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Oncogenic Neu/ErbB-2 Increases Ets, AP-1, and NF-κB-dependent Gene Expression, and Inhibiting Ets Activation Blocks Neu-mediated Cellular Transformation*

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Overexpression of Neu (ErbB-2/HER2) is found in ~20% of breast tumors. Activation of Neu by a point mutation (Neu_T) causes constitutive tyrosine kinase activity of this transmembrane receptor and transforming activity in fibroblasts. To identify downstream targets of Neu, we have analyzed the ability of Neu to activate gene expression. Expression of Neu_T, but not normal Neu, caused transcriptional activation of Ets, AP-1, or NF-κB-dependent reporter genes. Dominant inhibitory Ras or Raf mutants blocked the Neu-mediated transcriptional activation, confirming that Ras signaling pathways were required for this activation. Analysis with Ets2 mutants indicated that activation of Ets2 transcriptional activity mediated by Neu_T or oncogenic Ras required phosphorylation of the same Ets2 residue, threonine 72. Cotransfection of dominant inhibitory Ets2 mutants specifically blocked Neu_T-mediated activation of Ets-dependent reporter genes. Furthermore, in focus formation assays using NIH 3T3 cells, the transforming activity of Neu_T was inhibited 5-fold when Neu_T was cotransfected with a dominant negative Ets2 mutant. However, parallel colony formation assays showed that the Ets2 dominant negative mutant did not inhibit the growth of normal cells. Together, these data show that Neu_T activates a variety of transcription factor families via the Ras signaling pathway and that Ets activation is required for Neu_T-mediated cellular transformation. Thus, downstream targets of Neu, including Ets transcription factors, may be useful points for therapeutic intervention in Neu/ErbB-2-associated cancers.

The c-*neu* oncogene product (also called ErbB-2 or HER2) is a 185-kDa transmembrane receptor tyrosine kinase that belongs to the epidermal growth factor family (1–3). Overexpression of Neu/ErbB-2 is found in 20–30% of human breast cancers and is also seen in ovarian, lung, and gastric adenocarcinomas (4–8). A number of potential activating ligands for Neu/c-ErbB-2 have been studied (8), and this receptor can be constitutively activated by a point mutation in the transmembrane domain (9) or by deletions in the extracellular domain (10, 11). Constitutive activation by mutation causes Neu to exhibit strong transforming activity, both in cultured cells and in transgenic mice (9, 12). Expression of very high levels of normal Neu can transform fibroblasts *in vitro* (13). Overexpression of normal Neu in transgenic mice also leads to tumor formation, but because these tumors are infrequent and focal, it appears that a second event is required for tumor formation (14, 15). Cellular transformation is associated with alterations in the expression of multiple genes, many of which are likely regulated by the abundance or activity of specific transcription factors. Although downstream targets of Neu have been identified, like other receptor tyrosine kinases, the details of the pathways for cellular transformation remain unclear (16, 17).

Neu that has been activated by a point mutation in the transmembrane domain (called Neu_T) has enhanced tyrosine kinase activity (9) and has been found to activate a variety of signaling pathway components (18). Neu_T-activated signaling components include phospholipase Cγ and phosphatidylinositol 3′-kinase (19, 20), Src (21, 22), Shc, and Grb2/SOS (23–25). More downstream signaling components activated by Neu_T also include Ras, mitogen-activated protein kinases, and AP-1 activity (24, 26, 27). Therefore, although mutations in *ras* are rarely found in breast cancer, the Neu-mediated activation of Ras signaling pathway components suggests that this pathway may nonetheless play an important role in breast cancers (28).

We have focused on transcription factor activation by oncogenes (29, 30), because there is a close correlation between the ability of non-nuclear oncogenes to activate gene expression and to transform cells (31, 32). We previously examined the requirements for promoter DNA binding sites that could function as Ras responsive elements, and found that at the right spacing and orientation, two or more binding sites for members of either the Ets, AP-1 or NF-κB transcription factor families are sufficient to confer Ras responsiveness (29). The importance of oncogene-mediated transcription factor activation is revealed by the findings that inhibition of either AP-1 or Ets activation blocks Ras-mediated cellular transformation (33–36). In the present study, we have determined that Neu_T increases the activities of several different families of transcription factors, including Ets, AP-1, and NF-κB, and that activation of these downstream targets is mediated by the Ras signaling pathway. We further show evidence that Neu_T and oncogenic Ras share a common molecular target for activating transcription, by mediating the phosphorylation of Ets2 threonine 72, leading to increased Ets2 transactivation activity. Finally, to examine the biological significance of Ets activation, we have used dominant negative Ets mutants to show that Ets activation is required for Neu_T-mediated cellular transformation.

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EXPERIMENTAL PROCEDURES

Plasmid Construction—The minimal promoter reporter plasmid Δ56FosdE-CAT,[1] and its derivatives containing the E.18 (two overlapping head-to head Ets2 binding sites), Py (overlapping Ets and AP-1 binding sites), 6AP-1 (six adjacent AP-1 binding sites), or HIV NF-κB (two adjacent NF-κB binding sites) oncogene response elements have been described previously (29). The 2CRE reporter gene was constructed by inserting two tandem copies of a double-stranded oligonucleotide containing the somatostatin CRE (tcgaTACGGTGACGTCAGAGAG) into Δ56FosdE-CAT. The expression vector for normal Neu (pSV2*neu*N) and for oncogenic Neu (pSV2*neu*T) have been described previously (9), as has the expression constructs for oncogenic Ras, pZIP*ras*H(61L) (37), and dominant inhibitory Ras, pZIP*ras*H(17N) (38), and dominant inhibitory Raf, Raf-N3 (39).

The FNpcDNA3 expression vector was constructed using the cytomegalovirus promoter-driven expression vector pcDNA3 (Invitrogen, San Diego, CA), and inserting between the *Hin*dIII and *Bam*HI sites a peptide leader sequence encoding MDYKDDDDKPKKKRKVGS, preceded by a consensus translational start site (GCCACC). This leader sequence contains the FLAG epitope tag and the SV40 nuclear localization signal. The Ets2 coding sequence was modified to insert into this vector by standard polymerase chain reaction mutagenesis, by addition of an in-frame *Bam*HI site (GGA TCC) at the 5′ end and a *Sma*I site just 3′ of the termination codon. The *Bam*HI-*Sma*I cut Ets2 coding sequence was inserted into the *Bam*HI-*Eco*RV cut FNpcDNA3 vector, to create FN-Ets2. The Ets2 coding sequence inserted directly into pcDNA3 gave the same results as the epitope tagged FN-Ets2 construct.[2] Mutant FN-Ets2 constructs with altered residue 72 were constructed using the Chameleon site-directed mutagenesis kit (Stratagene, La Jolla, CA) as recommended by the manufacturer. The Ets dominant negative mutants were constructed using the FNpcDNA vector in the same manner as full-length Ets2. E2TAD contains Ets2 residues 1–331, and E2DBD contains Ets2 residues 332–468. Similarly, E1TAD contains Ets1 residues 1–305, and E1DBD contains residues Ets1 residues 306–466.

DNA Transfections and CAT Assay—The growth of NIH 3T3 cells and their transfection by the calcium phosphate method, as well as assays for reporter gene expression and normalization, have been described previously (29, 30). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, and for transient transfections, were split to 1×10^6 cells/60-mm dish the day prior to transfection. This cell density gave a maximal transactivation response with Neu_T. The day after transfection, the cells were refed with Dulbecco's modified Eagle's medium containing 0.5% calf serum and incubated for an additional 48 h prior to harvest for the TLC-based CAT assay. For cAMP induction, 0.5 mM isobutylmethylxanthine and 10 μM forskolin (final concentrations) were added to the media the final 16 h prior to harvest. All of the CAT assay results are the average of at least three separate experiments quantitated using a phosphorimager (Bio-Rad), and the standard deviations for each experiment are shown. For analysis of Ets2 protein levels, the transfected cells were treated the same as for the CAT assay, except that for the 4 h prior to harvest, the cells were metabolically labeled with [^{35}S]methionine. The cell labeling and subsequent quantitation of the amount of epitope-tagged Ets2 protein following immunoprecipitation using the anti-FLAG M2 monoclonal antibody (Eastman Kodak Co.) and SDS-polyacrylamide gel electrophoresis, was performed as described previously (40).

Focus Formation Assay—Focus formation assays following calcium phosphate transfection of NIH 3T3 cells were performed as described previously (41), with 250 ng of pSV2*neu*N or pSV2*neu*T plasmid DNA. The focus formation results for each cotransfection are the average number of foci from two separate experiments on a total of six dishes. For the G418-resistant colony formation assay, 3 days after transfection, the cells were placed in media containing 400 μg/ml G418, and G418-resistant colonies were allowed to grow out as described previously (41).

RESULTS

Transforming Neu Activates Transcription of Ets, AP-1, and NF-κB-dependent Reporter Genes—To test the effects of normal and activated Neu/ErbB-2 on transcription factor activation, transient cotransfection experiments were performed in NIH 3T3 cells. Oncogene expression constructs or empty expression vectors were cotransfected with reporter plasmids containing oncogene response elements. We have previously characterized the requirements for several kinds of promoter elements that can confer Ras responsiveness, by placing synthetic transcription factor binding sites just upstream of a minimal promoter fused to the CAT gene. While the minimal promoter-CAT reporter gene (Δ56FosdE) is not transcriptionally activated by cotransfection with oncogenic Ras, expression of reporter plasmids containing multiple Ets, AP-1, or NF-κB binding sites is strongly activated by oncogenic Ras (29). We have now used these reporter plasmids to determine what families of transcription factors are activated by normal c-Neu (Neu_N) or transforming Neu (Neu_T), which contains an activating point mutation (Val^{664} to Glu) in the transmembrane domain (9).

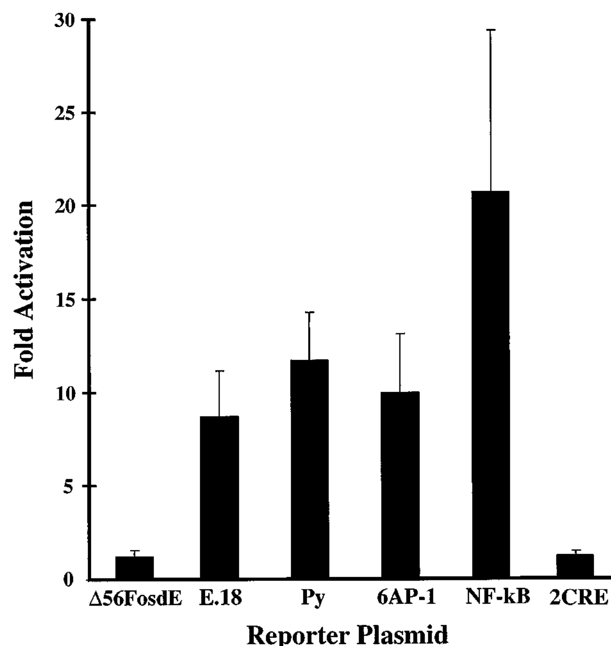

FIG. 1. **Neu_T-induced transcription activation of Ets, AP-1, and NF-κB-dependent reporter genes.** Reporter plasmid DNA (3 μg of Δ56FosdE or 1 μg or the other reporters) and either pSV2*neu*T or pSV2 (4 μg) were cotransfected in to NIH 3T3 cells, and the resulting CAT activity was determined. The fold activation is the ratio of the CAT activity from each reporter gene in the presence of pSV2*neu*T, relative to the CAT activity from the same reporter gene with empty pSV2 expression vector. The bar graph results display the average of at least three separate experiments, and the standard deviation is shown by *error bars*.

The results of cotransfection experiments (Fig. 1) showed that expression of Neu_T activated the transcription of the reporter genes containing synthetic binding sites for Ets (E.18), AP-1 (6AP-1), or NF-κB (NF-κB), and a reporter gene containing single overlapping Ets and AP-1 binding sites (Py). Expression of Neu_N did not significantly activate reporter gene expression (data not shown). The transcriptional activation for each reporter gene was measured by determining the ratio of the CAT activity from cells transiently cotransfected with Neu expression construct relative to cells cotransfected with empty pSV2 expression plasmid. The transcription of the minimal promoter-containing CAT reporter construct, Δ56FosdE, was not significantly activated by Neu_T (Fig. 1). However, the transcriptional activation by Neu_T of reporter genes containing Ets, AP-1, or NF-κB binding sites ranged from 8.6-fold for the E.18 reporter to 20.6-fold for the NF-κB reporter (Fig. 1). The difference in transactivation activity between the expression plasmids for Neu_N and Neu_T, which differ by only a single nucleo-

[1] The abbreviations used are: CAT, chloramphenicol acetyltransferase; DBD, DNA binding domain; TAD, transactivation domain.

[2] C. K. Galang, J. J. García-Ramírez, and C. A. Hauser, unpublished data.

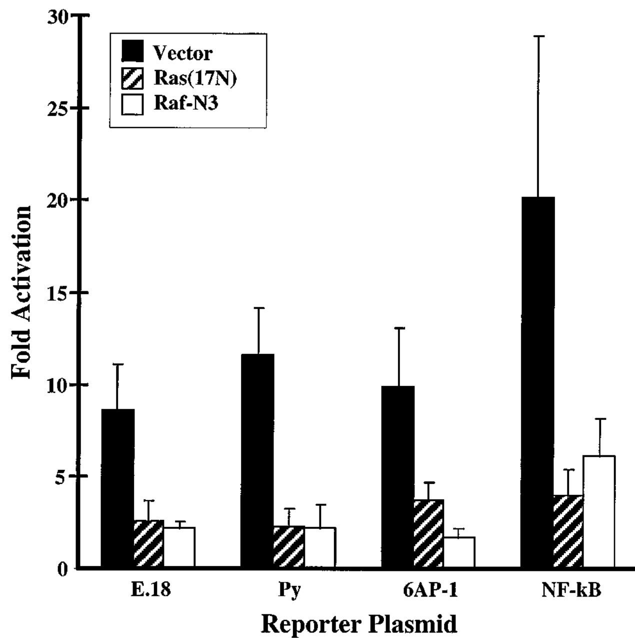

FIG. 2. **Neu_T-induced transcription activation is Ras- and Raf-dependent.** The CAT cotransfection assays and determination of fold activation by Neu_T were the same as for Fig. 1, except that 3 μg of an additional expression plasmid was cotransfected with the reporter and Neu expression plasmids. The added plasmids were either pZIP vector, (*solid bars*), pZIP*ras*H(17N) (*striped bars*), or Raf-N3 (*open bars*).

tide, parallels the difference in their transformation activity, because pSV2*neu*$_T$, but not pSV2*neu*$_N$, can transform NIH 3T3 cells (9). Under the conditions of this assay, oncogenic Ras activated the transcription of these reporter genes to an even higher level than Neu_T (data not shown). To demonstrate the specificity of Neu_T-mediated activation, we tested whether the expression of a reporter gene containing 2 copies of the somatostatin CRE was activated by Neu_T. This cAMP response element has been previously shown to confer cAMP-responsiveness, but not Ras-responsiveness (42). The 2CRE reporter gene was not transactivated by Neu_T (Fig. 1) or by oncogenic Ras, but was transactivated approximately 10-fold by treatment of the cells with forskolin and isobutylmethylxanthine to induce cAMP levels (data not shown). Similar to the results we previously found for oncogenic Ras (29), reporter genes containing the minimal *fos* promoter with only a single added binding site for Ets, AP-1, or NF-κB family members were not significantly transactivated by expression of Neu_T (data not shown), indicating that the presence of a single binding site for any of these transcription factors was not sufficient to create an Neu responsive promoter element.

Transcription Activation by Neu_T Uses the Ras Signaling Pathway—A variety of components of the Ras signaling pathway have been found to be activated by Neu_T. Therefore, to determine whether activation of Ras and Raf are involved in the observed transcription factor activation by Neu_T, expression constructs for dominant inhibitory mutants of Ras or Raf were cotransfected along with Neu_T and the reporter plasmids. Expression of the RasH(Asn17) mutant and the truncated Raf-N3 mutant proteins interfere with the activation of endogenous Ras and Raf, respectively (39, 43). The data in Fig. 2 show that the Neu_T-mediated activation of Ets, AP-1, or NF-κB transcription factor activity was strongly inhibited by either dominant negative Ras or Raf. These data suggest that activation of Ras and Raf are essential components of the signaling pathway for activation of these three families of transcription factors by Neu_T.

Ets2 Is a Downstream Target of Neu_T—We have previously found that Ets-2 is a target for oncogenic Ras signaling and that cotransfection of expression constructs for oncogenic Ras and Ets2 results in very high levels of the Ets-dependent E.18 reporter gene expression (29, 40). To determine whether Ets2 is a target of Neu_T signaling, we first tested whether NeuT could synergize with Ets2 to superactivate the E.18 reporter gene. Transfection of 100 ng of the FN-Ets2 expression construct alone activated the E.18 reporter gene expression 4-fold, and Neu_T activated E.18 expression 6-fold (Fig. 3*A*). However, when the same amount of Ets2 expression construct was cotransfected with Neu_T, E.18 expression was activated by 33 fold (Fig. 3*A*). Thus, Neu_T can synergistically activate transcription of the E.18 reporter when coexpressed with Ets2, suggesting Ets2 is indeed a target of Neu_T signaling. This Ras/Ets2 synergy, was termed "superactivation," and we have shown previously, using a combination of biochemical and genetic approaches, that Ras-dependent phosphorylation of Ets2 Thr^{72} is essential for superactivation by oncogenic Ras. Substitution of Ets2 Thr^{72} with Ala or Glu abolishes Ras superactivation of Ets2 activity, whereas substitution of Thr^{72} with Ser (which preserves the putative mitogen-activated protein kinase recognition site) has little effect (40). To determine if Neu_T activation of Ets2 activity has similar requirements to activation by oncogenic Ras, we tested the ability of Neu_T to superactivate E.18 expression in combination with Ets2 residue 72 mutants. Like Ras, Neu_T did not superactivate E.18 expression when cotransfected with Ets2 Ala^{72} or Glu^{72} mutants, but did superactivate expression when cotransfected with Ets2 Ser^{72} (Fig. 3*A*). To determine whether Neu_T alters the amount or the transcriptional activity of Ets2 and whether the mutant Ets2 proteins are expressed equivalently to the wild type, we measured the Ets2 protein levels in the cotransfected cells. Quantitation by phosphorimager of the immunoprecipitated labeled Ets2 signals shown in Fig. 3*B*, revealed that there was not a significant increase in Ets2 levels caused by coexpression of Neu_T and that there was only a 10% difference in the amounts of Ets2(Ala72) and wild type Ets2(Thr72) proteins present in the transfected cells. Furthermore, in this experiment, there was actually almost 2-fold more of the nonactivable Ets2(E72) mutant than the wild type. Therefore, the Neu_T-mediated superactivation of Ets2 activity resulted from altered transactivation activity and not increases in Ets2 protein levels. Furthermore, because this pattern of Neu_T-mediated transcriptional activation in combination with Ets2 mutants was the same as that we previously found mediated by oncogenic Ras (40), these results strongly suggest that phosphorylation of Ets2 Thr^{72} is a common molecular target of both Neu_T and oncogenic Ras signaling.

Two Types of Dominant Negative Ets2 Mutants Block Transcriptional Activation by Neu_T and Oncogenic Ras—The strong transcriptional activation by Neu_T of the E.18 reporter gene, whose promoter consists of two synthetic Ets2 consensus DNA binding sites inserted into a minimal promoter, suggested that Ets proteins are downstream targets of Neu_T signaling. We then tested whether two different types of Ets2 dominant negative mutants could interfere with Neu_T transactivation of Ets-dependent reporter genes. One of these inhibitory mutants contains the same portion of Ets2 as a previously described construct which suppresses Ras-mediated transactivation (35). This ETS domain-containing portion of Ets2 (see Fig. 4*A*) functions as the DNA binding domain, and we called this construct E2DBD. The corresponding Ets1 construct, E1DBD, was also tested for its activity. The E2DBD and E1DBD proteins were very potent inhibitors of Ets activity, as 50 ng of cotransfected E2DBD plasmid was enough to nearly abolish either Neu_T or

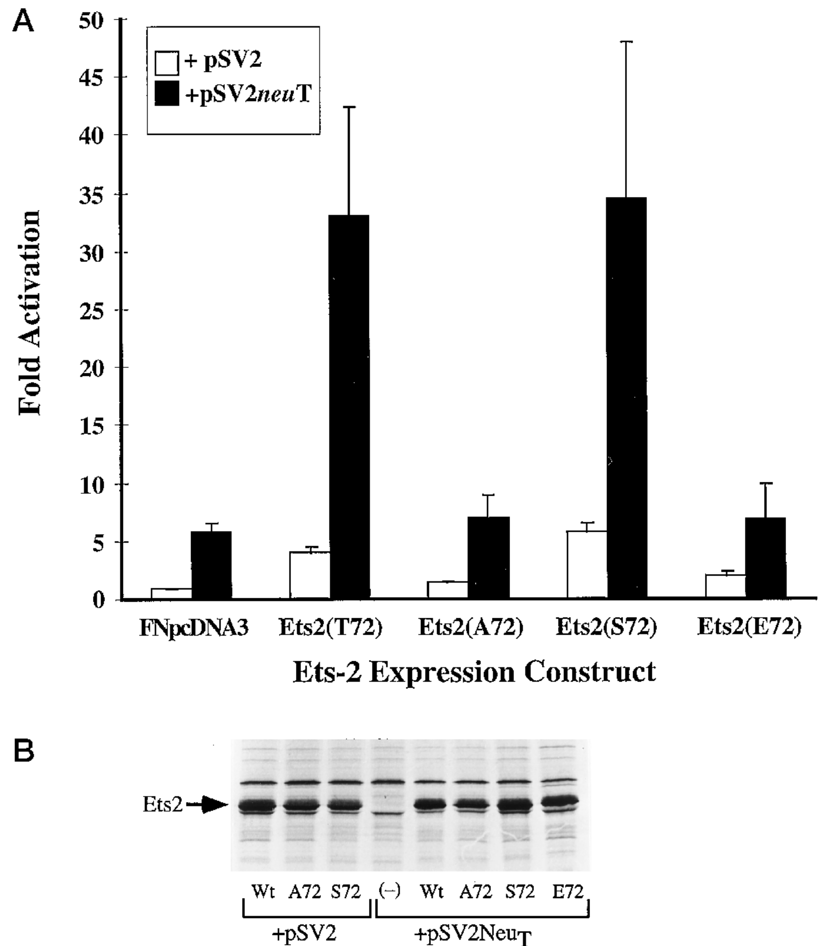

FIG. 3. **Neu$_T$-mediated enhancement of Ets2 activity targets Ets2 residue 72.** *A,* CAT cotransfection assays were performed using the E.18 reporter gene (1.5 μg) and 100 ng of the indicated Ets2 expression plasmid, along with 4 μg of empty expression vector (pSV2, *open bars*) or Neu$_T$ expression vector (pSV2-*neu*T, *closed bars*). The fold activation and *error bars* were determined as described in Fig. 1. *B,* immunoprecipitation analysis of Ets2 protein levels. Cells were cotransfected with the Ets2 expression construct indicated beneath each lane or with no expression vector denoted by "*(−)*," and either pSV2 or pSV2*neu*T. The cells were treated as in Fig. 3*A*, except that they were metabolically labeled with [^{35}S]methionine for 4 h prior to harvest. Subsequently, the cells were lysed and the transiently expressed epitope tagged Ets2 proteins were immunoprecipitated, separated by SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography (see "Experimental Procedures"). The specific Ets2 signal is indicated with an *arrow*.

oncogenic Ras-mediated transactivation of the E.18 reporter gene (Fig. 4, *B* and *C*), and of the Py reporter gene (data not shown).

The other type of Ets2 inhibitory mutant we tested consisted of the Ets2 sequences lacking the DNA binding domain (Fig. 4). This portion of Ets2 contains the transactivation domains of Ets2 (44, 45) including a predicted helix-loop-helix domain (46), and we called this construct E2TAD. The only known ETS family member to contain extensive identity to Ets2 in this region is Ets1, making this putative dominant negative mutant potentially more specific. We also tested the corresponding Ets1 transactivation domains construct, E1TAD. High level expression of either E2TAD or E1TAD (4 μg of cotransfected plasmid) also strongly inhibited E.18 reporter gene activation by either Neu$_T$ (Fig. 4*B*) or oncogenic Ras (Fig. 4*C*) and of the Py reporter gene (data not shown). Plasmid titration studies revealed that inhibition of Neu$_T$ or Ras-mediated transactivation by the E2TAD expression construct required 30–100 fold more expression plasmid DNA than was required with E2DBD, and Western blot analysis revealed that this difference was reflected in protein levels, as substantially more TAD protein than DBD protein was required for dominant negative function (data not shown). However, the observed inhibition of both types of Ets dominant negative mutants was specific, and furthermore, not due to inhibition of Neu expression, because control experiments showed that Neu-mediated activation of the AP-1 dependent reporter gene, 6AP-1, was not inhibited by either of the Ets dominant negative constructs (Fig. 4*B*). Similar specificity was seen with Ras-mediated activation, as Ets-dependent, but not AP-1-dependent reporter gene activation was strongly inhibited by Ets dominant negative mutants (data not shown).

Dominant Negative Ets2 Suppresses Cellular Transformation by Neu$_T$—When the *neu* coding sequence is placed in an expression plasmid containing the SV40 promoter, activated Neu$_T$, but not normal Neu$_N$, is highly transforming for NIH 3T3 cells (9). To determine whether the Neu$_T$-mediated activation of Ets-dependent transcription described above is an important component of the cellular transformation pathway of Neu$_T$, we tested the effect of a strong dominant negative Ets2 mutant on cellular transformation. NIH 3T3 cell focus formation assays showed that transfection of 250 ng of pSV2*neu*$_T$ DNA caused an average of 51 foci per dish, whereas the equivalent amount of pSV2*neu*$_N$ DNA or an empty vector pZIP caused no foci (Fig. 5). For the focus inhibition assay, 50 ng of DNA of either empty expression vector (FNpcDNA3), or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2*neu*$_T$ DNA. While cotransfection of the empty expression vector did not significantly reduce focus formation, dominant negative Ets1 and Ets2 reduced focus formation by 1.7- and 5.7-fold, respectively (Fig. 5). When 1 μg of dominant negative expression plasmid was used, there was some nonspecific inhibition of focus formation by FNpcDNA3 (2.1-fold) but strong inhibition of focus formation by E1DBD (5.7-fold) or E2DBD (8.8-fold). To determine whether the Ets dominant negative mutants were blocking focus formation by toxicity or growth inhibition, parallel colony formation experiments were carried out in NIH 3T3 cells. The FNpcDNA3 vector, with which the EtsDBD constructs were made, confers resistance to G418. The average number of G418-resistant colonies obtained with 50 ng of FNpcDNA3, Ets1DBD, or Ets2DBD was 12, 12,

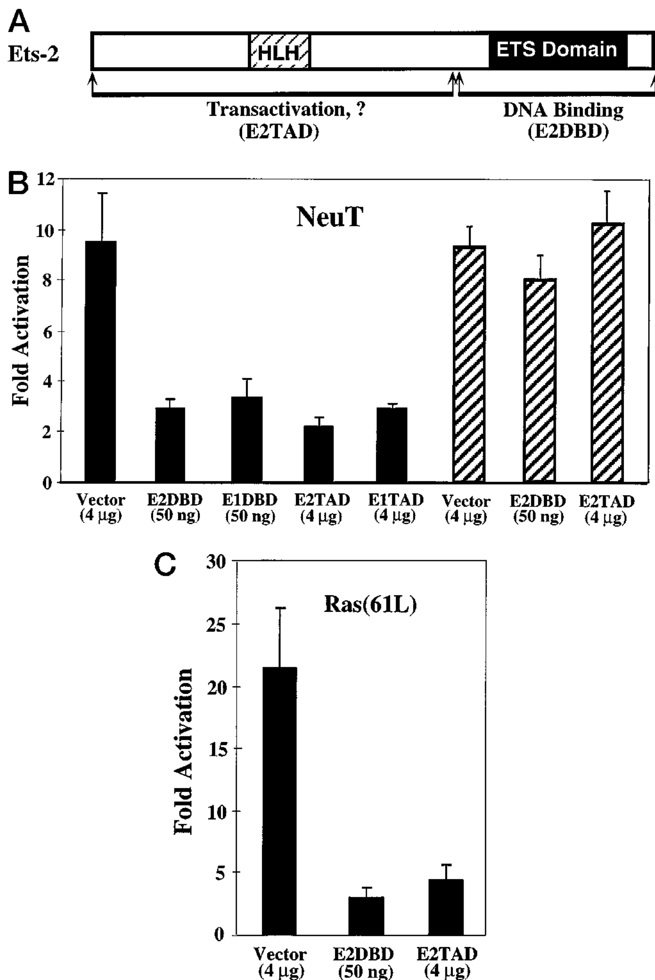

FIG. 4. **Dominant negative Ets mutants block E.18 transactivation by Neu or Ras.** *A*, schematic representation of the Ets2 protein, with the potential helix-loop-helix region denoted by *HLH*. The regions present in the truncated E2TAD and E2DBD mutants are indicated. *B*, CAT cotransfection assays were performed with 1 μg of E.18 reporter plasmid (*solid bars*) or of 6AP-1 reporter (*striped bars*) the indicated amounts of Ets dominant negative construct and 4 μg of either pSV2 or pSV2*neu*T expression construct. Fold activation was determined as in Fig. 1. *C*, CAT cotransfection assays as described in *B*, except that the E.18 reporter gene was cotransfected with the indicated amount of Ets2 dominant negative expression construct and either 2 μg of pZIP or pZIP*ras*H(L61).

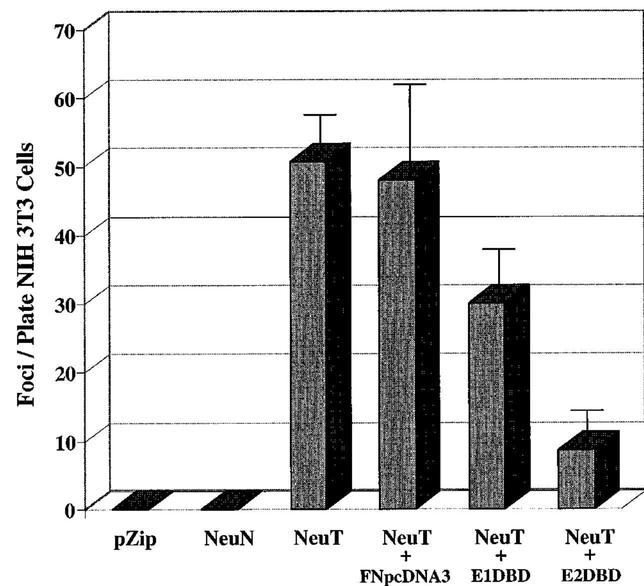

FIG. 5. **Dominant negative Ets mutants inhibit Neu$_T$-mediated cellular transformation.** Results of focus formation assay using 6-cm dishes of NIH 3T3 cells transfected with 250 ng of the indicated oncogene expression construct. Where indicated below, 250 ng of pSV2*neu*T was cotransfected with 50 ng of empty expression vector (FNpcDNA3) or dominant inhibitory Ets plasmids (E1DBD or E2DBD). Each *bar graph* shows the average of six plates from two separate experiments, and the standard deviation is indicated by *error bars*.

and 9 colonies/ng of plasmid DNA, respectively, with no observed difference in colony size. There was also not a significant difference between the number or size of G418 resistant colonies obtained using 1 μg of the empty vector compared with the dominant negative Ets constructs (data not shown). Together, the results of the focus inhibition and colony formation assays indicated that the Ets1 and Ets2 dominant negative mutants inhibited Neu$_T$-mediated cellular transformation without blocking normal cell growth.

DISCUSSION

The *neu* oncogene product has been reported to activate an array of signaling molecules, the most downstream of which is AP-1 transactivation activity, which results in increased AP-1-dependent gene expression (27). The purpose of the current study was to identify other downstream targets of Neu$_T$ by determining whether Neu$_T$ mediates activation of other transcription factor families, and to assess the biological significance of Neu$_T$-mediated transactivation. Therefore, we used cotransfection analysis with Neu$_T$ expression plasmids and reporter genes that we had shown previously to be activated by oncogenic Ras (29). These reporter genes contained synthetic transcription factor binding sites placed in front of a minimal promoter-CAT reporter gene and allow analysis of the activation of specific transcription factor families by oncogenes (29). This analysis showed that the transactivation activity of Ets and NF-κB transcription factors, as well as AP-1, is activated by Neu$_T$, but not Neu$_N$. These results form the basis for more detailed future studies to determine which individual members of these large transcription factor families are targets of Neu$_T$.

As an example of such analysis, we have shown here, using cotransfection studies with wild-type and mutant Ets2 proteins, that Ets2 is a downstream target of Neu$_T$. The observed Neu$_T$-mediated transcriptional activation was not simply a consequence of Neu$_T$ increasing cell growth or generally stimulating transcription, because the minimal promoter-CAT reporter gene alone (Δ56FosdE) or reporter plasmids containing nonfunctional oncogene response elements had clearly measurable basal levels of expression but were not transactivated by Neu$_T$. Furthermore, a reporter gene inducible by signals distinct from the Ras pathway, the cAMP-inducible reporter gene 2CRE, was also not transcriptionally activated by Neu$_T$. The finding that dominant negative Ras or Raf blocked transcription factor activation indicated that an essential component of Neu$_T$ signaling is the Ras signaling pathway. However, because transcriptional activation was measured after 2 days of coexpression, this inhibition by dominant negative Ras and Raf does not necessarily mean that there is a direct linear pathway from Neu through Ras and Raf. For example, it is possible that Neu behaves similar to Raf, which stimulates the production of autocrine factors which in turn activate other components of the Ras signaling pathway (47). The fact that Ets2 is a downstream target of Neu is intriguing, given the finding that there is a binding site for an unidentified ETS protein in the promoter of the HER/*neu* gene that influences its expression (48). Although we found that the three families of transcription factors examined were induced in parallel by Neu$_T$ and oncogenic Ras in NIH 3T3 cells, the transcription signaling pathways of these oncogenes do not appear to be identical. Two

examples of differential gene regulation by Ras and Neu that are found in Ras transformed cells, but not in the same cell lines transformed with Neu_T, are the elevation of the transcription of the parathyroid hormone-related peptide gene (49) and decreased expression of the NF-1/CTF gene (50).

A question that arises from our transactivation results, is why overexpression of normal Neu, which is the defect in Neu/ErbB-2 associated with human cancers, did not significantly activate transcription factor activity in our assays. One possible explanation is that normal Neu requires its ligand to stimulate transcription, and this ligand is not present in the mouse fibroblasts. In fact, studies where the normal Neu/ErbB-2 receptor was stimulated with antibodies or by using a hybrid epidermal growth factor-Neu receptor and epidermal growth factor or with heregulin and cotransfected ErbB-3, the same activation of signal transduction (including AP-1 activation), is seen as with Neu_T (51–55). A second partial explanation may be that while previous studies of human breast tumors found no activating Neu/ErbB-2 mutations (56, 57), many activated Neu mutants may not have been detected. Recent work with transgenic mice overexpressing normal Neu has revealed that in 65% of the tumors examined, the *neu* gene contained an activating mutation, but outside of the previously examined sequences encoding the transmembrane domain (11). Another potential reason that Neu_N was not active in the transactivation assay is that the pSV2*neu*N expression construct does not sufficiently overexpress Neu to achieve the amplified levels of Neu/ErbB-2 found in human tumors. In support of this idea is the finding that transfection of pSV2*neu*N does not transform cells (Ref. 9 and see Fig. 5), whereas transfection of an expression plasmid that causes much higher levels of normal Neu expression can transform NIH 3T3 cells (13).

Because oncogene expression can have widespread effects on cells, it was important to establish whether Neu_T-mediated activation of Ets transcription factors is an necessary component of Neu_T-mediated cellular transformation. For this analysis, we made the dominant inhibitory Ets mutants. The truncated Ets mutants that expressed only the Ets1 or Ets2 DNA binding domains (DBD) were potent inhibitors of Ets-dependent transcription activation by Neu_T or Ras. Presumably these mutant proteins act by binding to Ets binding sites, and blocking the function of endogenous Ets proteins. These mutant proteins may effectively compete with the endogenous protein due to the loss of the postulated intramolecular inhibition of DNA binding present in full-length Ets1 and Ets2 (58, 59). It is likely that E2DBD or E1DBD inhibit the activity of most ETS family members, due to the similarity of ETS family DNA binding sites (60). The potential for cross-inhibition of ETS family members was illustrated by a recent study in which overexpression of either of two of the most divergent ETS domains, Ets1 or PU.1, has similar inhibitory effects on Ras-mediated transactivation and transformation (36). We found that overexpression of the transactivation domains (TAD) of Ets1 or Ets2 also blocked Neu_T-mediated activation of Ets-dependent transcription, but much less efficiently than the DBD mutants. We postulate that the TAD mutants act by titrating out some limiting Ets interaction partner. However, the inhibition of transactivation by TAD is not a generalized squelching of all transcription or by inhibiting Neu_T expression, because neither the TAD nor the DBD mutants efficiently blocked the Neu_T or Ras-mediated activation of an AP-1-dependent reporter gene.

The potential biological importance of the activation of Ets-dependent transcription by Neu_T was revealed by the observation that the Ets dominant negative mutant Ets2DBD specifically inhibited Neu_T-mediated focus formation over 5-fold in an NIH 3T3 cell cotransfection assay (Fig. 5). Thus, Ets mutants that blocks Neu_T-mediated transcriptional activation of an Ets-dependent reporter gene also block cellular transformation. We do not yet understand why the Ets1DBD mutant inhibited Neu_T-mediated focus formation less efficiently than Ets2DBD, but this inhibition was still significant. We have further found that both Ets1DBD and Ets2DBD inhibit focus formation by oncogenic Ras and that the Ets2DBD mutant also inhibits this focus formation more efficiently than Ets1DBD.[3] The results of the colony formation assay, in which equivalent numbers and size of G418-resistant colonies were obtained with empty expression vector or EtsDBD mutants indicated that these dominant negative mutants did not block focus formation by growth inhibition or toxicity. Thus, with the appropriate expression of an Ets dominant negative protein, it appears that Neu_T-mediated cellular transformation can be blocked without interfering with normal cell growth. It has previously been found that Ras transformed cells can actually be reverted to normal morphology and growth characteristics upon expression of dominant negative Ets (35, 36), and it will now be of great interest to determine whether introduction of dominant negative Ets mutants can revert Neu_T transformed cell lines or human breast cancer cell lines back to nontransformed cells. Previous experimental approaches to inhibiting Neu/ErbB-2-mediated cellular transformation and tumor growth have largely been based on directly interfering with Neu/ErbB-2 function, either with dominant negative ErbB-2 mutants, antibodies against ErbB-2, or ErbB-2 antisense oligonucleotides (61–65). The results described here suggest that inhibiting targets well downstream of Neu/ErbB-2, including activation of transcription factors, may complement other approaches for therapy in tumors associated with Neu/ErbB-2.

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