Binding at and transactivation of the *COX-2* promoter by nuclear tyrosine kinase receptor ErbB-2

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Summary

Pathological expression of human ErbB-2 protein, also known as HER-2, is common in many types of cancer. ErbB-2 is a member of the EGF receptor tyrosine kinase family and has been rigorously studied as a signaling molecule on the cell membrane. Here, we report that ErbB-2 is also expressed in the nucleus in cultured cells as well as primary tumor tissues. Nuclear ErbB-2 was found to associate with multiple genomic targets in vivo, including the cyclooxygenase enzyme *COX-2* gene promoter. ErbB-2 forms a complex at a specific nucleotide sequence of the *COX-2* promoter and is able to stimulate its transcription. This study demonstrates the presence of ErbB-2 in the nucleus and identifies the function of ErbB-2 as a transcriptional regulator.

Introduction

The proto-oncogene *ErbB-2* encodes a 185 kDa tyrosine kinase receptor that belongs to the epithelial growth factor receptor (EGFR) family (Yarden and Sliwkowski, 2001). There are four members in the EGFR family, namely ErbB-1 (EGFR), ErbB-2 (HER-2/*neu*), ErbB-3, and ErbB-4. Each of these four receptors contains an extracellular domain, a hydrophobic transmembrane domain, and an intracellular domain. Except for ErbB-3, the intracellular domains of the EGFR proteins contain intrinsic tyrosine kinase activity. Upon stimulation, these receptors form homodimers or heterodimers with other EGFR members and become activated through tyrosine phosphorylation. Although no known ligand binds to ErbB-2 with high affinity, ErbB-2 itself is a preferred coreceptor for other members of the EGFR family to form high-affinity complexes for different ligands (Graus-Porta et al., 1997). The activated receptor complexes consequently recruit other signaling molecules and activate numerous downstream signaling cascades, such as the mitogen-activated protein kinases (MAPKs) and the phosphatidylinositol 3-OH kinase (PI3K) pathways. Through these signaling cascades, ErbB-2 and its family members regulate a variety of cellular functions, ranging from proliferation, differentiation, survival, and motility to adhesion. In breast cancer, ErbB-2 overexpression can be observed in 15%–30% of invasive ductal carcinoma. Elevated ErbB-2 expression is associated with promoted proliferation potential, enhanced resistance to apoptotic stimuli, and lymph node metastasis (Holbro et al., 2003). Due to its important role in tumor development, targeting ErbB-2 has become a critical area for breast cancer therapy (Arteaga, 2003; Yarden and Sliwkowski, 2001; Mendelsohn and Baselga, 2000).

The cyclooxygenase enzyme COX-2 catalyzes the conversion of lipids to inflammatory prostaglandins. It is well documented that COX-2 expression contributes to increased anti-apoptotic, proangiogenic, and metastatic potential in cancer cells and COX-2 deregulation is correlated with tumor progression (Rajnish et al., 2001; Turini and DuBois, 2002). Transcriptional upregulation of the *COX-2* gene by ErbB-2 expression has been found in cultured cancer cells (Half et al., 2002; Howe et al., 2001; Subbaramaiah et al., 2002; Vadlamudi et al., 1999) and in a *c-erbB-2* transgenic animal model (Kiguchi et al., 2001). Consistently, significant correlation of COX-2 and ErbB-2 expression has been reported in several human cancer types, including cholangiocarcinoma, colon cancer, and breast cancer

SIGNIFICANCE

Many membrane receptor tyrosine kinases (RTKs), including the EGF receptor proteins, VEGF receptor, FGF receptor, insulin receptor, and NGF receptor, have recently been found in the nucleus. However, the nuclear functions of these RTKs are largely unknown due to the lack of knowledge on their nuclear targets. In the current study, we demonstrate that human ErbB-2, the clinically important RTK associated with multiple types of human cancer, is also located in the nucleus, and we identify multiple genomic targets of the nuclear ErbB-2, including the cyclooxygenase enzyme *COX-2* promoter. The same experimental strategy, which combines molecular and bioinformatic approaches, could be applied to other membrane RTKs expressed in the nucleus and may open a novel avenue to understanding their nuclear functions.

(Endo et al., 2002; Howe et al., 2001; Vadlamudi et al., 1999). Indeed, a recent large-scale clinical investigation involving 1576 patients with breast cancer revealed a significant correlation between COX-2 and ErbB-2 expression in primary tumors (Ristimäki et al., 2002).

Although it is commonly accepted that tyrosine kinase receptors such as the EGFR family proteins function as signaling initiators on the cell membrane, there is accumulating evidence indicating nuclear expression of growth factors and their receptors (Carpenter, 2003; Clevenger, 2003; Keresztes and Boonstra, 1999; Wells and Marti, 2002). In addition to the EGFR family proteins ErbB-1, ErbB-3, and ErbB-4 (Lin et al., 2001; Ni et al., 2001; Offterdinger et al., 2002; Xie and Hung, 1994), nuclear translocation of other tyrosine kinase receptors, including vascular endothelial growth factor (VEGF) receptor (Feng et al., 1999), nerve growth factor (NGF) receptor (Rakowicz-Szulczynska et al., 1988), and fibroblast growth factor (FGF) receptor (Maher, 1996; Reilly and Maher, 2001), have been reported. However, the potential nuclear functions of these receptors/ligands remain elusive due to the fact that a systemic approach to identify their nuclear targets has not been established. To this regard, it is known that the carboxy-terminal acidic regions of EGFR and ErbB-4 contain an intrinsic transactivation function and are able to transactivate a luciferase-based report system (Lin et al., 2001; Ni et al., 2001). Indeed, we previously demonstrated that the nuclear EGFR functions as a transcription factor to stimulate cyclin D1 expression (Lin et al., 2001). Like EGFR and ErbB-4, the carboxyl terminus of ErbB-2 is also highly acidic, and this feature is conserved across different species, including human, mouse, and rat (Xie and Hung, 1994).

In the current study, we provide evidence for nuclear expression and function for human ErbB-2 protein and identify important genomic targets of ErbB-2 using a strategy combining chromatin immunoprecipitation, molecular cloning, and bioinformatic analysis. We demonstrate that nuclear ErbB-2 associates with a specific sequence in the *COX-2* promoter and upregulates expression of the *COX-2* gene.

Results

Nuclear localization of ErbB-2 protein

We detected nuclear ErbB-2 in ErbB-2-expressing human breast cancer cell lines using different monoclonal antibodies against the amino (N) and the carboxyl (C) terminus of ErbB-2. In addition to staining of the plasma membrane and perinuclear cytoplasm, both antibodies clearly showed punctate ErbB-2 expression in the nucleus (the nuclear green spots marked by white arrows in Figures 1B and 1D–1F). To rule out the possibility that the nuclear ErbB-2 was contaminated from the endoplasmic reticulum (ER), we stained cells with antibodies against the ER markers calreticulin (Figures 1C, 1E, and 1F) and calnexin (data not shown). Whereas the perinuclear ErbB-2 mainly colocalized with ER markers, the nuclear ErbB-2 did not (Figures 1B, 1C, 1E, and 1F). Consistantly, when ErbB-2 expression was stably reconstituted in the MCF-7/HER cells, nuclear immunoreactivity of ErbB-2 was also detected (see Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/6/3/251/DC1). Confirming this phenomenon, nuclear expression of ErbB-2 in the MCF-7/HER cells was also demonstrated by electron microscopy (Figure 1G). Using this method, only minimal nuclear ErbB-2 could be detected in the parental MCF-7 cells that expressed a basal level of ErbB-2 (data not shown). Consistent with these results, ErbB-2 protein was detected in the nuclear fraction of ErbB-2-expressing breast cancer cells by cellular fractionation and immunoblot analysis (Figure 1H). It is known that tyrosine kinase activity of tyrosine kinase receptors is required for endocytosis, so we also examined nuclear expression of ErbB-2 in the presence of a ErbB-2-specific kinase inhibitor AG825 (Levitzki and Gazit, 1995). The result indicated that the kinase inhibitor blocked nuclear expression of ErbB-2, suggesting that tyrosine kinase activity is required for nuclear translocation of ErbB-2 (Figure 1I). In addition to cell lines, we also detected nuclear ErbB-2 in multiple types of primary human tumor tissue by immunohistochemical staining (Figure 6). Thus, the ErbB-2 thought to be a cell surface membrane receptor can be detected in the nucleus by four different approaches, including fluorescence microscopy, electron microscopy, biochemical fractionation, and immunohistochemistry in tumor tissues (Figure 6). These results also suggest that nuclear ErbB-2 is an intact molecule, as it can be detected by antibodies against both N- and C-terminal epitopes of ErbB-2 protein and the size of nuclear ErbB-2 is the same as that of cytoplasmic ErbB-2.

Binding of nuclear ErbB-2 to a specific DNA sequence of the *COX-2* promoter

To elucidate the function of nuclear ErbB-2, we explored the potential role of ErbB-2 as a gene regulator by investigating the genomic targets associated with ErbB-2 using chromatin immunoprecipitation (ChIP). DNA fragments associated with ErbB-2 were processed, cloned, and identified by sequencing, as depicted in Figure 2A. The promoter regions or introns identified by these clones were further verified by ChIP. Using this protocol, the genomic sequences of *PRPK* (Abe et al., 2001), *MMP-16* (Will and Hinzmann, 1995), *DDX-10* (Savitsky et al., 1996), and *COX-2* (Jones et al., 1993) associated with ErbB-2 were identified (Supplemental Figure S2A). We chose to analyze the interaction between ErbB-2 and the *COX-2* promoter in detail because overexpression of both ErbB-2 and COX-2 has been implicated in tumorigenesis of many human cancer types (Howe et al., 2001; Yarden and Sliwkowski, 2001). The cloned DNA fragment, which we named HER-2-associated sequence (HAS), is located 1750 nucleotides upstream from the transcriptional start site in this gene (Figure 2B). By using primers flanking the HAS, we detected association of ErbB-2 with the *COX-2* promoter in multiple breast cancer cell lines, including SK-BR-3, BT474 (Figure 2C), and MCF-7/HER (Figure 2D), as well as in the ovarian cancer cell line SK-OV-3 (Figure 2C). In a parallel ChIP experiment to test the specificity of chromatin association (Figure 2D), estrogen receptor was recruited to the *pS2* promoter, a documented target of estrogen receptor (Berry et al., 1989), but not the HAS of *COX-2* promoter. Conversely, ErbB-2 did not associate with the *pS2* promoter or a distant genomic sequence upstream from the *COX-2* promoter. The same ErbB-2-DNA complex could be detected using ErbB-2 antibodies against different epitopes (data not shown). A quantitative ChIP assay using real-time PCR further validated the recruitment of ErbB-2 in the *COX-2* promoter (Figure 2E). Significant ErbB-2 recruitment to the *COX-2* promoter was detected in MCF-7 stable clones expressing ectopic ErbB-2 but not the MCF-7 parental cells, indicating that *COX-2* occupancy was indeed ErbB-2 dependent (Figures 2F and 2G). In addition, radiolabeled HAS oligonucleotide formed a specific complex with nuclear

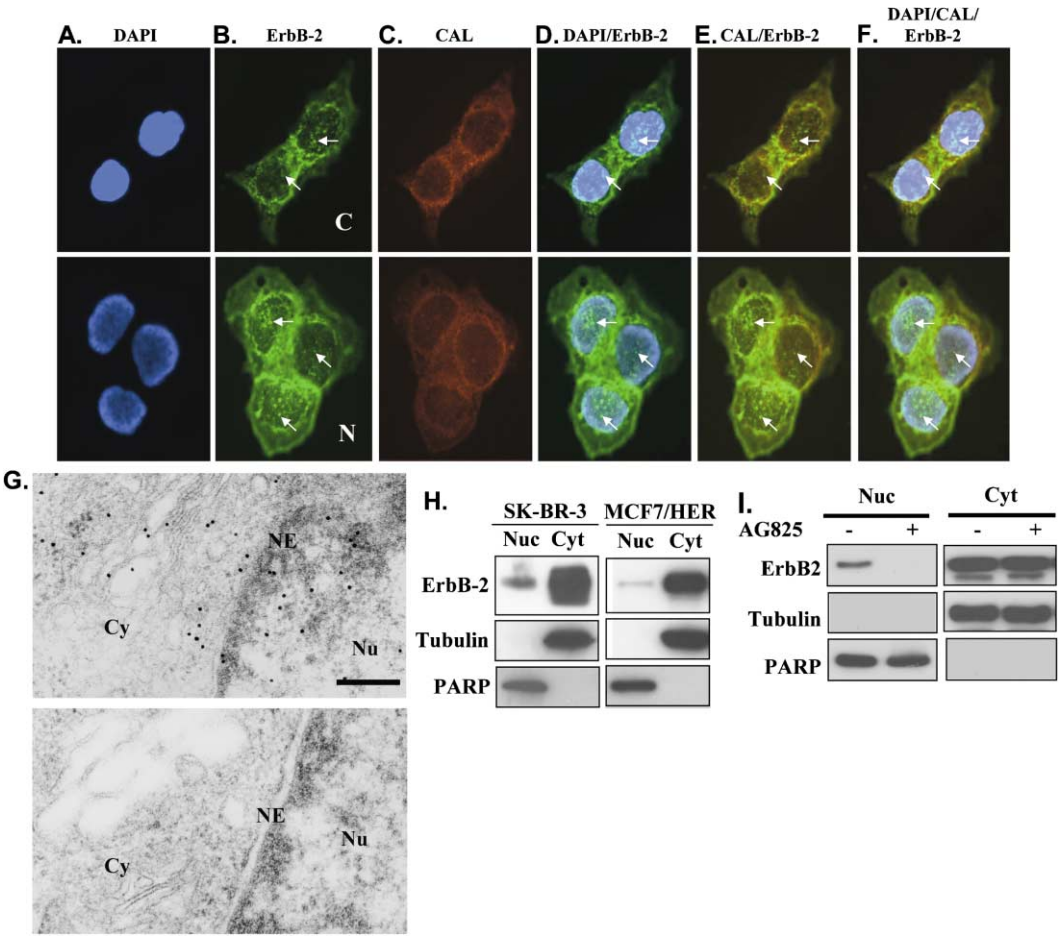

Figure 1. Nuclear expression of ErbB-2 in BT474 cells

A–C: BT474 cells were stained for nucleus with DAPI (**A**, blue), ErbB-2 using antibodies recognizing the carboxyl (C) (upper row) or amino (N) terminus (lower row) (**B**, green) of ErbB-2, and calreticulin (**C**, red). See Experimental Procedures for details of the antibodies.

D–F: Image superimpositions are indicated. Authentic ErbB-2 immunoreactive nuclear spots are shown in green (examples indicated by white arrows), and the perinuclear ErbB-2 colocalized with calreticulin is shown in yellow (**E**, primarily in the perinuclear region). The staining was visualized by immunofluorescence microscopy with a deconvolution program.

G: Detection of nuclear ErbB-2 by transmission electron microscopy (TEM). MCF-7/HER cells were processed for immuno-EM, sectioned, and labeled using monoclonal antibody to the N terminus of ErbB-2 (upper) or mouse IgG (lower) followed by anti-mouse antibody conjugated with 15 nm gold. Scale bar equals 200 nm. Cy, cytoplasm; Nu, nucleus; NE, nuclear envelope.

H: The nuclear (Nuc) and cytoplasmic (Cyt) fractions of two cell lines (SK-BR-3 and MCF-7/HER cells) were subjected to immunoblot analysis. α-tubulin and poly(ADP-ribose) polymerase (PARP) were used as the cytoplasmic and nuclear markers, respectively.

I: MCF-7/HER cells were treated with the ErbB-2-specific inhibitor AG825 (80 μM) for 6 hr. Cytoplasmic (Cyt) and nuclear (Nuc) fractions were then prepared from the cells, and the expression of ErbB-2, tubulin, and PARP was analyzed by Western blotting.

ErbB-2, which was abolished by cold HAS or anti-ErbB-2 antibodies but not by the consensus binding sequence of the transcription factor AP2 or Sp1, an antibody against CD44, or normal mouse IgG (Figure 2H). The ErbB-2 antibodies had no effect on the DNA binding activity of other transcription factors (data not shown). These results demonstrate that nuclear ErbB-2 associates with a specific genomic DNA sequence in the *COX-2* promoter and that the ChIP cloning strategy can be used to identify target DNA sequences for nuclear receptor tyrosine kinases.

Functional ErbB-2 is required for formation of ErbB-2-*COX-2* promoter complex

It is known that ErbB-2 can be downregulated by Herceptin, which has been used in clinic to target ErbB-2-overexpressing cancers by inducing ErbB-2 internalization and subsequent degradation (Klapper et al., 2000). Consistently, our results showed that Herceptin treatment triggered ErbB-2 protein depletion and mitigated the association between nuclear ErbB-2 and the *COX-2* promoter (Figure 3A).

As mentioned above (Figure 1I), the intrinsic tyrosine kinase activity of ErbB-2 is critical for its nuclear expression. Consistently, the wild-type ErbB-2 targeted the *COX-2* promoter more efficiently than did the kinase-deficient (kd) mutant, as demonstrated by stable transfectants established in the MCF-7 cell line (Figure 3B). Similar results were obtained using transient transfection, in which the wild-type ErbB-2 also bound DNA more efficiently than the kd mutant (Supplemental Figure S3A). In addition, treating cells with the ErbB-2-specific tyrosine ki-

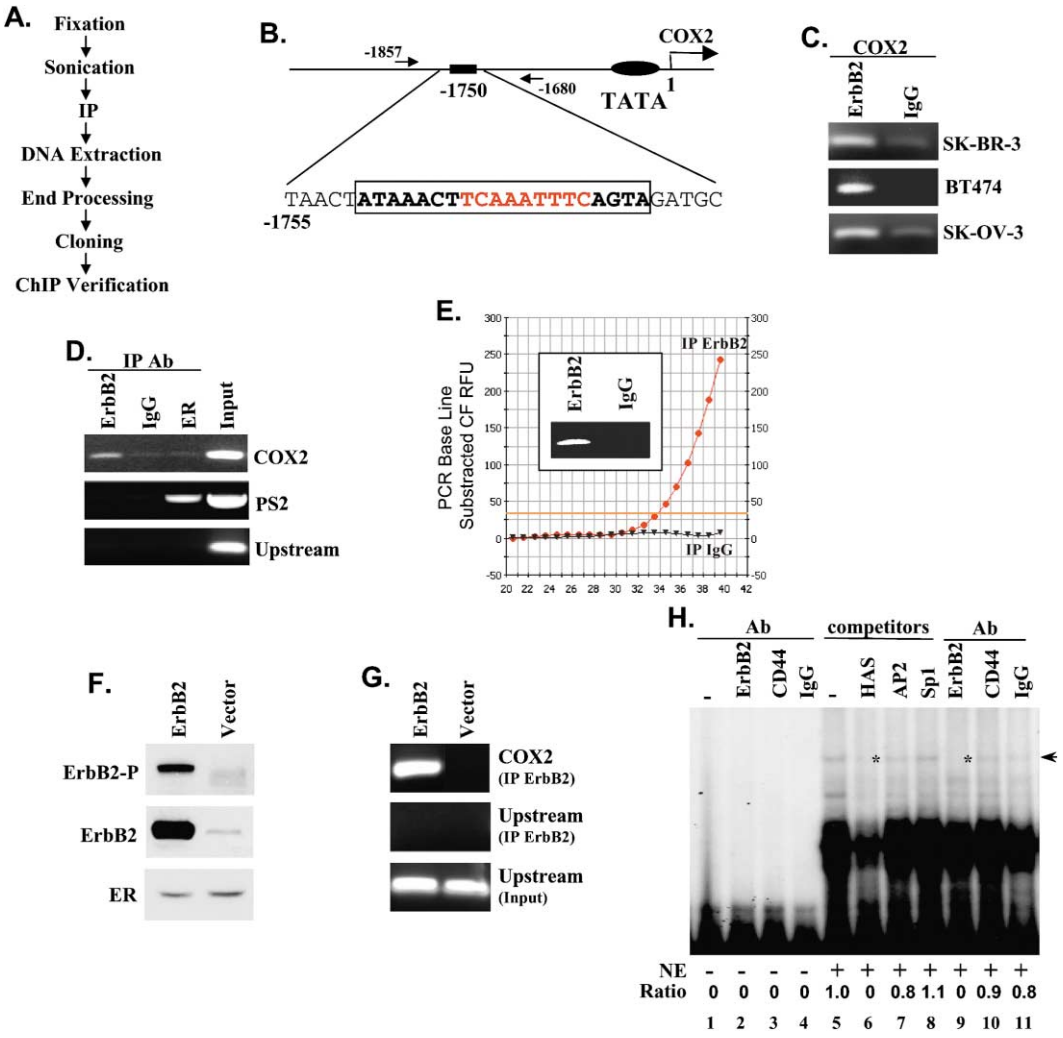

Figure 2. Nuclear ErbB-2 in association with the *COX-2* promoter

A: A simplified flow chart of the ChIP-cloning procedure. Nuclei from the fixed ErbB-2-expressing breast cancer cells BT-474 were sonicated to fragment the genomic DNA. The ErbB-2-DNA complexes were next immunoprecipitated by an anti-ErbB-2 antibody. Copurified DNA fragments were cloned and the DNA was identified by sequencing. Genomic locations of the identified DNA sequences were determined by querying the GenBank.

B: The sequence in the *COX-2* promoter associated with ErbB-2 (boxed sequence). The nucleotide sequence required for ErbB-2 association is in red (see Figure 4 for details). The PCR primers for ChIP analysis are shown by small arrows with the coordinates indicated.

C: ErbB-2-*COX-2* association was confirmed in breast (SK-BR-3, BT474) and ovarian (SK-OV-3) cancer cell lines. ChIP was performed using either monoclonal anti-ErbB-2 antibody or IgG as the negative control. The target DNA was amplified by PCR using flanking primers as diagrammed in **B**.

D: The *pS2* promoter and the distal upstream region of the *COX-2* promoter (Upstream) were examined by ChIP for the occupancy of ErbB-2 and estrogen receptor (ER) using anti-ErbB-2, anti-ER, or IgG antibody.

E: Quantitative real-time PCR was used to verify the ChIP result obtained by conventional PCR as shown in the inset.

F: Immunoblotting analysis was performed to verify the expression and phosphorylation of ErbB-2 in freshly established MCF-7 cell lines with (ErbB2) or without (Vector) ectopic ErbB-2 expression.

G: ChIP analysis using anti-ErbB-2 antibody on the same MCF-7 derivatives characterized in **F**. Either the *COX-2* promoter (COX2) or the upstream region of the *COX-2* promoter (Upstream) was tested as described in **C** and **D**. The input was also amplified using the upstream primers as a positive control (Input).

H: The radiolabeled HAS oligonucleotide (the boxed sequence in Figure 2B) was used as the probe for electrophoretic mobility shift assay (EMSA). Lanes 1–4, the labeled probe was incubated with no antibody (lane 1) or with antibodies against ErbB-2 (lane 2), CD44 (lane 3), or IgG (lane 4). Lanes 5–8, competition assay using the cold oligonucleotides as indicated. Lanes 9–11, the binding reactions containing nuclear extracts and the labeled probe was challenged by adding different antibodies. The arrow indicates the ErbB-2/oligonucleotide complex. The stars indicate the disappeared ErbB-2-oligonucleotide complex competed by the cold HAS or the ErbB-2 antibodies. Relative intensities (ratio) of the indicated bands were shown at the bottom of the gel. NE, nuclear lysate.

nase inhibitor AG825, which prevented nuclear translocation of ErbB-2 (Figure 1I), consistently inhibited the association between ErbB-2 and the target *COX-2* promoter (Supplemental Figure S3B). Thus, suppressing the tyrosine kinase activity of ErbB-2 inhibited its binding to the *COX-2* promoter. These results together indicate that formation of the ErbB-2-*COX-2* promoter complex depends on ErbB-2 expression and requires tyrosine kinase activity of ErbB-2.

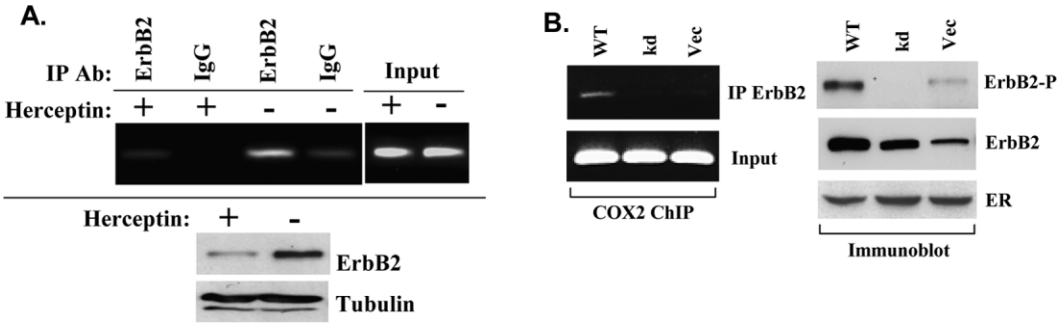

Figure 3. Formation of the ErbB2-*COX2* promoter complex required functional, kinase-competent ErbB-2 protein

A: The MCF-7 stable cell line expressing wild-type ErbB-2 was treated with or without Herceptin (20 μg/ml) for 84 hr, then processed for ChIP and Western blot analysis using ErbB-2 antibody. Tubulin expression is shown as loading control.

B: Left, MCF-7 cell stable clones of wild-type ErbB-2 (wt), the kinase-deficient mutant of ErbB-2 (kd), or the vector (Vec) were tested for the occupancy of ErbB-2 on the *COX-2* promoter by ChIP using ErbB-2 antibody; Right, immunoblot analysis for phosphorylated (ErbB2-P) and the total expression of ErbB-2 (ErbB2) and estrogen receptor (ER), which was used as an internal control.

Transactivation of the *COX-2* promoter by ErbB-2 through HAS DNA sequence

To further investigate functionality of nuclear ErbB-2, we tested whether ErbB-2 associated with transactivation activity. To this end, we found that the C-terminal domain (CD) of ErbB-2 contained an intrinsic transactivation activity, as the Gal4-CD fusion protein induced a dramatic activation function toward a reporter gene under the control of a Gal4 binding sequence in the promoter (Figure 4A). To assess the transactivation potential of ErbB-2 in vivo, a 200 bp region containing the HAS was subcloned to control a luciferase reporter system. ErbB-2 cotransfection with the HAS-luciferase receptor induced the reporter activity (Figure 4B). We reasoned that, if the HAS mediates ErbB-2-dependent gene activation, abolishing the ErbB-2-HAS interaction should therefore inhibit the activation. To test this hypothesis, we first determined whether the recruitment of ErbB-2 depends on specific nucleotide residues on the HAS. The nucleotides of the HAS were mutated in series by transversion-transition (A to C or T to G, for example) in 5-base windows. The resultant double-strand mutant oligonucleotides were then used to compete with the wild-type HAS in EMSA assays. The mutants lost their competitive activity, which would suggest that the mutated sequences are critical for ErbB-2 association. Thus, we identified a subsequence critical for ErbB-2 association, with region C being the most important, followed by the flanking regions B and D (Figure 4C; Table 1). A more detailed permutation analysis in regions B and D demonstrated that the TCAAATTTC sequence (residues marked in red in Figure 2B) was required for ErbB-2 association. Mutations within this region of the HAS abolished competivity of the oligonucleotides (Figure 4D). Further analysis by EMSA showed that the "core" oligonucleotide, which contains the TCAAATTTC sequence flanked by randomly chosen sequences (Table 1), formed a specific complex with the nuclear ErbB-2 (Figure 4E). Formation of this complex was blocked by competition with the cold HAS, the core oligonucleotide or anti-ErbB-2 antibodies, but not the AP2 binding sequence or the anti-CD44 control antibody. Consistently, biotinylated wild-type, but not the mutant, HAS oligonucleotide was able to associate with ErbB-2 protein in an oligonucleotide pull-down assay (Figure 4F).

To test whether the identified core sequence is critical for ErbB-2-mediated *COX-2* transactivation, *COX-2* full-length promoter containing the wild-type or the corresponding mutated sequence at the HAS was cotransfected with ErbB-2 into MCF-7 cells. Expression of ErbB-2 was able to induce higher reporter gene expression from the wild-type than the HAS-mutated full-length promoter (Figure 5A). Thus, the HAS on the COX-2 promoter plays a significant role in responding to the nuclear ErbB-2 transactivation function. However, the full-length promoter also contains other transcription factor binding elements that can be modulated through the traditional signal transduction pathways. To avoid the interference from the rest of the promoter sequence, the trimeric repeats of either the wild-type HAS or the corresponding mutant in the identified core sequence were subcloned into a plasmid to control a luciferase reporter. Cotransfection of the ErbB-2-expressing plasmid induced the reporter activity driven by the wild-type, but not the mutant, HAS (Figure 5B). On the other hand, the HAS-mediated reporter activity responded only to the wild-type ErbB-2 but not to the mutant ErbB-2, which can not translocate to the nucleus (Supplemental Figure S2C). Thus, these results show that ErbB-2 formed a complex with the *COX-2* promoter in the nucleus and transactivated the *COX-2* gene promoter. Consistently, ectopic expression of ErbB-2 enhanced the mRNA and protein expression from the endogenous *COX-2* gene (Figure 5C). In addition to *COX-2*, functional verification of the *PRPK* promoter, which was also identified by ChIP cloning (Supplemental Figure S2A), demonstrated that ErbB-2 was able to transactivate the full-length wild-type promoter, but not the mutant promoter in which the ErbB-2-associating DNA motif was mutated, in a kinase activity-dependent manner (Supplemental Figure S2B). This observation further supports the role of ErbB-2 as a transactivator in the nucleus.

Correlation of COX-2 expression with nuclear ErbB-2 and ErbB-2-*COX-2* promoter complex in human tumor tissues

To explore the physiological function of ErbB-2 in the nuclei of human breast tumor cells, a cohort of 30 archived primary breast cancer tissues were stained by immunohistochemistry for ErbB-2 and COX-2 expression (Figure 6A; Supplemental Figure S4A). ErbB-2 nuclear expression closely correlated with total ErbB-2

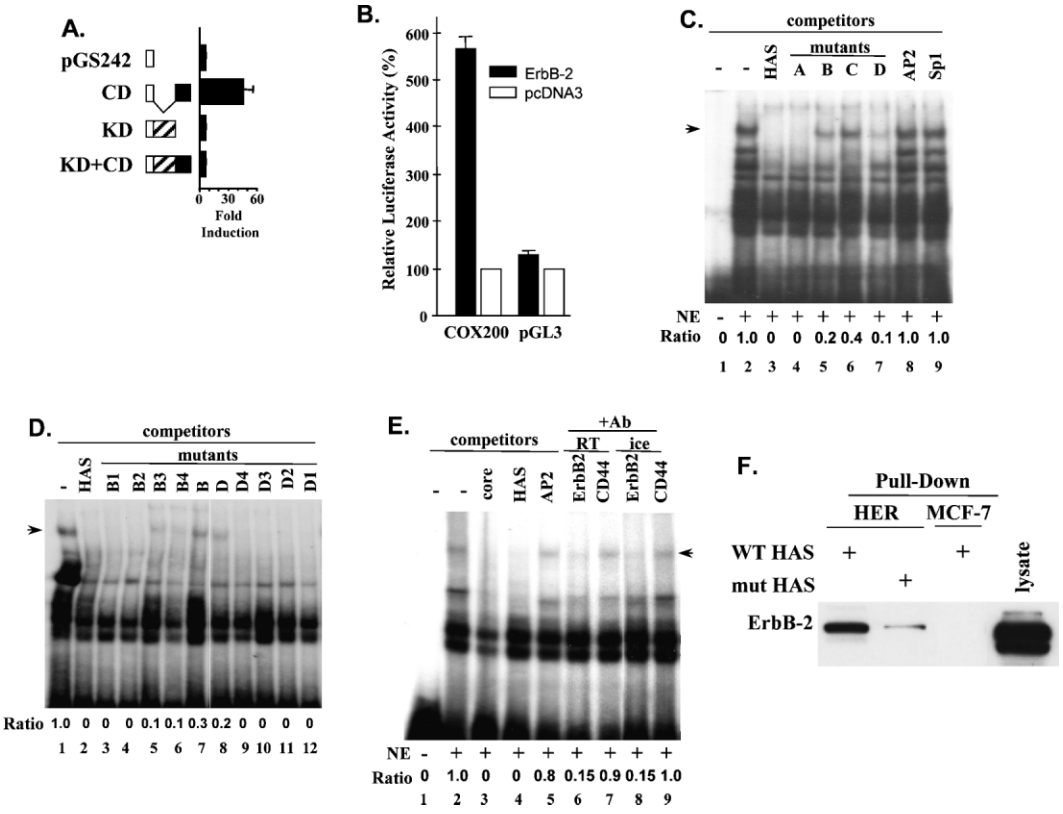

Figure 4. The ErbB2-*COX2* complex was associated with transactivation

A: NIH3T3 cells were cotransfected with 1.8 μg of the indicated Gal4 fusion constructs and 0.2 μg of the Gal4-luciferase reporter plasmid. The fold induction of luciferase activity compared with that produced by Gal4 alone was plotted. CD, C-terminal domain (filled box); KD, kinase domain (hatched box).

B: Reporter construct of the 200-bp region (COX200) surrounding the HAS was cotransfected into NIH3T3 cells with an ErbB-2-expressing plasmid (ErbB-2) or the control vector (pcDNA3). After incubation, cells were collected and luciferase activity was measured.

C: The radiolabeled wild-type HAS probe was incubated in the absence (lane 1) or presence (lanes 2–9) of nuclear lysate, and the resulting ErbB-2-HAS complex (indicated by an arrow) was challenged with cold HAS (lane 3), mutant oligonucleotides (A)–(D) (lanes 4–7), or control oligonucleotides containing AP2 (lane 8) or Sp1 (lane 9) binding motif sequence. Relative intensities (ratio) of the indicated bands were shown at the bottom of the gel. NE, nuclear extract. The sequences of mutant oligonucleotides are listed in Table 1.

D: An EMSA was performed as described in **C**. Unlabeled wild-type HAS (lane 2) or the indicated mutant oligonucleotides (lanes 3–12) were used to compete against the radiolabeled ErbB-2-HAS complex (indicated by an arrow). Relative intensities (ratio) of the indicated bands were shown at the bottom of the gel.

E: The radiolabeled ErbB-2-core complex (lane 2, indicated by an arrow) was challenged with the cold "core" (lane 3), HAS (lane 4), or AP2 (lane 5) oligonucleotides. Antibodies against ErbB-2 (lanes 6 and 8) or CD44 (lanes 7 and 9) were used to confirm the specific involvement of ErbB-2 in the protein-DNA complexes. The antibodies were incubated with the binding mixture either at room temperature (lanes 6 and 7) or on ice (lanes 8 and 9), and similar results were obtained. Relative intensities (ratio) of the indicated bands were shown at the bottom of the gel. NE, nuclear extract.

F: The "core" sequence of the HAS is required for association with ErbB-2 protein. Cellular proteins isolated from MCF-7 and MCF-7/HER cells were precipitated by biotinylated double-stranded wild-type HAS oligonucleotide or the mutant oligonucleotide in which the core TCAAATTTC motif was mutated. The resulted protein-DNA complexes were then pulled down by streptavidin-agarose beads, and the ErbB-2 protein associated with the oligonucleotide was eluted and detected using immunoblot analysis.

expression ($p = 0.008$). While total ErbB-2 expression was associated with COX-2 expression (Pearson correlation coefficient $r = 0.399$, $p = 0.029$), nuclear expression of ErbB-2 was more significantly correlated with COX-2 expression (Pearson correlation coefficient $r = 0.582$, $p = 0.001$). The status of total and nuclear ErbB-2 expression as well as COX-2 expression in the tumor tissues is summarized in Supplemental Figure S4B. To test whether ErbB-2 can be recruited to the *COX-2* promoter in primary tumor tissues, a cohort of fresh surgically excised tumors from different breast cancer patients were processed for ChIP analysis. As demonstrated by the representative result shown in Figure 6B, association of ErbB-2 with the *COX-2* promoter was detected in a ErbB-2-positive tumor but not in another ErbB-2-negative tumor, which was included as a negative control. These results demonstrate ErbB-2 nuclear expression and that the *COX-2* gene is a target of the nuclear ErbB-2 in human primary breast tumors. In addition to breast cancer, nuclear expression of ErbB-2 was also detected in other cancer types, including small bowel, esophagus, and kidney, suggesting that nuclear ErbB-2 may be a general phenomenon in multiple cancer types (Figure 6C). To further address whether the enhanced COX-2 expression by ErbB-2 is required for ErbB-2-mediated malignancy, we compared the effect of COX-2 inhibition on the invasion activity between MCF-7 and its ErbB-2 transfectants. As mentioned earlier, the ErbB-2 transfectant expressed higher level of COX-2 than the MCF-7 parental cells

Table 1. Sequences of the oligonucleotides used in the EMSA experiments

Oligonucleotides	Nucleotide sequences	Competitiveness[a]
HAS	aactATAAACT*TCAAATTTC*AGTAgat	+
Mutant A	aactCGCCCCTTCAAATTTCAGTAgat	+
Mutant B	aactATAAAAGGACAATTTCAGTAgat	−
Mutant C	aactATAAACTTCACCGGGCAGTAgat	−
Mutant D	aactATAAACTTCAAATTTACTGCgat	−
Mutant B1	aactATAAAATTCAAATTTCAGTAgat	+
Mutant B2	aactATAAAAGTCAAATTTCAGTAgat	+
Mutant B3	aactATAAAAGGCAAATTTCAGTAgat	−
Mutant B4	aactATAAAAGGAAAATTTCAGTAgat	−
Mutant D1	aactATAAACTTCAAATTTCAGTCgat	+
Mutant D2	aactATAAACTTCAAATTTCAGGCgat	+
Mutant D3	aactATAAACTTCAAATTTCATGCgat	+
Mutant D4	aactATAAACTTCAAATTTCCTGCgat	+
core	gcctgccgagTTCAAATTTCAcccgat	+

The corresponding *COX-2* promoter sequence complexed with ErbB-2 (as the sequence boxed in Figure 2B) is in capital letters. The mutated nucleotides are underlined. The identified essential residues for ErbB-2 complex formation are in italics.

[a]The compatibility was defined empirically by the EMSA competition assay as shown in Figures 4C–4E, in which the particular cold oligonucleotide was scored "+" if it was able to diminish the ErbB-2-HAS complex. Thus, the mutants B2/B3 and D/D4 defined, respectively, the 5′ and 3′ boundaries of the HAS sequence required for ErbB-2-HAS complex formation.

(Figure 5C). Treatment by two classic COX-2-specific inhibitors in clinic, celecoxib (Celebrex) and rofecoxib (Vioxx) (Flower, 2003), preferentially suppressed the invasion activity of the ErbB-2-expressing cells (Figure 6D). The decreased invasion of the ErbB-2-expressing cells was not due to preferential cell growth inhibition of the ErbB-2-expressing cells, as the inhibitors had similar effect on both cell lines (Supplemental Figure S5). Similar results were observed when the breast cancer cell line MDA-MB-435 and its ErbB-2 transfectant were tested (data not shown). Thus, these results indicate that the ErbB-2-induced COX-2 expression is critical for its malignancy.

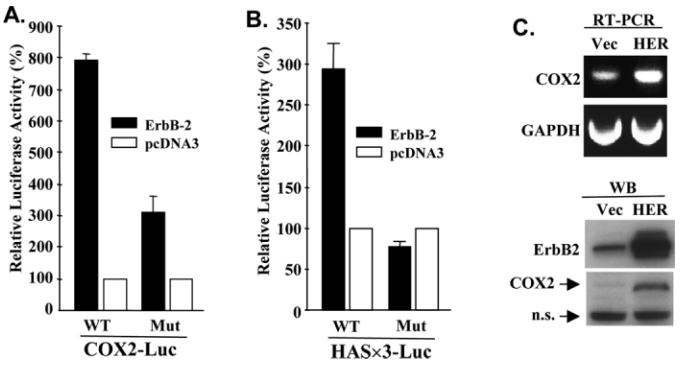

Figure 5. ErbB-2 transactivated the COX-2 promoter through the HAS motif

A: Full-length *COX-2* promoter containing the wild-type (wt) or mutated (mut) HAS was cotransfected with *ErbB-2* cDNA or the vector control (pcDNA3) into MCF-7 cells, and the relative luciferase reporter activity was measured.

B: A luciferase reporter controlled by the wild-type (wt) or mutant (Mut) HAS as trimeric repeat was cotransfected into MCF-7 cells with an *ErbB-2*-expressing plasmid or the vector pcDNA3, and the relative luciferase activity was determined.

C: RNA and protein expression of the *COX-2* gene was determined for the freshly established stable clone of the MCF-7 cells expressing ErbB-2 (HER) or the vector control (Vec). RT-PCR, reverse transcriptase-polymerase chain reaction; WB, Western blotting; n.s., nonspecific bands. The same cell lines were also used in a functional assay as described in Figure 6D.

Discussion

Nuclear expression of other human EGFR family proteins, namely ErbB-1 (Lin et al., 2001), ErbB-3 (Offterdinger et al., 2002), and ErbB-4 (Ni et al., 2001), was reported recently. The identification of the ErbB-2 protein in the nucleus as described in this report makes nuclear expression a phenomenon common to all EGFR family members. However, the EGFR family proteins appear to adopt different mechanisms for nuclear translocation. Although EGFR and ErbB-3 translocate to the nucleus in full length, ErbB-4 is first processed by membrane proteinases and the resulting intracellular fragment is then transported to the nucleus. In the case of ErbB-2, like EGFR and ErbB-3, the membrane form of the protein seems to be identical to its nuclear form, as judged by the different antibodies against epitopes at the amino- and carboxy-terminal regions in immunostaining, as well as the Western blot analysis that shows that the nuclear ErbB-2 migrates at the same position as does the total ErbB-2 (Figure 1; Supplemental Figure S1). These results indicate that ErbB-2 translocates to the nucleus in its full-length form. However, the possibility that the same receptor may use different mechanisms for nuclear translocation under different conditions should not be excluded.

By using an approach combining molecular cloning and bioinformatic analysis, we demonstrate that the nuclear ErbB-2 targets several gene promoters, including the *COX-2* gene promoter, to mediate gene expression. The interaction between ErbB-2 and the chromatin provides functional relevance for ErbB-2 nuclear expression. However, since ErbB-2 lacks any known structural signatures required for DNA association, it is likely that ErbB-2, and possibly other EGFR family members, does not directly interact with genomic DNA. Rather, they may associate with other DNA binding nuclear factors. In this regard, it has been shown previously that Schwannoma-derived growth factor (SDGF), a rodent ligand for EGFR, can directly bind to AT-rich DNA sequences (Kimura, 1993), which are similar to the sequences associated with nuclear EGFR (Lin et al., 2001). In addition, it is worth mentioning that the association of EGFR

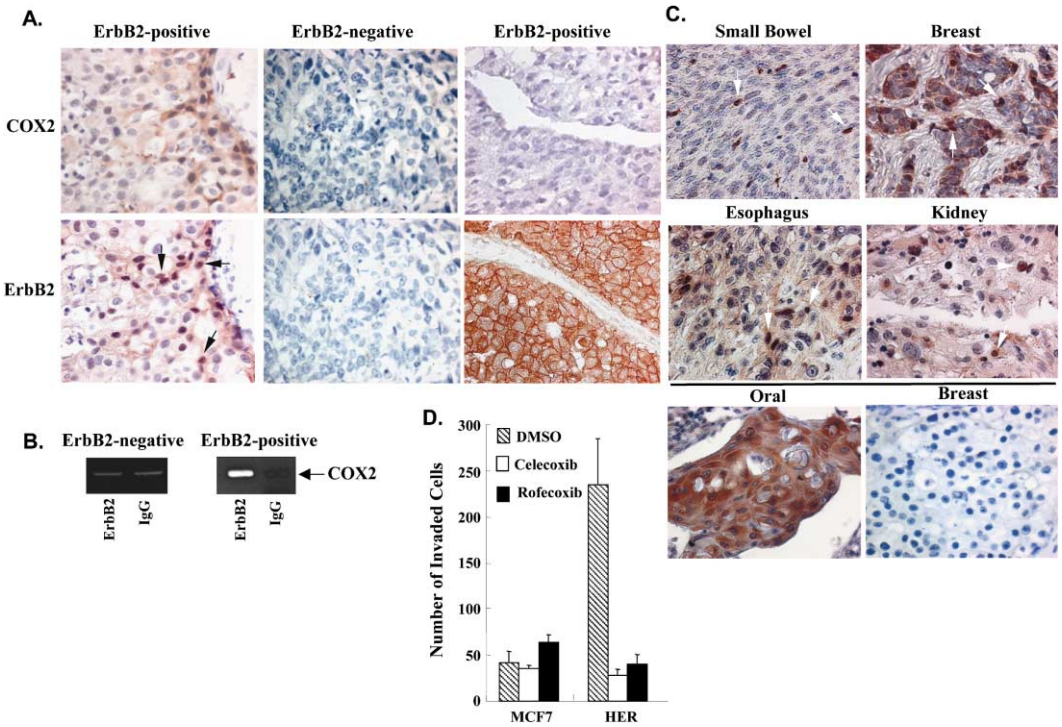

Figure 6. ErbB-2 associated with COX-2 expression in primary human breast tumors

A: 30 archived primary breast cancer tissues were analyzed by immunohistochemistry staining using antibodies against ErbB-2 and COX-2 as described in the Experimental Procedures. In addition to membrane-cytoplasm localization, ErbB-2 expression was also observed in the nucleus in certain areas (examples indicated by arrows). Statistic analysis confirmed that the nuclear expression of ErbB-2 is significantly correlated with COX-2 expression in the neighboring tumor tissue sections (see text).

B: ChIP analysis was done for fresh surgically excised tumor samples from breast cancer patients. The DNA-protein complex was pulled down by an anti-ErbB-2 monoclonal antibody (Ab-5, Oncogene) or IgG, and the resulting DNA was PCR amplified by the primers on the *COX-2* promoter as described in Figure 2. Representative results are shown.

C: Tissue sections derived from different tumor types, including small bowel, breast, esophagus, kidney, and oral tumors were stained with anti-ErbB-2 antibody as described in the Experimental Procedures. White arrows indicate examples of nuclear ErbB-2 expression. Bottom: the oral cancer tissue, although it expressed high level of ErbB-2 in the cytoplasm, had a lack of nuclear ErbB-2 expression. Another breast cancer tumor tissue was also shown at the bottom as an ErbB-2-negative example.

D: COX-2 inhibition suppressed invasion activity in ErbB2-expressing breast cancer cells. The enhanced invasion activity of ErbB-2-expressing cancer cells (HER) was blocked by specific COX-2 inhibitor, celecoxib (50 μM) and rofecoxib (100 μM), when compared with the parental cells (MCF-7). The invasion chambers were incubated for 45 hr and the filter was fixed and then stained with DAPI. The number of invaded cells was determined by counting multiple areas (>3) under a fluorescence microscope at 100× amplification power.

with a histone 3 isoform has been reported recently (Blagoev et al., 2003). One can therefore envision that the tyrosine kinase receptors in the nucleus may have a more global genomic function. In line with this prospect, nuclear ErbB-2 forms punctate subnuclear structure, which partially overlaps with the promyelocytic leukaemia (PML) protein (Supplemental Figure S6). PML, either in the form of matrix-associated discrete nuclear speckles or in the form of soluble nonmatrix distribution, has been linked to a wide range of nuclear functions including transcriptional regulation (Zhong et al., 2000). Whether and how the nuclear tyrosine kinase receptors are functionally involved in chromatic modulating and gene regulation becomes a fundamental biological question which should be further explored.

The cyclooxygenase enzyme COX-2 is one of the major contributors in tumor development. Increased expression of COX-2 occurs in multiple cells within the tumor microenvironment and has an impact on angiogenesis, invasiveness, and antiapoptotic potential (Gately, 2000; Takahashi et al., 1999; Tsujii et al., 1997). Hence, targeting COX-2 activity and expression is becoming an important issue in cancer chemoprevention and therapy (Howe et al., 2001; Turini and DuBois, 2002). In breast cancer, high level of COX-2 expression has a tight correlation with ErbB-2 expression (Ristimäki et al., 2002; Subbaramaiah et al., 2002), and the current study described here provides an unexpected mechanism in which the nuclear ErbB-2 protein activates the *COX-2* promoter in breast cancer tissues as well as in cultured cancer cells. Besides the tumor types mentioned above, we analyzed a gastric cancer cDNA microarray data set deposited online by others and found that significant correlation between *ErbB-2* and *COX-2* expression can also be extended to gastric cancer (Supplemental Figure S7; Chen et al., 2003). Interestingly, the analysis showed that the *ErbB-2-COX-2* correlation becomes more significant in metastatic tumor tissues, suggesting that the interaction between the ErbB-2 and COX-2 pathways is correlated with the advance of tumor development in certain cancer types. It remains to be tested whether nuclear ErbB-2 preferentially occurs in metastatic tumor tissues in gastric and other types of cancer. In addition to *COX-2*, several

different promoter sequences with clustered HAS-like motifs were identified by searching a promoter database using a HAS-derived DNA sequence matrix. Among these potential ErbB-2 targets, two genes, namely the *Fanconi anemia complementation group C* (*FANCC*) gene and the nuclear receptor *NR4A3* gene, were verified by ChIP analysis (Supplemental Figure S8A). ErbB-2 expression resulted in transactivation of the *FANCC* promoter (Supplemental Figure S8B). The FANCC protein is critically involved in the repair of DNA damage generated from oxidative stress, and expression of FANCC prevents apoptosis (Ahmad et al., 2002; Pagano and Youssoufian, 2003). Thus, when combined with appropriate bioinformatic programs and databases, the described ChIP-cloning approach was also able to identify multiple nuclear targets sharing similar binding motifs.

The function of tyrosine kinase receptors as signaling molecules on the cell membrane has been well documented to play a major role in fundamental cell biology in the last decades. Despite the fact that many tyrosine kinase receptors and their cognate growth factors were found to be located in the nucleus, the functionality of tyrosine kinase receptors in nucleus is virtually unknown. This is partly due to the lack of an efficient strategy to identify their nuclear targets. In the current study, we developed a cloning strategy involving ChIP to identify specific DNA sequences as nuclear targets for the nuclear ErbB-2 in vivo. The strategy used in the current study is simpler and more physiologically relevant than the casting method that we previously used to identify DNA sequences for EGFR nuclear targets (Lin et al., 2001). Thus, it may serve as a general approach to identify nuclear targets for other tyrosine kinase receptors that were detected in the nucleus and to facilitate understanding of the functions of nuclear tyrosine kinase receptors that have been overlooked for decades.

Experimental procedures

Cell culture, antibodies, and tissue slide

All cell lines were maintained in DMEM/F12 with 10% fetal bovine serum. The following antibodies were purchased: ErbB-2 (Oncogene, San Diego, CA; DAKO); CD44, calnexin, and PML (Santa Cruz); COX-2 (Oxford Biochemical); phosphotyrosine (Upstate); PARP (BD Biosciences); and α-tubulin (Sigma). The tissue slide including multiple types of human tumors was purchased from Imgenex.

Plasmids and reporter assay

The cytoplasmic region (amino acids 727–1234), kinase domain (amino acids 727–986), and C-terminal region (amino acids 987–1234) of ErbB-2 (Yamamoto et al., 1986) were amplified by PCR and cloned in-frame into the pSG424 vector. The constructs were cotransfected with the pG5-TK-Luc reporter plasmid to assess the transactivation activity of the fusion proteins. The ErbB-2-associated DNA fragments were cloned into the pGL3-promoter vector and transfected along with the pRL-TK reporter plasmid (Promega). Luciferase assays were carried out using the Dual-Luciferase assay kit (Promega). The pG5-TK-Luc and pSG424 plasmids were generous gifts from Dr. Marilyn Szentirmay. The wild-type full-length *COX-2* promoter-luciferase construct was provided by Dr. Miguel Angel Iñiguez.

Cellular fractionation

The cytoplasmic and nuclear fractions were prepared as described (Maher, 1996), except that the nuclei were lysed in RIPA buffer (150 mM NaCl, 20 mM Na_2 HPO_4, 1% TritonX-100, 100 mM NaF, 2 mM Na_3VO_4, 5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.5 mg/ml benzamidine) by sonication (Sonics Vibra Cell, amplitude 20). The fractionation efficiency was assessed by antibodies against α-tubulin and PARP.

Immunofluorescence

Cultured cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Tween-20 for 15 min, and then immunostained with primary antibodies (1:200 dilution in PBS with 1% BSA) for 1 hr at 37°C. For ErbB-2, the primary antibodies used were Ab-3 (Oncogene; targeting the carboxy-terminal region) and Ab-5 (Oncogene; targeting the extracellular region). After three washes with PBS, the FITC-conjugated secondary antibody was then applied for 45 min at room temperature. The nucleus was stained with DAPI (fluorescence) or TOPRO 3 (confocal) before mounting. For immunofluorescence microscopy, the images were captured with a Zeiss AxioPlan2 fluorescence microscope with a digital camera. The confocal microscopy was performed by a Zeiss CLM510 laser microscope.

Chromatin immunoprecipitation

Cells were fixed with 1% formaldehyde for 10 min, washed, and lysed in cell lysis buffer (5 mM HEPES [pH 8.0], 85 mM KCl, 0.5% NP-40) at 4°C for 30 min. The nuclei were released by a Dounce homogenizer and then lysed in 100–200 μl of nuclei lysis buffer (Tris-HCl 50 mM [pH 8.0], EDTA 10 mM, SDS 1%). The lysate was sonicated on ice, and the supernatant was diluted 10-fold with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 6.8], 167 mM NaCl). 1 μg of antibody was added to 0.5–1 ml of the lysate and rotated at 4° for overnight. The immunocomplexes were then pulled down by protein G-conjugated magnetic Dynabeads (Dynal Biotech). The beads were washed with wash buffer (0.1 M sodium phosphate buffer [pH 6.8], 0.1% Tween-20) for 4 times, and the bound protein was eluted twice with 30 μl 0.1 M citrate (pH 3.0). Then, 240 μl extraction buffer (0.1% SDS, 50 mM $NaHCO_3$, 5 μl of 10 mg/ml RNase, 18 μl of 5 M NaCl) was added to the pooled elutent and incubated at 65°C overnight. The reverted DNA was purified with a miniprep spin column (Qiagen) and then eluted in 50 μl of 10 mM Tris-HCl (pH 8.0). The COX-2 promoter region was amplified by conventional PCR (with primers CTTCAAAATAAGCTTGAATT CAGGATTGTAATG and CTTTTTGATAATTTAATAATTTCAATCTTCTGTTTC) or a real-time PCR (with primers CAATAAATAGGAGTGCCATAAATG and ACGGATGACTAAAATTCCATCT and the probe 5′-FAM-AAGCCTTTCTCC TCCTCTAGTCAT-BHQ-3′). The PCR program was: 95°C for 15 min, followed by 30 to 35 cycles at 95°C for 30 s, 53.5°C for 1 min, and 72°C for 1 min. For real-time PCR, the program was 95°C for 3.5 min, followed by 40 cycles of 95°C for 15 s and 50°C for 30 s. The real-time PCR was performed using the iCycler system (Bio-Rad).

Cloning of the ChIP fragments

The immunoprecipitated DNA was processed and cloned as described previously (Weinmann et al., 2001). Briefly, the DNA isolated from ChIP was heated at 68°C for 5 min then cooled to 37°C. One to two units of T4 DNA polymerase was added to the DNA and reaction mix containing the repair buffer (18 mM ammonium sulfate, 66 mM Tris [pH 8.0], 6.6 mM $MgCl_2$, 50 mM β-mercaptoethanol, and 0.5 mM of each nucleotide) and incubated at 37°C for 15 min. The reaction was terminated by 1 μl of 0.5 M EDTA for a 50 μl reaction mix. The processed DNA was cloned into pBluescript vector, which was linearized by Eco321 (Fermentas) and dephosphorylated by calf intestine phosphatase.

Electrophoretic mobility shift assay

Double-stranded oligonucleotides (Genosys/Sigma-Aldrich) were end labeled by α-^{32}P using T4 polynucleotide kinase (Promega) and then purified by phenol:chloroform:isoamyl ethanol (25:24:1) extraction. 10 μg of nuclear extracts were incubated with 5 ng radiolabeled probes in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 4% glycerol, 0.5 mM EDTA, 0.5 mM DDT, and 1.25 μg poly(dI-dC).poly(dI-dC) (Amershan Pharmacia Biotech). After 15 min at room temperature, the reaction was terminated by adding 10× gel loading buffer (250 mM Tris-HCl [pH 7.5], 40% glycerol) and separated by electrophoresis at 4°C in a 5% nondenaturing polyacrylamide gel (acrylamide:bis-acrylamide, 50:1) containing 0.5× TBE and 2.5% glycerol. In the competition experiments, nuclear extracts were preincubated with a 200-fold excess of a cold competitive oligonucleotide for 15 min before ^{32}P-labeled probes were added. To verify the involvement of ErbB-2 in the complex, 0.5–2.0 μg of antibodies against ErbB-2 (a cocktail of Ab-3 and Ab-5 from Oncogene and Ab-11 from NeoMarker) or equal amount of anti-CD44 antibody (Santa Cruz) or mouse IgG was added to the mixture containing the nuclear extracts

and radiolabeled probes and incubated at room temperature or on ice for an additional 10 min.

Oligonucleotide pull-down assay

Precipitation of biotinylated oligonucleotide-associated proteins was performed as described previously (Hata et al., 2000). Double-stranded oligonucleotide derived from the wild-type (5′-ATAAACTTCAAATTTCAGTA-3′) and the mutant (5′-ATAAACTGACCCGGGAAGTA-3′) HAS were used. The underlines indicate the 9-nucleotide difference between the wild-type and mutant oligonucleotides.

Immunohistochemistry

ErbB-2 and COX-2 were detected by the immunoperoxidase staining method. Slides with fixed paraffin-embedded sections (5 μm) were deparaffinized in serial grades of xylene followed by rehydration in sequentially increasing dilutions of ethanol following the standard procedure. Antigen retrieval was then performed using microwave heating in 10 M sodium citrate buffer (pH 6.0) for 8 min (1000 W for 2 min, 200 W for 6 min), cool down at room temperature for 30 min, then washed twice by PBS. Slides were then trypsinized by 0.05% trypsin (GIBCO-BRL) in PBS at room temperature for 15 min, then washed three times with PBS, 5 min each time. The endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol for 15 min at room temperature, followed by three 5 min washes in PBS. The slides were then blocked with rabbit serum (10% in PBS) in a humid chamber at room temperature for 30 min. The serum was wiped out from around the sections, and primary antibody, either the rabbit polyclonal anti-COX-2 (Oxford Biochemical, Cat. Number PG27B; 1:200) or the rabbit polyclonal anti-ErbB-2 (DAKO, Cat. Number A0485; 1:300), was applied and incubated at 4° for overnight and then washed three times with PBS followed by incubation with biotinylated secondary antibody (1:200) at room temperature for 1 hr. After three washes with PBS, the avidin-biotin-horseradish peroxidase complex (1:100; Vector Laboratories) was applied and incubated at room temperature for 1 hr in a humid chamber followed by three washes with PBS. Antibody detection was performed with the AEC chromogen substrate kit (Vector Laboratories) for 5–10 min and then washed with several changes of distilled water. The slides were counterstained with Mayer's hematoxylin and mounted in aqueous mountant (Lerner Laboratories).

Transmission electron microscopy

The cells were scraped, washed with filtered PBS, syringed to get single-cell suspension, spun down, and fixed in 0.4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 hr at room temperature. After washing three times with PBS, cells were osmicated in 2% osmium tetroxide in PBS and dehydrated in ascending series of ethanol and sequentially infiltrated with araldite:ethanol (1:1) for 2 hr, araldite:ethanol (4:1) overnight, araldite for 45 min at 45°C, araldite for 45 min at 55°C, and finally araldite to a depth of 1.5–2 mm before polymerization at 60°C for 24 hr. Ultrathin sections of cells were prepared with ultramicrotome (Reichert E), mounted on the copper grids, and treated with sodium metaperiodate for 30 min followed by 5% normal goat serum treatment for 30 min, and then presaturated with mouse immunoglobulin before staining with the primary antibody against ErbB-2 (Ab-5, Oncogene). They were then incubated for 45 min with 15 nm gold particle-conjugated goat anti-mouse secondary antibody (Amersham Biosciences) at 1:20 dilution. Sections were washed and stained with uranyl acetate for 2 min and Reynolds's lead citrate for 1 min before examination in a Jeol 1200EX microscope.

Statistical analysis

Statistic analyses were carried out using the SPSS 10.0 program for Windows. The Pearson correlation coefficient (r) was used to compare the correlation between ErbB-2 and COX-2 immunostaining in tumor tissues. Two-sided t test was used to evaluate the statistical significance. A p value <0.05 was set as the criteria for statistical significance.

Invasion assay

The ability of cell invasion was examined as described previously (Tan et al., 1997). In brief, 6×10^4 cells were plated in 24-well invasion chambers (BioCoat Transwell; Becton Dickinson Labware) coated with diluted matrigel (1:70). The invasion was carried out at 37°C for 45 hr. After incubation, the filter was fixed with 3% glutaraldehyde in PBS and then stained with DAPI. The cell number on the filter was scored under a fluorescence microscope with the aid of an image processing software (alphaEaseFC; α Innotech).

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