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Regulation of *Her2/neu* Promoter Activity by the ETS Transcription Factor, ER81

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Abstract Overexpression of the HER2/Neu receptor is correlated to a poor prognosis in tumor patients and leads to stimulation of mitogen-activated protein kinase (MAPK) signaling pathways, which in turn activate transcription factors, such as the ETS protein ER81. Here, we have analyzed whether, on the other hand, ER81 may regulate the *Her2/neu* gene. Indeed, ER81, together with its co-activators, p300 and CBP, activates the *Her2/neu* promoter, and this activation is enhanced upon stimulation of MAPK pathways as well as by oncogenic HER2/Neu protein. Furthermore, ER81 interacts with one ETS binding site in the *Her2/neu* promoter, whose mutation decreases ER81-mediated transcription. Activation of the *Her2/neu* promoter is also diminished upon mutation of MAPK-dependent phosphorylation sites in ER81 or upon deletion of ER81 transactivation domains. In addition, the ER81 DNA-binding domain on its own functions as a dominant-negative molecule, effectively repressing any stimulation of the *Her2/neu* promoter. Altogether, our results show that ER81 is a component of a positive regulatory feedback loop, in which the HER2/Neu protein activates ER81, as well as p300/CBP via MAPKs causing the upregulation of the *Her2/neu* gene. J. Cell. Biochem. 86: 174–183, 2002. © 2002 Wiley-Liss, Inc.

Key words: cancer; CBP; ER81; HER2/Neu; MAP kinase; p300; transcription

The HER2/Neu protein, also called ErbB2, is a transmembrane receptor tyrosine kinase that is closely related to the epidermal growth factor receptor, EGFR/ErbB1. No ligand for HER2/Neu has hitherto been discovered, however, it heterodimerizes with EGFR/ErbB1 and the neuregulin receptors, ErbB3 and ErbB4, and serves the function of a co-receptor. Overexpression of HER2/Neu, due to gene amplification and/or enhanced gene transcription, has been observed in breast, ovarian, lung, and gastric tumors and correlates with an increased metastatic potential of cancer cells. Furthermore, overexpression of HER2/Neu in mouse breast tissue results in the development of breast tumors, clearly indicating the oncogenic potential of HER2/Neu [Hynes and Stern, 1994; Hung and Lau, 1999; Olayioye et al., 2000]. Therefore, various strategies to antagonize HER2/Neu overexpression are presently pursued in cancer treatment, including inactivation of HER2/Neu by the Herceptin antibody or by specific tyrosine kinase inhibitors, as well as transcriptional repression of the *Her2/neu* gene [Menard et al., 2000; Yu and Hung, 2000].

Analysis of the human *Her2/neu* gene promoter has revealed various DNA-binding sites for transcription factors. For instance, Sp1, the family of AP-2 proteins and RBPJκ, all bind to the *Her2/neu* promoter and can stimulate its activity [Hollywood and Hurst, 1993; Chen and Gill, 1994; Bosher et al., 1995, 1996; Chen et al., 1997]. In addition, a DNaseI hypersensitive site in the *Her2/neu* promoter encompasses a potential binding site for ETS proteins [Scott et al., 1994], a family of transcription factors characterized by an 85-amino acid long

Abbreviations used: GST, glutathione *S*-transferase; HA, hemagglutinin; MAPK, mitogen-activated protein.

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conserved DNA-binding domain [Graves and Petersen, 1998]. Three ETS proteins, PEA3, ESX, and Elf-1, have subsequently been shown to bind to this site, and thereby, activate the *Her2/neu* promoter [Benz et al., 1997; Chang et al., 1997a; Scott et al., 2000].

Another ETS protein, the transcription factor ER81 [Brown and McKnight, 1992], is expressed in human breast tumor specimens and has been shown to be overexpressed in a subset of breast tumor cell lines, as well as in *Her2/neu*-induced mouse mammary tumors [Baert et al., 1997; Bosc et al., 2001; Shepherd et al., 2001]. Furthermore, ER81 is activated by HER2/Neu via mitogen-activated protein kinases (MAPKs), and the *Her2/neu* gene itself is inducible by an activated form of the HER2/Neu protein [Benz et al., 1997; Bosc et al., 2001], prompting us to investigate whether ER81 regulates the *Her2/neu* promoter.

MATERIALS AND METHODS

Transfection of Cells

RK13 cells were seeded in 6-cm dishes and grown to 30% confluency. Then, the cells were transiently transfected by the calcium phosphate co-precipitation method. Briefly, 1 μg of luciferase reporter plasmid, 0.2 μg of the β-galactosidase expression plasmid pEQ176, and indicated mammalian expression vectors were cotransfected. To achieve a total amount of 9 μg of DNA per transfection, the carrier pBluescript KS^+ (Stratagene) was employed. The following amounts of expression vectors were utilized: 0.6 μg of full-length 6Myc-$ER81_{2-477}$ [Papoutsopoulou and Janknecht, 2000], truncations, thereof, or the respective empty vector $pCS3^+$-6Myc, 0.1 μg of Gal4 fusion proteins [Janknecht and Nordheim, 1996], 1.5 μg of HER2/Neu-V664E, 1.5 μg of BXB [Bruder et al., 1992], 3 μg of MKK7a [Holland et al., 1997], 0.5 μg of MEK6(DD) [Stein et al., 1997], 5 μg of p300-HA [Eckner et al., 1994], and 20 ng of 12S E1A [Liu et al., 2000]. Ten hours after addition of the precipitate, cells were washed twice with 2-ml phosphate-buffered saline, and then grown for another 36 h, after which the cells were lysed and luciferase activity measured [Pearson et al., 1999].

Electrophoretic Mobility Shift Assay (EMSA)

$ER81_{249-477}$ (0.01–0.1 μl), which was obtained by employing the IMPACT-CN system (New England Biolabs) [Bosc et al., 2001], was incubated with ^{32}P-labeled oligonucleotides in 10 μl of 20 mM HEPES, pH 7.4, 25 mM NaCl, 2 mM DTT, 0.5 mM EDTA, 12% glycerol, 0.01% Tween-20, 0.1 μg/μl bovine serum albumin, 0.05 μg/μl poly(dI-dC)*poly(dI-dC) (sonicated to an average length of $\sim$500 bp). Where indicated, competitor oligonucleotides or 0.5 μl of α-ER81 antibody (ETV1-C-20, Santa Cruz Biotechnology) were included. After 1 h on ice, the samples were loaded onto a 4.5% polyacrylamide gel and electrophoresed for 4 h at 100 mV in 0.5 × TBE at 4°C. Gels were dried and then exposed to film.

To obtain double-stranded, ^{32}P-labeled oligonucleotides, the following respective sense and antisense oligonucleotides (ETS core sequence is in bold type) were hybridized before filling-in overhanging ends with Klenow enzyme in the presence of α-^{32}P-dATP:

−116 (sense): 5′-CTCCCAGACTTGTT**GGAA**TGCAGTTGG-3′
−116 (antisense): 5′-CTCCAACTGCA**TTCC**AACAAGTCTGGG-3′
−31 (sense): 5′-AGGGCTGCTTGA**GGAA**GTATAAGA-3′
−31 (antisense): 5′-CATTCTTATAC**TTCC**TCAAGCAGCCCT-3′
E74 (sense): 5′-AGCTTCTCTAGCTGAATAACC**GGAA**GTAACTCATCG-3′
E74 (antisense): 5′-TCGACGATGAGTTAC**TTCC**GGTTATTCAGCTAGAGA-3′

Kinase Assay

RK13 cells were transfected with expression vectors for hemagglutinin (HA)-tagged MAPK (200 ng in case of ERK1, 200 ng in case of JNK1, and 50 ng in case of p38-2). Where indicated, BXB, MKK7a, or MEK6(DD) were cotransfected as described above. Thirty-six hours after removal of the calcium phosphate–DNA coprecipitate, cells were washed with 2 ml phosphate-buffered saline and then lyzed at 4°C for 5 min with 600 μl of 10 mM Tris/30 mM $Na_4P_2O_7$ pH 7.1, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 0.5 mM Na_3VO_4, 0.2 mM DTT, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM PMSF, 2.5 mM sodium β-glycerophosphate. The lysate was scraped off the dish, transferred to a 1.5-ml tube, vortexed for 15 s, and then tumbled for 45 min at 4°C. After centrifugation (20,800*g*, 10 min), the

supernatant was pre-cleared with 30 μl of protein A agarose beads (Repligen). After 45 min, the beads were pelleted by centrifugation (20,800*g*, 10 min) and the supernatant incubated with 0.5 μl of α-HA mouse monoclonal antibody (12CA5). Twenty-five microliters of protein A agarose bead slurry were added after 2 h followed by another hour of incubation with continuous tumbling. Beads were recovered by centrifugation (960*g*, 1 min), washed three times with 0.5 ml lysis-buffer, and two times with 0.5-ml kinase-buffer (20 mM HEPES pH 7.4, 25 mM NaCl, 10 mM $MgCl_2$, 10 mM sodium β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM DTT, 0.2 mM PMSF, 10 μM ATP). After resuspension in 40 μl of kinase-buffer, 7.5 μl of this slurry were incubated with 1 μg of glutathione *S*-transferase (GST) fusion protein and 2 μCi of γ-^{32}P-ATP (3,000 Ci/mmol) in a total volume of 15 μl for 20 min at 30°C. Samples were then mixed with 15 μl of 2 × Laemmli sample buffer, boiled for 5 min, and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Gels were dried and respective autoradiograms obtained.

RESULTS

Activation of the *Her2/neu* Promoter by Oncogenic HER2/Neu Protein

Previously, it has been reported that the *Her2/neu* gene is induced upon overexpression of an activated form of the HER2/Neu protein [Benz et al., 1997]. In order to map the region of the *Her2/neu* promoter that is responsive to overexpression of the HER2/Neu protein, we expressed an oncogenic version of HER2/Neu, the V664E mutant [Ben-Levy et al., 1994], and analyzed its effect on progressive truncations of the *Her2/neu* promoter fused to a luciferase gene in RK13 cells. As shown in Figure 1, the full-length *Her2/neu* promoter (–496/+30) was >100-fold more active than the parental luciferase reporter plasmid pGL2-Basic, and oncogenic HER2/Neu induced the activity of the full-length *Her2/neu* promoter by ~2.5-fold. Truncation of up to 357 bp of distal promoter sequences (see −301/+30 and −139/+30, Fig. 1) barely influenced promoter activity. However, further deletion of 55 bp of the *Her2/neu* promoter (−84/+30 construct) markedly reduced inducibility by oncogenic HER2/Neu, and the –50/+30 construct had dramatically lost promoter activity. Based on these results,

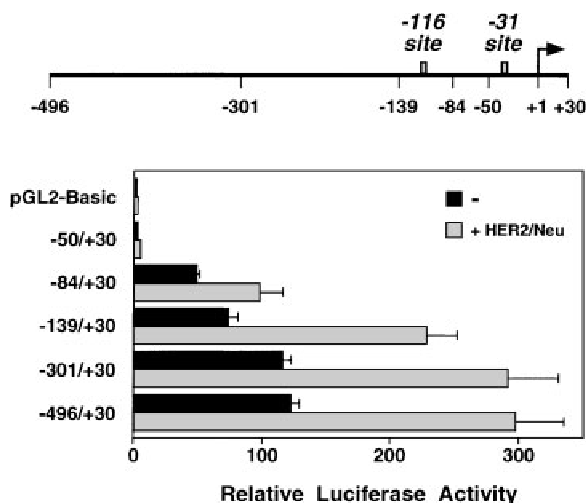

Fig. 1. Activation of the *Her2/neu* promoter by the HER2/Neu receptor. The indicated *Her2/neu* promoter constructs or the parental luciferase reporter pGL2-Basic were transfected into RK13 cells and luciferase activities measured. Where indicated, oncogenic HER2/Neu protein was cotransfected. The top panel gives a schematic sketch of the *Her2/neu* promoter, with +1 indicating the major transcription initiation site and the −116 and −31 ETS core sites being highlighted.

we decided to focus our investigation on the smallest functional promoter construct, −139/+30.

Binding of ER81 to the *Her2/neu* Promoter

ER81 belongs to the family of ETS transcription factors, which bind to DNA targets encompassing a core sequence, GGA^{A}/$_{T}$ [Graves and Petersen, 1998]. Analysis of the *Her2/neu* promoter between −139 and the transcription initiation point revealed the presence of two potential ETS binding sites. These two core sites range from −116 to −113 and from −31 to −28, have both a GGAA type core, and will be referred to from here on as −116 and −31 ETS sites, respectively (see Fig. 1). We next analyzed whether ER81 can bind to the *Her2/neu* promoter. To this end, recombinant ER81 protein was incubated with ^{32}P-labeled oligonucleotides encompassing the −31 or −116 ETS core sites of the *Her2/neu* promoter, or as a control with the ^{32}P-labeled E74 site, a paradigmatic high-affinity binding-site for ETS proteins [Urness and Thummel, 1990; Rao and Reddy, 1992; Janknecht, 1996; Bredemeier-Ernst et al., 1997; Greenall et al., 2001]. As reported before [Janknecht, 1996], ER81 was capable of interacting strongly with the E74 site (Fig. 2A). Inclusion of an antibody directed against the C-terminus of ER81 resulted in a supershift, but

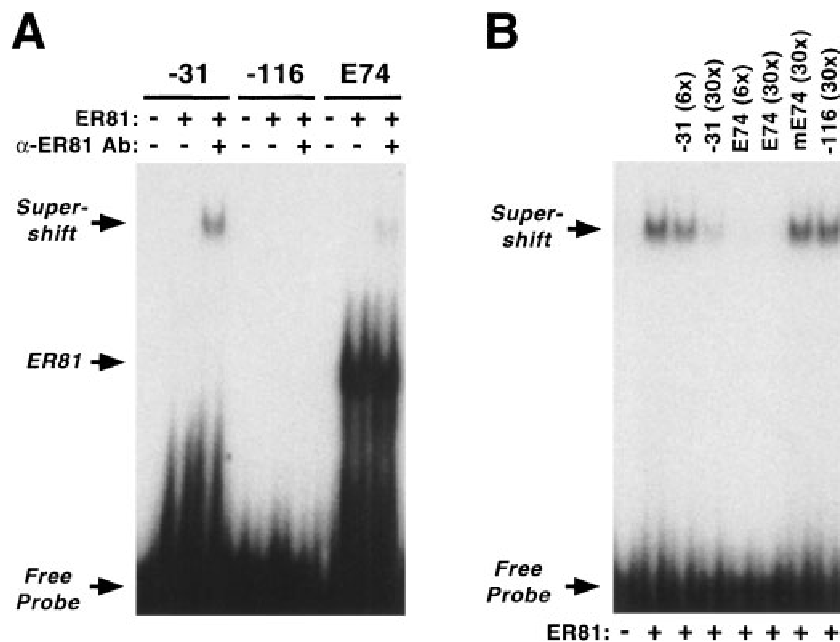

Fig. 2. ER81 binding to the *Her2/neu* promoter. **A**: EMSA with the indicated ^{32}P-labeled oligonucleotides, recombinant ER81, and α-ER81 antibodies. **B**: Analogous suppression of complex formation between ER81, α-ER81 antibodies, and ^{32}P-labeled −31 oligonucleotide by the indicated molar excesses of unlabeled competitor oligonucleotides.

this supershift formation was very inefficient. In stark contrast, no interaction of ER81 with the −31 and −116 oligonucleotides was observed; however, inclusion of the α-ER81 antibody resulted in supershift formation with the −31 oligonucleotide (Fig. 2A). These data suggest that DNA binding of ER81 to the E74 site and the −31 site employ different modes: only binding to the latter site necessitates a conformational change that is induced by the α-ER81 antibody. A comparison of the −31 ETS site (5′-TGAGGAAGTA-3′) to the E74 site (5′-ACCGGAAGTA-3′) revealed that they differ in the nucleotides 5′ of the GGAA core sequence. These nucleotides are critical determinants of the DNA-binding specificity of ETS proteins [Graves and Petersen, 1998], which may require ER81 to adopt different conformations in order to bind to these different DNA sequences.

The formation of the complex consisting of ER81, α-ER81 antibody, and ^{32}P-labeled −31 oligonucleotide could be suppressed by increasing amounts of unlabeled −31 or E74 oligonucleotides (Fig. 2B). In contrast, unlabeled −116 and mutated E74 oligonucleotides were unable to compete with the ^{32}P-labeled −31 oligonucleotide for complex formation. These data demonstrate that the supershift formation was DNA-binding site specific. Altogether, ER81 may bind to the −31 ETS core site in the *Her2/neu* promoter, possibly only when a conformational change is induced in vivo by, for example, interacting proteins or post-translational modification.

Activation of the *Her2/neu* Promoter by ER81

Next, we studied the impact of ER81 expression on *Her2/neu* promoter activity. Expression of ER81 led to ~3.5-fold activation of the *Her2/neu* promoter (Fig. 3). Importantly, whereas oncogenic HER2/Neu elicited only a ~2.5-fold activation of the *Her2/neu* promoter, ER81 and HER2/Neu jointly led to > 10-fold activation, indicating that ER81 and HER2/Neu synergize. Mutation of the −31 ETS site reduced basal transcription mediated by ER81 by 48%, and luciferase activity obtained with the mutated *Her2/neu* promoter reporter was 38% less than with the wild-type construct in the simultaneous presence of ER81 and oncogenic HER2/Neu (Fig. 3). These results suggest that ER81 binding to the *Her2/neu* promoter via the −31 ETS core site contributes to, but is not absolutely required for ER81-dependent activation.

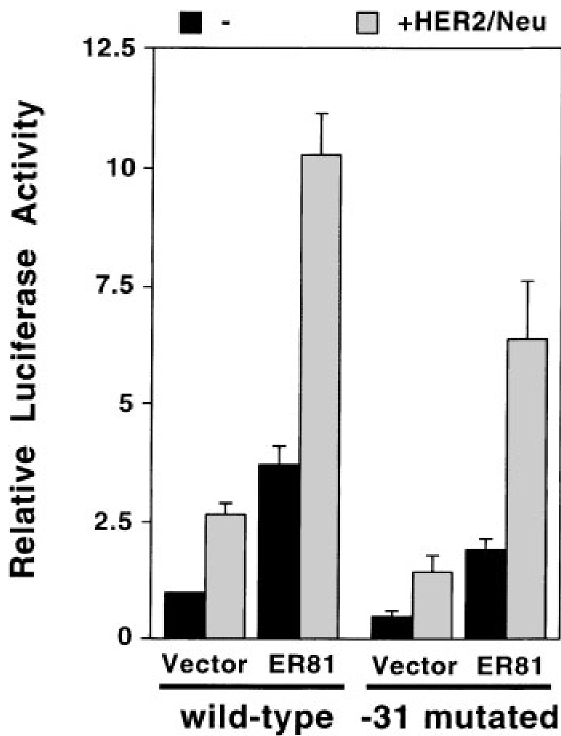

Fig. 3. Stimulation of the −139/+30 *Her2/neu* promoter luciferase construct (wild-type or mutated at the −31 ETS core site) by ER81 and oncogenic HER2/Neu.

Involvement of MAPKs in *Her2/neu* Promoter Activation

The HER2/Neu protein is known to activate MAPK signaling pathways [Hynes and Stern, 1994; Hung and Lau, 1999; Olayioye et al., 2000], of which three major ones exist in the mammalian cell: the ERK, JNK, and p38 MAPK pathways [Cobb, 1999; Chang and Karin, 2001]. Therefore, we investigated which of these pathways may activate the *Her2/neu* promoter via ER81. To this end, we utilized specific upstream activators of ERK, JNK, and p38 MAPKs, namely BXB [Bruder et al., 1992], MKK7a [Holland et al., 1997], and MEK6(DD) [Stein et al., 1997], respectively. All of these MAPK activators caused a less than 1.7-fold stimulation of the *Her2/neu* promoter in the absence of overexpressed ER81, whereas they enhanced ER81-mediated transcription by two-to four-fold (Fig. 4A).

Interestingly, MKK7a proved to be significantly less stimulatory than BXB and MEK6(DD). We wondered whether this is a consequence of a low propensity of the MKK7a downstream effector, JNK MAPK, to phosphorylate ER81. In order to analyze this, we immunoprecipitated ERK, JNK, or p38 MAPKs and utilized them in in vitro kinase assays. As

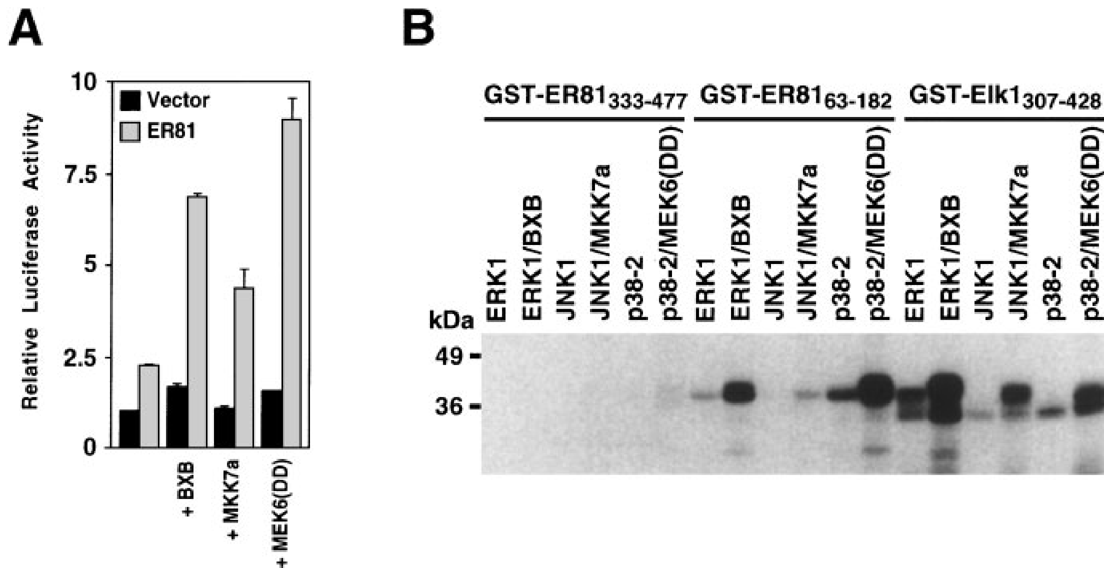

Fig. 4. MAPK phosphorylation of ER81. **A**: Stimulation of ER81 by the MAPK activators BXB, MKK7a or MEK6(DD). Luciferase activity derived from the −139/+30 *Her2/neu* promoter is depicted under all conditions tested. **B**: In vitro kinase assays. The indicated GST fusion proteins were employed as substrates for immunoprecipitated ERK1, JNK1, or p38-2. Where indicated, the MAPKs were immunoprecipitated from cells co-expressing BXB, MKK7a, or MEK6(DD).

shown in Figure 4B, BXB activated ERK1 MAPK and MEK6(DD) activated p38-2 MAPK, leading to efficient phosphorylation of ER81 amino acids 63–182 that harbor all in vivo MAPK phosphorylation sites [Bosc et al., 2001]. In contrast, MKK7a did not elicit much of ER81 phosphorylation by JNK1 MAPK. As a control, we utilized a paradigmatic MAPK target, the C-terminal amino acids 307–428 of the transcription factor, Elk1 [Cahill et al., 1996; Wasylyk et al., 1998]. Here, we observed that stimulation of ERK1 resulted in highest phosphorylation, followed by stimulated JNK1 and p38-2 (Fig. 4B), indicating that the different MAPKs have a different propensity to utilize ER81 and Elk1 as substrates. As a negative control, we employed the C-terminal amino acids of ER81 (amino acids 333–477) that are not phosphorylated by MAPKs [Janknecht, 2001]. Overall, the degree of in vitro phosphorylation of ER81 correlates with the level of *Her2/neu* promoter activation upon stimulation of the three different MAPK subclasses, implicating that direct MAPK phosphorylation of ER81 is involved in the activation of *Her2/neu* transcription.

ER81 possesses five MAPK-dependent phosphorylation sites regulating its activity [Bosc et al., 2001], mutation of which reduced the ability of ER81 and oncogenic HER2/Neu to activate the *Her2/neu* promoter by half, yet did not abolish it (data not shown). These data suggest that HER2/Neu activates ER81-mediated transcription by at least two means, only one of which involves MAPK-dependent phosphorylation of ER81.

Involvement of ER81 Coactivators in *Her2/neu* Promoter Regulation

Since our results indicated that oncogenic HER2/Neu is able to activate ER81-dependent transcription even when its in vivo MAPK-induced phosphorylation sites are mutated, we suspected that HER2/Neu might also target coactivators of ER81. Hitherto, two coactivators of ER81 have been identified, the highly homologous proteins p300 and CBP [Papoutsopoulou and Janknecht, 2000]. Thus, we analyzed whether HER2/Neu could activate CBP. To this end, different portions of CBP, which have been shown to contain transactivation domains [Kwok et al., 1994; Janknecht and Nordheim, 1996], were fused to the Gal4 DNA-binding domain and assayed with a Gal4 binding-site driven luciferase reporter construct. As expected, the N- and C-termini of CBP (amino acids 1–451 and 1891–2441) acted as very potent transactivation domains, and amino acids 451–721 were still ~20-fold activating luciferase activity (Fig. 5A). Importantly, coexpression of HER2/Neu resulted in three- to five-fold enhancement of luciferase activities,

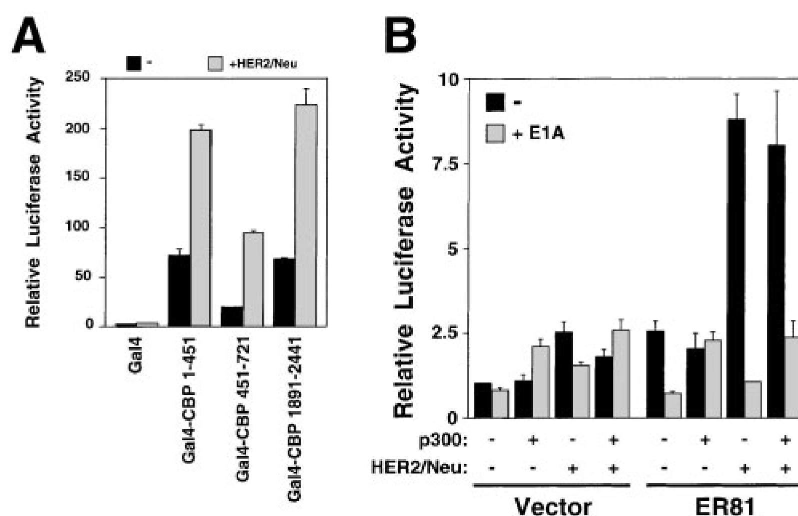

Fig. 5. Impact of p300/CBP on *Her2/neu* promoter activity. **A**: Activation of a Gal4 binding site-driven luciferase reporter plasmid by the indicated Gal4 fusion proteins in the absence or presence of oncogenic HER2/Neu protein. **B**: Stimulation of the −139/+30 *Her2/neu* promoter luciferase reporter by ER81, oncogenic HER2/Neu, p300, and/or E1A.

suggesting that HER2/Neu stimulates all activation domains of CBP.

Next, we investigated whether p300/CBP overexpression may augment ER81-dependent activation of the *Her2/neu* promoter. However, p300 overexpression did not stimulate the *Her2/neu* promoter in the absence or presence of ER81 and/or oncogenic HER2/Neu (Fig. 5B). This could be due to the fact that endogenous levels of p300 and CBP are already sufficiently high to accommodate maximal *Her2/neu* transcription in RK13 cells. If so, depletion of available p300/CBP should reduce ER81-dependent transcription. One way of depleting endogenous p300/CBP is by overexpression of the adenoviral protein E1A that interacts with, and can thereby, sequester p300/CBP [Arany et al., 1995; Lundblad et al., 1995]. Thus, we expressed E1A in the presence or absence of ER81. Whereas, E1A did not greatly affect *Her2/neu* promoter activity in the absence of ER81, E1A suppressed ER81-dependent basal promoter activity by 3.7-fold and reduced HER2/Neu-stimulated ER81 function by even 8.5-fold (Fig. 5B). Importantly, coexpression of p300 completely relieved E1A-mediated repression of basal ER81-dependent transcription, and also HER2/Neu-stimulated ER81 activity was significantly less repressed by E1A (Fig. 5B), suggesting that E1A is indeed inhibiting ER81 function by competing for p300/CBP. Altogether, these results indicate that ER81-mediated activation of the *Her2/neu* promoter is, at least partially, dependent on the coactivators p300/CBP.

Suppression of *Her2/neu* Promoter Activity by Dominant-Negative ER81

ER81 is a modular protein consisting of two activation domains, one inhibitory domain, and the ETS DNA-binding domain (see Fig. 6A) [Janknecht, 1996]. In order to test the impact of these domains on ER81-dependent activation of the *Her2/neu* promoter, we analyzed several truncations of ER81. Deletion of the N-terminal activation domain of ER81 abolished any enhancement of basal transcription (Fig. 6B, see 182–477). Furthermore, in the presence of oncogenic HER2/Neu, no increase in transcriptional activation was observable with this N-terminal truncation, and luciferase activity was more than three-fold less compared to the vector control. These results indicate that $ER81_{182-477}$ is a dominant-negative molecule suppressing HER2/Neu-stimulated, and also MAPK-induced (data not shown), transcription from the *Her2/neu* promoter.

In addition, deletion of the small, and less potent [Janknecht, 1996], C-terminal activation domain did not reduce the ability of ER81 to activate basal *Her2/neu* promoter activity, yet it nearly abolished activation by oncogenic HER2/Neu (Fig. 6B, see 2–429). A protein that nearly solely consists of the DNA-binding domain, $ER81_{333-429}$, behaved similarly as $ER81_{182-477}$. Furthermore, the N-terminal activation domain of ER81 alone had barely any effect (Fig. 6B, see 1–182), indicating that it can only activate *Her2/neu* transcription when fused to the DNA-binding portion of ER81. In conclusion, ER81 can suppress the HER2/Neu-triggered stimulation of the *Her2/neu* promoter in a dominant-negative fashion upon deletion of its N-terminal activation domain.

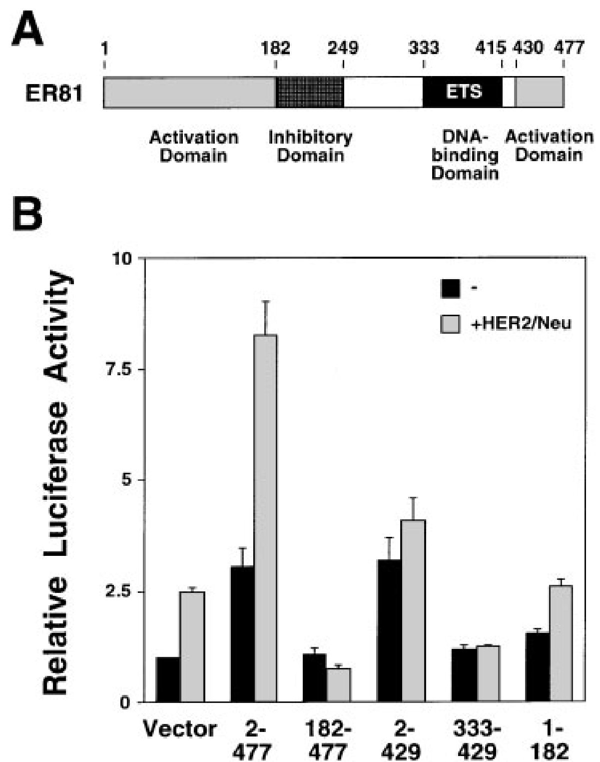

Fig. 6. Dominant-negative ER81 molecules. **A**: Sketch of murine ER81. **B**: The indicated ER81 truncations, full-length ER81 (2–477), or empty vector were transfected into RK13 cells. Stimulation of the −139/+30 *Her2/neu* promoter luciferase plasmid was measured in the absence and presence of oncogenic HER2/Neu protein.

DISCUSSION

HER2/Neu is an essential protein tyrosine kinase, whose absence leads to severe cardiac and neurological defects [Lee et al., 1995]. On the other hand, elevated HER2/Neu levels can cause cancer and are, especially in breast tumor patients, correlated to a poor prognosis [Hynes and Stern, 1994]. Thus, *Her2/neu* gene activity has to be tightly controlled under normal circumstances, and our results suggest that the ETS transcription factor ER81 may be involved in this strict regulation of *Her2/neu* gene transcription.

We have shown that ER81 can activate both basal, as well as MAPK-stimulated *Her2/neu* promoter activity. Mutating all relevant in vivo phosphorylation sites in ER81 does not fully block its ability to stimulate the *Her2/neu* promoter, indicating that MAPK-induced phosphorylation of ER81 is not the sole means of activation. Rather, ER81 may recruit other transcription factors or coactivators to the *Her2/neu* promoter, which themselves are targets for MAPKs. Indeed, the ER81 coactivators CBP/p300 are phosphorylated by MAPKs [Janknecht and Nordheim, 1996; Ait-Si-Ali et al., 1999; Liu et al., 1999]. Consistently, we have shown that three domains within CBP are activated by oncogenic HER2/Neu, presumably via MAPKs that are well-known downstream effectors of HER2/Neu [Hynes and Stern, 1994; Hung and Lau, 1999; Olayioye et al., 2000].

Analysis of 139-bp upstream of the major *Her2/neu* transcription initiation site revealed two ETS binding sites, only one of which (at position −31) is capable of interacting with ER81 and also reportedly with other ETS proteins [Benz et al., 1997; Chang et al., 1997a; Scott et al., 2000]. Mutation of the −31 ETS site does reduce, but not abolish, the ability of ER81 to activate *Her2/neu* transcription. This suggests that ER81 may also bind to cryptic sites in the *Her2/neu* promoter, is recruited to the *Her2/neu* promoter by other DNA-binding proteins, or indirectly affects *Her2/neu* promoter activity. Whatever the mechanism, our data indicate that ER81 is part of a positive regulatory feedback loop: HER2/Neu activates the ER81 protein that in turn stimulates *Her2/neu* gene transcription leading to even more HER2/Neu activity.

Interestingly, ER81 binding to the −31 ETS site was not detectable in our EMSA without an α-ER81 antibody that recognized the C-terminus of ER81. Probably, this antibody induces a conformational change in ER81, similarly as it has been reported for the related ETS protein PEA3 [Bojovic and Hassell, 2001], thereby allowing ER81 to interact with the −31 ETS site. If so, binding of ER81 in vivo would necessitate a conformational change, which could be induced by interacting proteins or post-translational modifications.

The adenoviral protein, E1A, has been reported to suppress basal *Her2/neu* transcription [Yu and Suen, 1990], and in line with this result, we found that E1A repressed enhancement of ER81-mediated basal *Her2/neu* promoter activity. In addition, E1A abolished enhancement of *Her2/neu* promoter activity mediated by ER81 upon stimulation with oncogenic HER2/Neu. One mechanism how E1A represses ER81 function may be via sequestration of the cofactors, p300/CBP [Arany et al., 1995; Lundblad et al., 1995], two essential proteins that perform multiple functions in cell growth and development [Goodman and Smolik, 2000; Janknecht, 2002]. Consistently, our results have shown that p300 overexpression abrogates or alleviates this E1A-mediated repression of the *Her2/neu* promoter. Similar to E1A, interferon-γ suppresses *Her2/neu* gene activity in prostate cancer cells, most likely by inducing phosphorylation of STAT1, which results in interaction with and thus, sequestration of p300 [Kominsky et al., 2000]. A previous report described the dependence of *Her2/neu* promoter activity on p300 and implicated a DNA region upstream of −139 as the target sequence for p300 [Chen and Hung, 1997]. However, our results were obtained with the −139/+30 promoter construct excluding these sequences, suggesting that p300 may affect *Her2/neu* promoter activity via several DNA target sequences bound by p300/CBP-interacting transcription factors.

Deletion of the N-terminal activation domain of ER81 resulted in the generation of a dominant-negative molecule, which completely abrogated the stimulation of the *Her2/neu* promoter by oncogenic HER2/Neu or MAPKs. These results suggest that dominant-negative ER81 competes with endogenous ER81 or ER81-related proteins and prevents them from stimulating the *Her2/neu* gene. Accordingly, delivery of dominant-negative ER81 to HER2/Neu overexpressing tumor cells may be a potential therapeutic approach, similarly as E1A expression

has been successfully employed in animal tumor models to suppress *Her2/neu* gene expression [Yu et al., 1992, 1995; Zhang et al., 1995; Chang et al., 1997b].

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