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

(Received for publication, October 5, 1995, and in revised form, January 17, 1996)

Christina K. Galang†, José J. García-Ramírez‡, Patricia A. Solski‡, John K. Westwick‡, Channing J. Der†, Nickolay N. Neznanov, Robert G. Oshima, and Craig A. Hauser†

From the La Jolla Cancer Research Foundation, La Jolla, California 92037-1063 and the University of North Carolina at Chapel Hill, Department of Pharmacology, Chapel Hill, North Carolina 27599-73465

Overexpression of Neu (ErbB-2/HER2) is found in ~20% of breast tumors. Activation of Neu by a point mutation (NeuT) 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 NeuT, 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 NeuT or oncogenic Ras required phosphorylation of the same Ets2 residue, threonine 72. Cotransfection of dominant inhibitory Ets2 mutants specifically blocked NeuT-mediated activation of Ets2-dependent reporter genes. Furthermore, in focus formation assays using NIH 3T3 cells, the transforming activity of NeuT was inhibited 5-fold when NeuT 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 NeuT activates a variety of transcription factor families via the Ras signaling pathway and that Ets2 activation is required for NeuT-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 oncoprotein 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/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 NeuT) has enhanced tyrosine kinase activity (9) and has been found to activate a variety of signaling pathway components (18). NeuT-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 NeuT 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 dose 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 NeuT 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 NeuT 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 NeuT-mediated cellular transformation.

* This work was supported by National Institutes of Health Grants CA63130 (to C. A. H.), CA42978 (to C. J. D.), and CA42302 (to R. G. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a NATO postdoctoral fellowship.

‡ To whom correspondence should be addressed: La Jolla Cancer Research Foundation, 10901 North Torrey Pines Rd., La Jolla, CA 92037-1063. Tel.: 619-455-6460 (ext. 3655); Fax: 619-455-1048; E-mail: chauser@ljcrf.edu.
Plasmid Construction—The minimal reporter plasmid plasmid Δ56FosdE-CAT, and its derivatives containing the E1.8 (two overlapping head-to-head Ets2 binding sites), Py (overlapping Ets and AP-1 binding sites), GAP-1 (six adjacent AP-1 binding sites), or HIV NF-xB (two adjacent NF-xB 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 (5'-TACGTGAAGCAGTC-3') into Δ56FosdE-CAT. The expression vector for normal Neu (pSV2neuN) and for oncogenic Neu (pSV2neuT) have been described previously (9, 39), as has the expression constructs for oncogenic Ras, p21RasH(61L) (37), and dominant inhibitory Ras, p21RasH(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 HindIII and BamHI sites a peptide leader sequence encoding MDYKDDDDKPPKRRKVG, 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 BamHI site (GGAATTC) at the 5' end and a SmaI site just 3' of the termination codon. The BamHI-Smal cut Ets2 coding sequence was inserted into the BamHI-EcoRV 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. 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 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 x 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 reseeded 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 (Biorad, San Diego, CA), 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 [35S]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 pSV2neuN or pSV2neuT 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-xB-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 fusion. 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-xB 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 (NeuN) or transforming Neu (NeuT), which contains an activating point mutation (Val564 to Glu) in the transmembrane domain (9).

The results of cotransfection experiments (Fig. 1) showed that expression of NeuT activated the transcription of the reporter genes containing synthetic binding sites for Ets (E1.8), AP-1 (6AP-1), or NF-xB (NF-xB), and a reporter gene containing single overlapping Ets and AP-1 binding sites (Py). Expression of NeuN 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 NeuT (Fig. 1). However, the transcriptional activation by NeuN of reporter genes containing Ets, AP-1, or NF-xB binding sites ranged from 8.6-fold for the E.18 reporter to 20.6-fold for the NF-xB reporter (Fig. 1). The differences in transactivation activity between the expression plasmids for NeuN and NeuT, 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.
Ets2 Is a Downstream Target of NeuT—We have previously found that Ets-2 is a target for oncogenic Ras signaling and that co-transfection of expression constructs for oncogenic Ras and Ets-2 results in very high levels of the Ets-dependent E.18 reporter gene expression (29, 40). To determine whether Ets-2 is a target of NeuT signaling, we first tested whether NeuT could synergize with Ets-2 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 NeuT activated E.18 expression 6-fold (Fig. 3A). However, when the same amount of Ets-2 expression construct was co-transfected with NeuT, E.18 expression was activated by 33 fold (Fig. 3A). Thus, NeuT can synergistically activate transcription of the E.18 reporter when coexpressed with Ets-2, suggesting Ets-2 is indeed a target of NeuT 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 Thr72 is essential for superactivation by oncogenic Ras. Substitution of Ets2 Thr72 with Ala or Glu abolishes Ras superactivation of Ets2 activity, whereas substitution of Thr72 with Ser (which preserves the putative mitogen-activated protein kinase recognition site) has little effect (40). To determine if NeuT activation of Ets2 activity has similar requirements to activation by oncogenic Ras, we tested the ability of NeuT to superactivate E.18 expression in combination with Ets2 residue 72 mutants. Like Ras, NeuT did not superactivate E.18 expression when cotransfected with Ets2 Ala72 or Glu72 mutants, but did superactivate expression when cotransfected with Ets2 Thr72 mutants (Fig. 3A). To determine whether NeuT 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. 3B, revealed that there was not a significant increase in Ets2 levels caused by coexpression of NeuT 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(Thr72) mutant than the wild type. Therefore, the NeuT-mediated superactivation of Ets2 activity resulted from altered transcription activity and not increases in Ets2 protein levels. Furthermore, because this pattern of NeuT-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 Thr72 is a common molecular target of both NeuT and oncogenic Ras signaling.

Two Types of Dominant Negative Ets2 Mutants Block Transcriptional Activation by NeuT and Oncogenic Ras—The strong transcriptional activation by NeuT 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 NeuT signaling. We then tested whether two different types of Ets2 dominant negative mutants could interfere with NeuT transactivation of Ets2-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. 4A) 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 NeuT or...
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 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.

Dominant Negative Ets2 Suppresses Cellular Transformation by NeuT—When the neu coding sequence is placed in an expression plasmid containing the SV40 promoter, activated NeuT, but not normal NeuN, is highly transforming for NIH 3T3 cells (9). To determine whether the NeuT-mediated activation of Ets-dependent transcription described above is an important component of the cellular transformation pathway of NeuT, 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 DNA caused an average of 51 foci per dish, whereas the equivalent amount of pSV2 neu 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 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 DNA was used, there was some nonspecific inhibition of focus formation by FNpcDNA3, or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2 neu 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 DNA was used, there was some nonspecific inhibition of focus formation by FNpcDNA3, or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2 neu 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 DNA was used, there was some nonspecific inhibition of focus formation by FNpcDNA3, or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2 neu 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 DNA was used, there was some nonspecific inhibition of focus formation by FNpcDNA3, or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2 neu 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 DNA was used, there was some nonspecific inhibition of focus formation by FNpcDNA3, or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2 neu 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 DNA was used, there was some nonspecific inhibition of focus formation by FNpcDNA3, or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2 neu 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 DNA was used, there was some nonspecific inhibition of focus formation by FNpcDNA3, or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2 neu 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 DNA was used, there was some nonspecific inhibition of focus formation by FNpcDNA3, or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2 neu 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 DNA was used, there was some nonspecific inhibition of focus formation by FNpcDNA3, or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2 neu 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 DNA was used, there was some nonspecific inhibition of focus formation by FNpcDNA3, or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2 neu 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 DNA was used, there was some nonspecific inhibition of focus formation by FNpcDNA3, or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2 neu 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 DNA was used, there was some nonspecific inhibition of focus formation by FNpcDNA3, or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2 neu 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 DNA was used, there was some nonspecific inhibition of focus formation by FNpcDNA3, or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2 neu 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 DNA was used, there was some nonspecific inhibition of focus formation by FNpcDNA3, or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2 neu 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 DNA was used, there was some nonspecific inhibition of focus formation by FNpcDNA3, or dominant negative Ets1 (E1DBD), or dominant negative Ets2 (E2DBD) expression constructs were cotransfected along with 250 ng of pSV2 neu 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).
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 NeuT-mediated cellular transformation without blocking normal cell growth.

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. 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 pSV2NeuT 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 pZiprasH(L61).

FIG. 5. Dominant negative Ets mutants inhibit NeuT-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 pSV2NeuT 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 NeuT-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 NeuT by determining whether NeuT mediates activation of other transcription factor families, and to assess the biological significance of NeuT-mediated transactivation. Therefore, we used cotransfection analysis with NeuT 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 NeuT, but not NeuN. These results form the basis for more detailed future studies to determine which individual members of these large transcription factor families are targets of NeuT.

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 NeuT. The observed NeuT-mediated transcriptional activation was not simply a consequence of NeuT 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 NeuT. Furthermore, a reporter gene inducible by signals distinct from the Ras pathway, the cAMP-inducible reporter gene 2CRE, was also not transcriptionally activated by NeuT. The finding that dominant negative Ras or Raf blocked transcription factor activation indicated that an essential component of NeuT 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 NeuT 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 NeuT, 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, are the elevation of the transcription of the parathyroid hormone-related peptide gene (49) and deregulated 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 NeuT (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 NeuN was not active in the transactivation assay is that the pSV2/SNeu 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/SNeu 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 NeuN-mediated activation of Ets transcription factors is a necessary component of NeuN-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 NeuN 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 NeuN-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 transactivation or by inhibiting NeuN expression, because neither the TAD nor the DBD mutants efficiently blocked the NeuN or Ras-mediated activation of an AP-1-dependent reporter gene.

The potential biological importance of the activation of Ets-dependent transcription by NeuN was revealed by the observation that the Ets dominant negative mutant Ets2DBD specifically inhibited NeuN-mediated focus formation over 5-fold in an NIH 3T3 cell cotransfection assay (Fig. 5). Thus, Ets mutants that blocks NeuN-mediated transcriptional activation of an Ets-dependent reporter gene also block cellular transformation. We do not yet understand why the Ets1DBD mutant inhibited NeuN-mediated focus formation less efficiently than Ets2DBD, but this inhibition was still significant. We have further observed 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. 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 NeuN-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 NeuN 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.

REFERENCES

1. Bargmann, C. I., Hung, M. C., and Weinberg, R. A. (1986) Nature 319, 226–230.
2. Coussens, L., Yang-Feng, T. L., Liao, Y. C., Chen, E., Gray, A., McGrath, J., Seeburg, P. H., Libermann, T. A., Schlessinger, J., and Francke, U. (1985) Science 230, 1132–1139.
3. Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., Saito, T., and Toyoshima, K. (1986) Nature 319, 230–234.
4. King, C. R., Kraus, M. H., and Aaronson, S. A. (1985) Science 229, 974–976.
5. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. (1987) Science 235, 177–182.
6. van de Vijver, M. J. van de, Bertooli, R., Deviere, P., Cornélis, C., Peters, J., and Nusse, R. (1987) Mol. Cell. Biol. 7, 2019–2023.
7. Singleton, T. P., and Strickler, J. G. (1992) Pathol. Annu. 27, 165–190.
8. Hynes, N. E., and Stern, D. F. (1994) Biochim. Biophys. Acts 1196, 185–189.
9. Bargmann, C. I., Hung, M. C., and Weinberg, R. A. (1986) Cell 45, 649–657.
10. Bargmann, C. I., and Weinberg, R. A. (1988) EMBO J. 7, 2043–2052.
11. Siegel, P. M., Dangott, D. L., Hardy, W. R., and Muller, W. J. (1994) Mol. Cell. Biol. 14, 7068–7077.
12. Muller, W. J., Sinn, E., Pattengale, P. K., Wallace, R., and Leder, P. (1988) Cell 54, 105–115.
13. Di Marco, E., Pierce, J. H., Knicely, C. L., and Di Fiore, P. P. (1990 Mol. Cell. Biol. 10, 3247–3252.
14. Bouchard, L., Lamarre, L., Tremblay, P. J., and Jolicoeur, P. (1989) Cell 57, 531–536.
15. Guy, C. T., Webster, M. A., Schaller, M., Parsons, T. J., Cardiff, R. D., and Muller, W. J. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 10578–10582.
16. Schlessinger, J., and Ullrich, A. (1992) Neuron 9, 383–391.
17. Pawson, T., and Hunter, T. (1994) Curr. Opin. Genet. Dev. 4, 1–4.
18. Dougall, W. C., Qian, X., Peterson, N. C., Miller, M. J., Samanta, A., and Greene, M. I. (1994) Oncogene 9, 2109–2123.
19. Peles, E., Ben-Levy, R., and Ullrich, A., and Yarden, Y. (1991) EMBO J. 10, 2077–2086.
20. Peles, E., Lampecht, R., Ben-Levy, R., Tzahar, E., and Yarden, Y. (1992) J. Biol. Chem. 267, 12267–12274.
21. Luttrell, D. K., Lee, A., Lansing, T. J., Crosby, R. M., Jung, K. D., Willard, D., Luther, M., Rodriguez, M., Berman, J., and Gilmer, T. M. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 83–87.
22. Muduswanyu, S. K., Siegel, P. M., Dangott, D. L., Webster, M. A., and Muller, W. J. (1994) Mol. Cell. Biol. 14, 735–743.
23. Segatto, O., Pelici, G., Giulii, S., Digiesi, G., Di Fiore, P. P., McGlade, J., Dawson, T., and Pelici, P. G. (1994) Oncogene 8, 2105–2112.
24. Janes, P. W., Daly, R. J., DeFazio, A., and Sutherland, R. L. (1994) Oncogene 9, 3601–3608
25. Xie, Y., Li, K., and Hung, M. C. (1995) Oncogene 10, 2409–2413
26. Satoh, T., Endo, M., Nakafuku, M., Akiyama, T., Yamamoto, T., and Kaziro, Y. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7926–7929
27. Ben-Levy, R., Paterson, H. F., Marshall, C. J., and Yarden, Y. (1994) EMBO J. 13, 3302–3311
28. Clark, G. J., and Der, C. J. (1995) Breast Cancer Res. Treat. 35, 133–144
29. Galang, C. K., Der, C. J., and Hauser, C. A. (1994) Oncogene 9, 2913–2921
30. Hauser, C. A., Westwick, J. K., and Quilliam, L. A. (1995) Methods Enzymol. 255, 412–426
31. Bortner, D. M., Langer, S. J., and Ostrowski, M. C. (1993) Mol. Cell. Biol. 13, 5355–5362
32. Aoyama, A., and Klemenz, R. (1993) Crit. Rev. Oncog. 4, 137–160
33. Lloyd, A., Yancheva, N., and Wasylyk, B. (1995) Mol. Cell. Biol. 15, 395–412
34. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., et al. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 899–903
35. Nebl, G., Mermod, N., and Cato, A. C. B. (1994) J. Biol. Chem. 269, 6263–6266
36. Seth, A., and Papas, T. S. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3302–3311
37. Der, C. J., Weissman, B., and MacDonald, M. J. (1988) Mol. Cell. Biol. 2, 535–547
38. Der, C. J., Weissman, B., and MacDonald, M. J. (1988) Mol. Cell. Biol. 2, 99–105
39. Brtva, T. R., Drugan, J. K., Ghosh, S., Terrell, R. S., Campbell-Burk, S., Bell, R. M., and Der, C. J. (1995) J. Biol. Chem. 270, 7111–7116
40. Yang, B.-S., Hauser, C. A., Henkel, G., Colman, M. S., Van Beveren, C., Stacey, K. J., Hume, D. A., Maki, R. A., and Ostrowski, M. C. (1996) Mol. Cell. Biol. 16, 538–547
41. Clark, G. J., Cox, A. D., Graham, S. M., and Der, C. J. (1995) Methods Enzymol. 255, 395–412
42. Gamett, D. C., and Cerione, R. A. (1994) Genes & Dev. 8, 19848–19858
43. Sliwkowski, M. X., Schaefer, G., Akita, R. W., Loefgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., III (1994) J. Biol. Chem. 269, 14661–14665
44. Motamedi, A., and Ben-Asher, I. (1994) J. Biol. Chem. 269, 1761–1767
45. Sliwkowski, M. X., Schaefer, G., Akita, R. W., Loefgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., III (1994) J. Biol. Chem. 269, 395–412
46. Grosschedl, R., and Hagman, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8889–8893
47. Seth, A., and Papas, T. S. (1990) Oncogene 5, 1953–1964
48. Scott, G. K., Daniel, J. C., Xiong, X., Maki, R. A., Kabat, D., and Benz, C. C. (1994) J. Biol. Chem. 269, 19848–19858
49. Grosschedl, R., and Hagman, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8889–8893
50. Bortner, D. M., Langer, S. J., and Ostrowski, M. C. (1993) Mol. Cell. Biol. 13, 5355–5362
51. Gannon, T. M., and Skolnick, P. (1994) Oncogene 10, 249–256
52. Galang, C. K., Der, C. J., and Hauser, C. A. (1994) Oncogene 9, 2913–2921
53. Scott, G. K., Dodson, J. M., Montgomery, P. A., Johnson, R. M., Sarup, J. C., Wong, W. L., Ulrich, A., Sheard, H. M., and Benz, C. C. (1991) J. Biol. Chem. 266, 14300–14305
54. Scott, G. K., Dodson, J. M., Montgomery, P. A., Johnson, R. M., Sarup, J. C., Wong, W. L., Ulrich, A., Sheard, H. M., and Benz, C. C. (1991) J. Biol. Chem. 266, 14300–14305
55. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., and Ulrich, A. (1989) Science 244, 707–712
56. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., and Ulrich, A. (1989) Science 244, 707–712
57. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., and Ulrich, A. (1989) Science 244, 707–712
58. Sliwkowski, M. X., Schaefer, G., Akita, R. W., Loefgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., III (1994) J. Biol. Chem. 269, 14661–14665
59. Grosschedl, R., and Hagman, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8889–8893
60. Bortner, D. M., Langer, S. J., and Ostrowski, M. C. (1993) Mol. Cell. Biol. 13, 5355–5362
61. Gannon, T. M., and Skolnick, P. (1994) Oncogene 10, 249–256
62. Gamett, D. C., and Cerione, R. A. (1994) FEBS Lett. 351, 335–339
63. Seth, A., and Papas, T. S. (1990) Proc. Natl. Acad. Sci. U. S. A. 89, 8889–8893
