 184B5/HER cells were treated with vehicle (lane 1) or 10 μM PD 98059 (lane 2) for 4.5 h. E, 184B5/HER cells were treated with vehicle (lane 1) or SB 202190 (1, 5, 10 μM; lanes 2–4) for 4.5 h. Cell lysate protein was loaded onto a 10% SDS-polyacrylamide gel, electroblotted, and subsequently transferred onto nitrocellulose. Immunoblots in D and E were probed for COX-2.

4°C, the mixture was then centrifuged at 3,000 × g for 5 min at 4°C. The supernatant was discarded. After washing the pellet four times with RIPA buffer, the pellet was resuspended. SDS-PAGE and immunoblotting were then performed as described below.

Western Blotting—Cell lysates were prepared by treating cells with lysis buffer (150 mM NaCl, 100 mM Tris (pH 8.0), 1% Tween 20, 50 mM diethylthiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 10 μg/ml leupeptin). Lysates were sonicated for 20 s on ice and centrifuged at 10,000 × g for 10 min to sediment the particulate material. The protein concentration of the supernatant was measured by the method of Lowry et al. (44). SDS-PAGE was performed under reducing conditions on 10% polyacrylamide gels as described by Laemmli (45). The resolved proteins were transferred onto nitrocellulose sheets as detailed by Towbin et al. (46). The nitrocellulose membrane was then incubated with primary antibodies. Secondary antibody to IgG conjugated to horseradish peroxidase was detected using a chemiluminescent ECL detection system (Amersham). Blots were visualized by exposure to X-ray film.
peroxidase was used. The blots were probed with the ECL Western blot detection system according to the manufacturer’s instructions.

_Northern Blotting_—Total cellular RNA was isolated from cell monolayers using an RNA isolation kit from Qiagen Inc. 10 μg of total cellular RNA per lane were electrophoresed in a formaldehyde-containing 1.2% agarose gel and transferred to nylon-supported membranes. After baking, membranes were prehybridized overnight in a solution containing 50% formamide, 5% sodium chloride/sodium phosphate/EDTA buffer (SSPE), 5% Denhardt’s solution, 0.1% SDS, and 100 μg/ml single-stranded salmon sperm DNA and then hybridized for 12 h at 42 °C with radiolabeled cDNA probes for human COX-2 and 18 S rRNA.

**Fig. 5.** HER-2/neu-mediated induction of COX-2 promoter activity is mediated via the cyclic AMP-response element. A, shown is a schematic of the human COX-2 promoter. B, 184B5 cells were transfected with 0.9 μg of a series of human COX-2 promoter deletion constructs ligated to luciferase (−1432/+59, −327/+59, −220/+59, −124/+59, −52/+59) alone (empty bars) or 0.9 μg of the indicated COX-2 promoter deletion construct plus 0.9 μg of expression vector for HER-2/neu (black bars). C, 184B5 cells were transfected with 0.9 μg of a series of human COX-2 promoter-luciferase constructs (−327/+59; KBM; ILM; CRM). The bars labeled HER-2/neu also received 0.9 μg of expression vector for HER-2/neu. KBM represents the −327/+59 COX-2 promoter construct in which the NF-κB site was mutagenized; ILM represents the −327/+59 COX-2 promoter construct in which the NF-IL6 site was mutagenized; CRM refers to the −327/+59 COX-2 promoter construct in which the CRE was mutated. B and C, all cells received 0.2 μg of pSVβgal. The total amount of DNA in each reaction was kept constant at 2 μg by using empty vector. Reporter activities were measured in cellular extract 24 h following transfection. Luciferase activity represents data that have been normalized to β-galactosidase activity. Columns, means; bars, S.D.; n = 6.

**Fig. 6.** Increased binding of AP-1 to the CRE of the COX-2 promoter is detected in HER-2/neu transformed cells. A, 184B5 cells were transfected with 0.9 μg of a human COX-2 promoter construct ligated to luciferase (−327/+59) (Control) or COX-2 promoter plus expression vector for HER-2/neu (0.4 μg) or COX-2 promoter, HER-2/neu plus decoy CRE (0.4 μg) or COX-2 promoter, HER-2/neu plus mismatch CRE (0.4 μg) or COX-2 promoter, HER-2/neu plus nonsense CRE (0.4 μg). All cells received 0.2 μg of pSVβgal. The total amount of DNA in each reaction was kept constant at 2 μg by using empty vector. Reporter activities were measured 24 h after transfection. Columns, means; bars, S.D.; n = 6. B, in lanes 1–3, 5 μg of nuclear protein was incubated with a 32P-labeled oligonucleotide containing the CRE of COX-2. Lane 1 represents nuclear protein from 184B5 cells; lane 2 represents nuclear protein from 184B5/HER cells; lane 3 represents nuclear protein from 184B5/HER cells incubated with a 50-fold excess of unlabeled oligonucleotide containing an AP-1 consensus site. C, 5 μg of nuclear protein was incubated with a 32P-labeled oligonucleotide containing the CRE of COX-2. Lane 1 represents nuclear protein from 184B5/HER cells; lane 2 represents nuclear extracts incubated with antibodies to ATF-2, c-Jun, and c-Fos, respectively. B and C, the protein DNA complex that formed was separated on a 4% polyacrylamide gel.

COX-2 and 18 S rRNA probes were labeled with [32P]CTP by random priming. After hybridization, membranes were washed twice for 20 min at room temperature in 2x SSPE, 0.1% SDS, twice for 20 min in 0.1x SSPE, 0.1% SDS at 55 °C. Washed membranes were then subjected to autoradiography.

_Nuclear Run-off Assay_—2.5 × 10⁶ cells were plated in four T150 dishes for each condition. Cells were grown in growth medium until ~60% confluent. Nuclei were isolated and stored in liquid nitrogen. For the transcription assay, nuclei (1.0 × 10⁵) were thawed and incubated in reaction buffer (10 mM Tris (pH 8), 5 mM MgCl₂, and 0.3 M KCl) containing 100 μCi of uridine 5′-32P-triphosphate and 1 μM unlabeled nucelotides. After 30 min, labeled nascent RNA transcripts were isolated. The human COX-2 and 18 S rRNA cDNAs were immobilized onto nitrocellulose and prehybridized overnight in hybridization buffer. Hybridization was carried out at 42 °C for 24 h using equal cpm/ml of labeled nascent RNA transcripts for each treatment group. The membranes were washed twice with 2x SSC buffer for 1 h at 55 °C and then...
treated with 10 mg/ml RNase A in 2× SSC at 37 °C for 30 min, dried, and autoradiographed.

Plasmids—The COX-2 promoter constructs (∼−1432/+59, −327/+59, −220/+59, −124/+59, −52/+59, KBM, ILM, CRM and CRM, ILM) were a generous gift of Dr. Tadashi Tanabe (National Cardiovascular Research Institute, Osaka, Japan) (47). Dr. Stephen M. Prescott (University of Utah, Salt Lake City, UT) generously provided the human COX-2 cDNA. The RSV-c-jun expression vectors were a gift of Dr. Tom Curran (Roche Laboratories, Nutley, NJ). Dr. Joan Heller Brown (University of Massachusetts, Worcester, MA). The HER-2/neu expression vectors were a gift of Dr. Roger Davis (University of Wurzburg, Wurzburg, Germany). Dr. Geoffrey Cooper (Harvard University, Cambridge, MA). The expression vectors for JNK and p38 were a gift of Dr. Melanie Cobb (Southwestern Medical Center, Dallas, TX). The expression vectors for the CRE of the COX-2 promoter were synthesized: 5′-AAACAAGTACATTCTGATCGAGCTGTC-3′ (sense), and 5′-AGCTGACGTCGTAATTCTGTCTT-3′ (antisense). An AP-1 consensus oligonucleotide was used: 5′-TCGAAGTACTGAGCTGTC-3′ (sense) and 5′-GGGAGAATCAGTCTGTC-3′ (antisense). The PEA3 antisense phosphorothioate primer used was 5′-CTAATCTGCGTCTTCTGTCGTT.

Electrophoretic Mobility Shift Assay—Cells were harvested and nuclear extracts were prepared. For binding studies, oligonucleotides containing the CRE or PEA3 sites of the COX-2 promoter were used. The complementary oligonucleotides were annealed in 20 mM Tris (pH 7.6), 50 mM NaCl, 10 mM MgCl2, and 1 mM dithiothreitol. The annealed oligonucleotide was phosphorylated at the 5′-end with [γ-32P]ATP and T4 polynucleotide kinase. The binding reaction was performed by incubating 5 μg of nuclear protein in 20 mM HEPES (pH 7.9), 10% glycerol, 300 μg of bovine serum albumin and 1 μg of poly(dI-dC) in a final volume of 10 μl for 10 min at 25 °C. The labeled oligonucleotide was added to the reaction mixture and allowed to incubate for an additional 20 min at 25 °C. The samples were electrophoresed on a 4% nondenaturing polyacrylamide gel. The gel was then dried and subjected to autoradiography at −80 °C.

Statistics—Comparisons between groups were made by the Student’s t test or the χ2 test of proportions. A difference between groups of p < 0.05 was considered significant.

RESULTS

COX-2 Is Overexpressed in HER-2/neu-transformed Human Mammary Epithelial Cells—Cell culture was used to investigate whether HER-2/neu regulated the expression of COX-2. PGE2 synthesis was increased by more than 10-fold in 184B5/HER cells compared with the 184B5 counterpart (Fig. 1A). Western blotting was carried out to determine whether the

FIG. 7. c-Jun is important for HER-2/neu-mediated activation of the COX-2 promoter. A, cells were transfected with 0.9 μg of a human COX-2 promoter construct ligated to luciferase (∼−327/+59) and 0.2 μg of pSVβgal. Cells received COX-2 promoter alone (Control) or COX-2 promoter construct and 0.4 μg of expression vectors for HER-2/neu, c-Jun, HER-2/neu plus c-Jun, or HER-2/neu plus c-Jun dominant negative. The total amount of DNA in each reaction was kept constant at 2 μg by using empty vector. Reporter activities were measured 24 h after transfection. Luciferase activity represents data that have been normalized with β-galactosidase activity. Columns, means; bars, S.D.; n = 6. B, cellular lysate protein was prepared from 184B5 (lane 1) and 184B5/HER cells (lane 2) and loaded (50 μg/lane) onto a 10% SDS-polyacrylamide gel. The immunoblot was probed with an antibody to c-Jun.

FIG. 8. PEA3 is important for HER-2/neu-mediated induction of COX-2. A, cellular lysate protein (25 μg/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto nitrocellulose. The immunoblot was probed with antibody specific for PEA3. Cell lysates were prepared from 184B5 (lane 1) and 184B5/HER (lane 2) cells. Lane 3 represents a PEA3 standard. B, 184B5 cells were transfected with 0.9 μg of a human COX-2 promoter construct ligated to luciferase (∼−327/+59) (Control) or COX-2 promoter plus expression vector for HER-2/neu (0.4 μg) or COX-2 promoter, HER-2/neu plus antisense to PEA3 (0.4 μg). The total amount of DNA in each reaction was kept constant at 2 μg by using empty vector. Reporter activities were measured 24 h after transfection. Luciferase activity represents data that have been normalized with β-galactosidase activity. Columns, means; bars, S.D.; n = 6.
differences in PGE_2 production were related to differences in amounts of COX-2. Fig. 1B shows that levels of COX-2 protein were much higher in 184B5/HER cells than in 184B5 cells. COX-1 was not detectable by immunoblotting in these cell lines. To elucidate further the mechanism responsible for the changes in amounts of COX-2 protein, we determined steady-state levels of COX-2 mRNA by Northern blotting. As shown in Fig. 1C, higher levels of COX-2 mRNA were also detected in 184B5/HER cells than in 184B5 cells. Differences in levels of mRNA could reflect altered rates of transcription. To investigate this possibility, nuclear run-offs were performed. Higher rates of synthesis of nascent COX-2 mRNA were observed in 184B5/HER cells than in 184B5 cells. Differences in levels of COX-2 mRNA were also detected in 184B5/HER cells with an excess of AP-1 cold probe (Fig. 6B). By contrast, binding to the NF-kB and NF-IL6 sites was similar in these two cell lines (data not shown). Supershift analysis identified c-Jun, c-Fos, and ATF-2 in the binding complex (Fig. 6C). Consistent with this finding, binding was also prevented by incubating nuclear extract from 184B5/HER cells with an excess of AP-1 cold probe (Fig. 6B). Transient transfections were performed to confirm the importance of AP-1 for mediating the induction of COX-2 by HER-2/neu. A downstream target of Ras is Raf-1. Hence, we determined whether Raf-1 mediated the inductive effects of HER-2/neu on COX-2. As shown in Fig. 2B, overexpressing Raf-1 led to a 3-fold increase in COX-2 promoter activity. The stimulation of COX-2 promoter activity by HER-2/neu was blocked by kinase-deficient Raf-1 (Fig. 2B).

Ras signaling can alter gene expression by three distinct MAPK cascades (49). It was important, therefore, to investigate whether increased MAPK activity contributed to the induction of COX-2 in HER-2/neu transformed cells. The activities of extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase (JNK) were higher in 184B5/HER cells than in 184B5 cells (Figs. 3, A–C). Subsequently, experiments were done to show that increased MAPK activity was linked to elevated levels of COX-2 in HER-2/neu transformed cells. In the first experiment, we utilized PD 98059, a specific inhibitor of MAPK kinase, which prevents activation of ERK1 and ERK2 (50). Treatment with PD 98059 caused a decrease in amounts of COX-2 in 184B5/HER cells (Fig. 3D). Similarly, SB 202190, a selective inhibitor of p38 MAPK (51), down-regulated amounts of COX-2 in these cells (Fig. 3E). To investigate further the importance of MAPK in mediating the induction of COX-2 in HER-2/neu transformed cells, a series of transient transfections was performed (Fig. 4). Transiently overexpressing ERK1, JNK, or p38 MAPKs led to severalfold increases in COX-2 promoter activity. The induction of COX-2 promoter activity by HER-2/neu was inhibited by dominant negatives for ERK1, JNK, and p38 (Fig. 4).

The Cyclic AMP-response Element and AP-1 Are Necessary for the Induction of COX-2 by HER-2/neu in Mammary Epithelial Cells—We next were interested in identifying the region of the COX-2 promoter that was important for mediating the inductive effects of HER-2/neu. Transient transfections were performed with a series of human COX-2 promoter-deletion constructs (Fig. 5A) in 184B5 cells. As shown in Fig. 5B, overexpression of HER-2/neu led to nearly a 3-fold increase in COX-2 promoter activity when a −1432/+59 COX-2 promoter construct was utilized. A stepwise decrease in basal COX-2 promoter activity was observed when shorter constructs were used. However, the magnitude of induction by HER-2/neu remained nearly 3-fold with all promoter deletion constructs except the −52/+59 construct (Fig. 5B). The −52/+59 COX-2 promoter construct was not stimulated by HER-2/neu. This result implies that one or more promoter elements lying between −53 and −123 is necessary for HER-2/neu-mediated induction of COX-2. A CRE is present between nucleotides −59 and −53 raising the possibility that this element could be involved in mediating the inductive effects of HER-2/neu. To test this notion, transient transfections were performed utilizing COX-2 promoter constructs in which specific known enhancer elements including the CRE were mutagenized. As shown in Fig. 5C, HER-2/neu-mediated stimulation of COX-2 promoter activity was abrogated by mutagenizing the CRE site. By contrast, mutagenizing the NF-kB and NF-IL6 sites had no effect on COX-2 promoter function. To confirm the importance of the CRE for mediating the induction of COX-2 by HER-2/neu, a separate series of transient transfections was performed. We examined the effects of a CRE-decoy oligonucleotide on HER-2/neu-mediated stimulation of COX-2 promoter activity. The CRE-decoy oligonucleotide effectively inhibited HER-2/neu-mediated activation of the COX-2 promoter (Fig. 6A). In contrast, neither a CRE mismatch oligonucleotide nor a non-sense-sequence palindromic blocked HER-2/neu-mediated induction of COX-2 promoter activity.

Electrophoretic mobility shift assays were performed to identify the transcription factor that contributed to the induction of COX-2 in HER-2/neu transformed cells. Increased binding of nuclear proteins to the CRE site of the COX-2 promoter was detected (Fig. 6B). By contrast, binding to the NF-kB and NF-IL6 sites was similar in these two cell lines (data not shown). Supershift analysis identified c-Jun, c-Fos, and ATF-2 in the binding complex (Fig. 6C). Consistent with this finding, binding was also prevented by incubating nuclear extract from 184B5/HER cells with an excess of AP-1 cold probe (Fig. 6B). Transient transfections were performed to confirm the importance of AP-1 for mediating the induction of COX-2 by HER-2/neu. A dominant negative form of c-Jun inhibited the induction of COX-2 promoter activity by HER-2/neu (Fig. 7A). Changes in either the amount or phosphorylation state of c-Jun can alter AP-1-mediated gene expression. Hence, we compared the amounts of c-Jun and phosphorylated c-Jun in 184B5/HER and 184B5 cells. Levels of c-Jun protein (Fig. 7B) and phosphorylated c-Jun protein (data not shown) were higher in 184B5/HER cells compared with its nontransformed 184B5 partner cell line. AP-1 activity was also increased in the transformed 184B5/HER cell line compared with its nontransformed 184B5 partner cell line (data not shown). Thus, in response to overexpression of HER-2/neu, increased MAPK signaling activates AP-1, which, in turn, contributes to enhanced COX-2 gene expression via the CRE in the COX-2 promoter.

PEA3 Is Also Necessary for HER-2/neu-mediated Induction of COX-2—Increased levels of PEA3 have been detected in more than 90% of HER-2/neu-overexpressing breast cancers (13). A variety of genes are regulated by closely spaced PEA3/ets and AP-1 sequences (52). Experiments were therefore carried out to investigate the potential role of PEA3 in HER-2/neu-mediated induction of COX-2. Higher levels of PEA3 were detected in 184B5/HER cells than in 184B5 cells (Fig. 8A). To investigate whether PEA3 is important for the induction of COX-2 by HER-2/neu, transient transfections were performed. As shown in Fig. 8B, overexpressing antisense to PEA3 blocked the stimulation of COX-2 promoter activity by HER-2/neu.

There are several possible PEA3 sites (GGAA) in the COX-2 promoter (53) (Fig. 9A). Site-directed mutagenesis was used to create COX-2 promoter constructs in which each of these candidate PEA3 sites (mut1–mut3) was altered. HER-2/neu stimulated COX-2 promoter activity except when PEA3 site 1 (−72/−75) was mutagenized (Fig. 9B). To evaluate further the importance of this site, electrophoretic mobility shift assays
were performed. Nuclear protein from both 184B5 and 184B5/HER cells was incubated with a labeled oligonucleotide containing PEA3 site 1 of the COX-2 promoter. Extracts from HER-2/neu transformed cells led to increased binding to PEA3 site 1 (Fig. 9C). This binding was abolished when an excess of unlabeled consensus PEA3 oligonucleotide was added. Super-shift analysis identified PEA3 in the binding complex (Fig. 9D).

Taken together, these results suggest that both PEA3 site 1 and the CRE are necessary for HER-2/neu-mediated induction of COX-2.

**DISCUSSION**

In the current study, we found that levels of COX-2 were increased in HER-2/neu-overexpressing human mammary epithelial cells and breast cancers. The induction of COX-2 by...
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HER-2/neu was mediated by the Ras pathway (Fig. 11). Ras can regulate gene expression by stimulating MAPK activities (49). Several lines of evidence suggest that HER-2/neu induced COX-2 via activation of ERK, JNK, and p38 MAPKs. First, the activities of ERK1/2, JNK, and p38 were increased in HER-2/neu transformed cells. Second, inhibitors of MAPK kinase and p38 decreased amounts of COX-2 in HER-2/neu transformed cells. Third, overexpression of dominant negatives for ERK1, JNK, and p38 suppressed the induction of COX-2 promoter activity by HER-2/neu.

We also report that the induction of COX-2 promoter activity by HER-2/neu is mediated through closely spaced PEA3/ets and AP-1 sites located 72 and 53 nucleotides upstream of the transcriptional start site, respectively. Several observations support a role for AP-1 in mediating the induction of COX-2 by HER-2/neu. Increased binding of AP-1 to the CRE of the COX-2 promoter was detected in HER-2/neu transformed cells (Fig. 6B); c-Jun, c-Fos, and ATF-2 were identified in the DNA binding complex (Fig. 6C). The functional importance of AP-1 was established because HER-2/neu-mediated activation of the COX-2 promoter was suppressed by mutagenizing the CRE or overexpressing dominant negative c-Jun (Fig. 7A). Our results are consistent with the findings of Xie and Herschman (54, 55). These investigators showed that, in response to expression of v-Src or treatment with platelet-derived growth factor, c-Jun induced murine COX-2 via the CRE site. Tumor-promoting phorbol esters also stimulate AP-1-mediated activation of COX-2 transcription via the CRE site (56). Several findings also suggest the involvement of the PEA3/ets-binding site in mediating the induction of COX-2 by HER-2/neu. First, levels of PEA3 were elevated in HER-2/neu transformed cells. Second, overexpression of antisense to PEA3 or by mutagenizing the PEA3 site has been described. In this context, it is noteworthy that PEA3 levels are elevated in 93% of HER-2/neu-positive breast cancers (13) suggests that the mechanism of regulation discussed above is likely to be operative in vivo. Recently, PEA3 subfamily Ets proteins were found to play an essential role in Neu-mediated mammary oncogenesis (12). Moreover, overexpression of COX-2 was sufficient to induce mammary cancer in multiparous transgenic mice (24). Our results suggest, therefore, that the interaction between PEA3 and COX-2 could be important for understanding Neu-induced tumor formation.

The results of this study provide other potentially significant insights. HER-2/neu induces the expression of vascular endothelial growth factor (64). COX-2-derived PGs enhance the production of vascular endothelial growth factor (65). It is reasonable to postulate, therefore, that the increased levels of vascular endothelial growth factor and angiogenesis in HER-2/neu-positive breast cancers are a consequence, in part, of HER-2/neu-mediated induction of COX-2 and PG biosynthesis. Another important issue concerns the role of nonsteroidal anti-inflammatory drugs, prototypic inhibitors of COX, in preventing cancer. The finding that COX-2 is undetectable in most cases of HER-2/neu-negative breast cancer may help to explain why nonsteroidal anti-inflammatory drugs have not been shown consistently to protect against breast cancer (66, 67). Our findings also imply that selective COX-2 inhibitors may be useful in preventing or treating the subset of cancers in which HER-2/neu is overexpressed. In support of this idea, treatment with a selective COX-2 inhibitor reduced the growth rate of a HER-2/neu-positive colon cancer cell line in vitro and in vivo (68). Future studies will be needed to determine whether selective inhibitors of COX-2 have a role in preventing or treating HER-2/neu-positive human breast cancers.

REFERENCES
1. Ullrich, A., and Schlessinger, J. (1990) Cell 61, 203–212
2. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and Press, M. F. (1989) Science 244, 707–712
3. Press, M. F., Bernstein, L., Thomas, P. A., Meisner, L. F., Zhou, J. Y., Ma, Y., Hung, G., Robinson, R. A., Harris, C., El-Naggar, A., Slamon, D. J., Phillips, R. N., Ross, J. S., Wolman, S. H., and Flom, R. J. (1997) J. Clin. Oncol. 15, 2894–2904
4. Seshadri, R., Figgaira, F. A., Forsfall, D. J., McCaul, K., Sethur, V., and Kitchen, P. (1995) J. Clin. Oncol. 13, 1936–1942

Fig. 11. Schematic of proposed mechanism by which HER-2/neu regulates the expression of COX-2. HER-2/neu activates COX-2 transcription by stimulating the Ras signal transduction pathway. Juxtaposed AP-1 and PEA3 sites are required for HER-2/neu-mediated activation of the COX-2 promoter.

MAPK

NFkB

NF-IL6

PEA3

AP-1

TATA

HER-2/neu

Ras

Raf-1

MEK

[Diagram showing the proposed mechanism]
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Pierce, J. H., Arnold, P. D., DiMarco, E., Artrip, J., Kraus, M. H., Lonardo, F., Di Fiore, P. P., and Aaronson, S. A. (1991) Oncogene 6, 1189–1194

Guy, C. T., Werer, M. A., Schaller, M. P., Parsons, T. J., Cardiff, R. D., and Muller, W. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10578–10582

Carter, P., Presta, L., Gorman, C. M., Ridgway, J. B., Henner, D., Wong, W. L., Rest-Dani, A. M., Kutas, C., Cancer Res. 59, 991–994

Baselga, J., Norton, L., Albanell, J., Kim, Y.-M., and Mendelsohn, J. (1998) Cancer Res. 58, 2825–2831

Parett, M. L., Harris, R. E., Joarder, F. S., Ross, M. S., Clausen, K. P., and Hwang, D., Scollard, D., Byrne, J., and Levine, E. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2809–2812

Shepherd, T., Kockeritz, L., Szrajber, M. R., Muller, W. J., and Hassell, J. A. (1997) J. Mammary Gland Biol. Neoplasia 2, 697–7678

Shepherd, T., Hassell, J. A. (2001) J. Mammary Gland Biol. Neoplasia 6, 129–140

Neil, G. P., Vickers, P. J., Evans, J. F., Mancini, J. A., and Robertson, F. M. (1997) Int. J. Oncol. 11, 185–120

Kulkarni, S., Rader, J. S., Zhang, F., Liapis, H., Koki, A. T., Masferrer, J. L., Dannenberg, A. J., Altorki, N. K., Boyle, J. O., Dang, C., Howe, L. R., Weksler, M. B., and Dannenberg, J. A. (1996) Cancer Res. 56, 4424–4429

Kutchera, W., Jones, D. A., Matsunami, N., Groden, J., McIntyre, T. M., Zimmerman, G. A., White, R. L., and Prescott, S. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4816–4820

Eberhart, C. E., Cofi, R. J., Radhika, A., Giardiello, F. M., Ferrenbach, S., and DuBois, R. N. (1994) Gastroenterology 107, 1183–1188

Kargman, S. L., O’Neil, G. P., Vickers, P. J., Evans, J. F., Manzie, J. A., and Jothy, S. (1995) Cancer Res. 55, 2556–2559

Ristimaki, A., Honkanen, N., Jankala, H., Sipponen, P., and Harkonen, M. (1995) Cancer Res. 55, 1276–1280

Parrett, M. L., Harris, R. E., Joarder, F. S., Ross, M. S., Claussen, K. P., and Robertson, F. M. (1997) Int. J. Oncol. 10, 503–507

Hwang, D., Scolard, D., Byrne, J., and Levine, E. (1998) J. Natl. Cancer Inst. 90, 455–460

Chen, G., Boyle, J. O., Yang, E. K., Zhang, F., Sacks, P. G., Shah, J. P., Edelestein, D., Soslow, R. A., Koki, A. T., Woerner, B. M., Masferrer, J. L., Dannenberg, A. J., and Subbaramaiah, K. (1999) Cancer Res. 59, 991–994

Kulkarni, S., Rader, J. S., Zhang, F., Liapis, H., Koki, A. T., Masferrer, J. L., Subbaramaiah, K., and Dannenberg, A. J. (2001) Clin. Cancer Res. 7, 429–434

Dannenberg, A. J., Alterki, N. K., Boyle, J. O., Dann, C., Howe, L. R., Wexler, B. B., and Subbaramaiah, K. (2000) Lancet Oncol. 1, 544–551

Liu, C. H., Chang, S. H., Narko, K., Trifan, O. C., Wu, M. T., Smith, E., Herring, C., Lane, T. F., and Hla, T. (2001) J. Biol. Chem. 276, 18563–18569

Oshima, M., Inokuchi, J., Koga, S., Yoshiya, H., Hori, M., and Ichihara, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7676–7680

Tian, H., Chulada, P., Spalding, J., Lee, C., Loftin, C., Mahler, J., Morham, S., and Langenbach, R. (1997) Proc. Am. Assoc. Cancer Res. 38, 1727

Kawamori, T., Rau, C. V., Seibert, K., and Reddy, B. S. (1998) Cancer Res. 58, 429–432

Bende, M., Milev, M., and H belongings

Fischer, S. M., Lo, H.-H., Gordon, G. B., Seibert, K., Kelloff, G., Lubet, R. A., and Conti, C. C. (1999) Mol. Carcinog. 25, 211–240

Sheng, H., Shao, J., Kirkland, S. C., Iacson, P., Cofey, R. J., Morrow, J., Beauchamp, R. D., and DuBois, R. N. (1997) J. Clin. Invest. 99, 2254–2259

Sawasaki, H., Kawano, S., Tsuji, S., Watanabe, E., Sato, H., Seiki, M., and Hori, M. (1998) Am. J. Pathol. 147, 1611–1615

Harris, R. E., Alshafie, G. A., Ashou-lasa, H., and Seibert, K. (2000) Cancer Res. 60, 2101–2103

Steinbach, G., Lynch, P. M., Phillips, R. K. S., Wallace, M. H., Haw, E., Gordon, B. W., Nakayashiki, N., Saunders, B., Shen, Y., Fujimura, T., Su, L. K., and Levin, B. (2000) N. Engl. J. Med. 342, 1946–1952

Sheng, H., Shao, J., Washington, M. K., and DuBois, R. N. (2001) J. Biol. Chem. 276, 18075–18081

Sheng, H., Shao, J., Washington, M. K., and DuBois, R. N. (2001) J. Biol. Chem. 276, 18075–18081

Sheng, H., Shao, J., Williams, C. S., Piscanec, P. I., Slukwinski, M. X., and DuBois, R. N. (2001) Gastroenterology 120, 1715–1719

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Kotha Subbaramaiah, Larry Norton, William Gerald and Andrew J. Dannenberg

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