Journal of Cellular Biochemistry 45:69–81 (1991)

Constitutively Activated *neu* Oncoprotein Tyrosine Kinase Interferes With Growth Factor-Induced Signals for Gene Activation

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Abstract The *neu* receptor oncoprotein tyrosine kinase, capable of transforming cultured fibroblasts and causing mammary carcinomas in transgenic mice, carries a point mutation in its transmembrane domain and shows a constitutive tyrosine kinase activity. We analyzed the *neu* tyrosine kinase and its substrates in transfected NIH 3T3 fibroblasts by phosphotyrosine immunoblotting. Tyrosine phosphorylated proteins were similar but not identical in epidermal growth factor (EGF)-stimulated cells expressing the human EGF receptor (EGFR) or a chimeric EGFR/*neu* receptor but differed from phosphotyrosyl proteins constitutively expressed in *neu* oncogene-transformed cells. The *neu* oncoprotein in the latter cells was phosphorylated in tyrosine in a ligand-independent manner and had a shortened half-life in comparison with the normal *neu* protein. Tumor promoter pretreatment inhibited ligand-induced receptor tyrosine phosphorylation and decreased tyrosine phosphorylated *neu* oncoprotein. Prolonged pretreatment with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) also prevented the induction of immediate early growth factor-regulated genes in response to *neu* activation. Expression of the *neu* oncogene but not the protooncogene in NIH 3T3 cells was associated with enhanced levels of the *jun* and *fos* oncoproteins and loss of serum growth factor induction of immediate early mRNA responses. The constitutively activated *neu* oncoprotein tyrosine kinase thus deregulates cellular genomic responses to growth factors.

Key words: epidermal growth factor, EGF, tumor promoter, receptor tyrosine kinase

The *neu* protooncogene encodes a 185 kD cell surface growth factor receptor-like protein [1–3]. $p185^{neu}$ possesses homology to the epidermal growth factor (EGF) receptor (EGFR) and has an intrinsic tyrosine kinase activity [1–6]. The *neu* oncogene is activated by a point mutation in its transmembrane region, where a glutamic acid residue replaces a valine residue at amino acid 664 [3,7,8]. The activated form of $p185^{neu}$ shows increased autophosphorylation and induces increased tyrosine phosphorylation of other cellular proteins [9,10]. Unlike the protooncogene, the *neu* oncogene transforms cells in vitro [11] and leads to the development of mammary carcinomas in transgenic mice when driven by a mouse mammary tumor virus long terminal repeat promoter [12,13]. The protooncogene transforms fibroblasts only when vastly overexpressed [6]. A significant correlation has been found between amplification of *neu* (the human counterpart is also called HER-2/*erb*B-2) and poor prognosis of human breast cancer [14,15].

We have studied *neu* effector functions in NIH 3T3 cells using a chimeric receptor consisting of EGFR extracellular, transmembrane, and protein kinase C-substrate domains linked to the intracellular tyrosine kinase and carboxyl terminal domains of the rat *neu* protein [16–22]. In the present study, we analyzed the *neu* receptor and oncoprotein tyrosine kinases and their effects on gene expression. We also examined the effect of the tumor promoters 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and okadaic acid on the phosphorylation of the EGFR/*neu* receptor and the *neu* oncoprotein. TPA is a potent activator of protein kinase C and okadaic acid has been shown to inhibit phosphatases 1

Received March 14, 1990; accepted August 17, 1990.

and 2A [23–25]. Particularly, we were interested in effects of *neu* on the growth factor induction of mRNAs characteristic of serum-stimulated fibroblasts in transit from quiescence to the cell cycle [26]. Comparison was made with EGFR and *neu* protooncogene-expressing cells. Also, we wanted to characterize the effect of the *neu* oncoprotein on the transduction of signals generated by growth factor receptors and to compare it with the effect of the c-Ha-*ras* oncogene, which is known to down-regulate growth factor-dependent genomic responses [27,28].

MATERIALS AND METHODS

Cells

NEN/HB cells [22] express the hygromycin B-resistance gene (pY3 [29]) and are derivatives of NEN37 cells which express chimeric EGFR/*neu* receptors under control of the simian virus 40 (SV40) promoter (pSVEGFR/*neu* [16] about 4×10^5 receptors per cell [20]). NEN/HB and neomycin-resistant NN6 cells expressing the pSV2*neo* plasmid [30] were used as control cells.

The N6 cells are derived from NIH 3T3 cells. They express the *neu* protooncogene (pLTR-*neu*N [17]), and the NT11 and NT12 cells express the *neu* oncogene (pSV2*neu*NT [4]). The Cl17 cells are NIH 3T3 derivatives expressing about 4×10^5 human EGFR per cell [31].

The NEN/SV-NT22 and NEN/LTR-NT7 cells, which express the pSV2*neu*NT [7] and pLTR-*neu*NT [17] plasmids, respectively, are *neu* oncogene-transformed derivatives of NEN37 cells [22]. The *neu* oncogene-transformed LTR-EN/NT2.4, and LTR-EN/NT2.7 cells [22] were obtained by transfecting the LTR-EN2 cells expressing the LTR-driven EGFR/*neu* receptors (about 1.4×10^6 receptors/cell; pLTREGFR/*neu*) with pLTR*neu*NT [17].

The c-Ha-*ras* oncogene-transformed NEN/EJ2 and NEN/EJ10 cells [22] are NEN37 derivatives transfected with the plasmid pEJ6.6 [32]. The c-Ha-*ras* protooncogene- and oncogene-expressing NEN/LTR-N29 and NEN/LTR-A34 cells were generated by transfecting the NEN37 cells with pLTRP*ras*(N) and pLTRP*ras*(A), respectively [22].

The cells were grown at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated newborn calf serum (NBCS). Prior to each growth factor-stimulation experiment the cells were washed with serum-free medium and incubated in 0.5–0.75% NBCS for 24–48 hr. The cells were routinely checked for *Mycoplasma* contamination using the Hoechst fluorochrome 33 258 [33], with negative results.

Reagents

Receptor-grade EGF from mouse submaxillary glands was obtained from Collaborative Research. [α-^{32}P]dCTP (400 Ci/mmol), [^{35}S]methionine (1,000 Ci/mmol), and [^{125}I]protein A were from Amersham; protein A-Sepharose from Pharmacia; hygromycin B from Calbiochem-Behring; and TPA from Sigma. All cell culture reagents were from Gibco Laboratories. Okadaic acid was a kind gift from Drs. Hirota Fujiki and Takashi Sugimura (National Cancer Center, Japan).

Antibodies

P-tyr antibodies were raised in rabbits that were immunized with azobenzylphosphonate (a phosphatase-resistant synthetic analogue of P-tyr), which was covalently coupled to keyhole limpet hemocyanin [34,35]. Antibodies were affinity purified and characterized as described [35]. The rabbit anti-*neu* antiserum has also been described previously [16]. Mouse monoclonal antibodies against the extracellular domain of the EGF receptor were from Amersham (RPN.513). Polyclonal antibodies against c-*jun* peptides were from Oncogene Science (PC06, PC07).

Molecular Probes

The following cDNA clones were used as molecular probes: p465.20 (*jun*B [36]), B10 (protein product as yet uncharacterized) and N10 (a putative nuclear receptor for an undefined ligand [37,38]), pRGAPDH-13 (rat glyceraldehyde phosphate dehydrogenase [39]). Nick translations of DNA were carried out according to the manufacturer's instructions (Amersham) and labeling by random priming method was performed as described by Feinberg and Vogelstein [40].

Analysis of RNA

Polyadenylated RNA was isolated by oligo(dT) chromatography from cell lysates [41]; 4–6 μg aliquots of RNA were electrophoresed in 1.2% formaldehyde-agarose gels, transferred to Biodyne nylon filters (Pall Corporation, Glen Cove, NY) in 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) and hybridized with nick-trans-

lated probes in 50% formamide at 42°C as described earlier [42]. For quantitation of the signals, the films were scanned with a densitometer (Helena Laboratories, Beaumont, TX).

For RNA dot blot analyses, the isolated poly(A)$^+$ RNA was labeled into complementary DNA using reverse transcriptase, oligo(dT) primers, and deoxynucleotide precursors including [α-^{32}P]dCTP. The cDNA probes were then hybridized to Gene Screen Plus filters (New England Nuclear Corp.) containing cDNA clones of serum-inducible genes [37]. Autoradiograms of dot blots were scanned into computer using a video camera and digitazing board in Macintosh II. The program Image 1.24 was used to quantitate the dots in eight-bit digital files.

Metabolic Labeling and Immunoprecipitation

For immunoprecipitation of *neu* and EGFR proteins, rabbit antisera against *neu* carboxyl terminal domain [16], and mouse monoclonal antibodies against the EGFR extracellular domain were used, respectively. The cells were metabolically labeled for 16 hr with [^{35}S]methionine (250 μCi/ml) in methionine-free minimal essential medium supplemented with 1% dialyzed fetal calf serum. For immunoprecipitation, the cells were lysed in 2 ml of 0.1% sodium dodecyl sulfate (SDS), 0.5% Triton X-100, 0.5% sodium desoxycholate, 20 mM Tris HCl, pH 7.5, and sonicated for 1 min at 300 W on ice. The lysates were centrifuged for 30 min, 10,000 rpm at 4°C; 1–10 μl of antibody was added into 1 ml of the supernatant and allowed to bind at 4°C for 1 hr. About 30 μl of a 50% v/v solution of protein A-sepharose (Pharmacia) was added to the antibody-containing lysates and the tubes were mixed gently at 4°C for 1 hr. For immunoprecipitation of the EGFR with mouse monoclonal antibodies, rabbit antimouse immunoglobulin-coated, washed protein A-Sepharose particles were used. The immune complexes were washed four times with the immunoprecipitation buffer; twice with PBS and once with 20 mM Tris HCl, pH 7.4; dissolved in the electrophoresis sample buffer containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 50 mM Tris HCl, pH 6.8; and boiled for 5 min. For immunoprecipitation of *jun* proteins the cells were labeled for 3 hr with [^{35}S]methionine (300 μCi/ml), after which the cell lysates were incubated with a mixture of the rabbit polyclonal antibodies Ab-1 and Ab-2 against *jun* protein and protein A-Sepharose particles.

The EGFR/*neu* and *jun* proteins were analyzed in 7.5% and 10% SDS-polyacrylamide gels, respectively, electrophoresed according to Laemmli [43]. After electrophoresis, the gels were fixed in 10% acetic acid, impregnated with Amplify (Amersham), dried onto filter paper, and fluorographed using X-Omat R film (Eastman Kodak) at −70°C.

For analysis of the degradation of the *neu* proteins and the *jun* proteins the cells were labeled for 16 and 6 hr, respectively. After labeling, the cells were washed and incubated for 0–12 hr in DMEM containing 1% FCS before lysis. The cells were then treated as above.

Phosphotyrosine Immunoblotting

The cells were plated on 6 cm plates at 0.8×10^6 cells/plate. At the end of various treatments, total cell proteins were extracted with 125 mM Tris (pH 6.8)/5% SDS. After heating for 3 min at 100°C, the samples were sonicated. The protein concentration of the samples were determined by the BCA protein assay reagent (Pierce); 300 μg of each sample was subjected to SDS electrophoresis according to Laemmli [43] as previously described. Proteins were blotted to nitrocellulose sheets (Bio-Rad). Blots were extensively washed in Tris-buffered saline (0.01 M Tris, pH 7.4/0.9% NaCl), then saturated with 5% bovine serum albumin (fraction IV, RIA grade from Sigma). Blots were then incubated with 9 μg/ml of affinity-purified rabbit phosphotyrosine antibody. After extensive washing, bound antibodies were revealed by ^{125}I-labeled protein A (Amersham). Dried blots were exposed to fluorography films with intensifying screens.

RESULTS

Tyrosine-Phosphorylated Proteins in EGFR/*neu* and *neu* Oncogene-Expressing Cells

As was reported earlier, anti-P-tyr antibodies allow the visualization of tyrosine phosphorylated proteins in Western blots [35]. To analyze the effect of EGF on the phosphorylation of proteins in receptor-expressing cells, we starved subconfluent cells for 24 hr and then stimulated them with 3 nM EGF for 30 min after a 15 min orthovanadate preincubation to inhibit phosphotyrosyl phosphatases [44]. The cells were extracted in SDS buffer and analyzed by SDS-PAGE and immunoblotting with phosphotyrosine antibodies.

In Figure 1 we show a comparison of proteins phosphorylated in tyrosine in untreated and EGF-stimulated cells expressing the chimeric EGFR/*neu* protein (LTR-EN2, NEN37), the *neu* oncogene (NT12), the *neu* protooncogene (N6), or the EGFR (Cl17) and in NIH 3T3 cells. The major proteins phosphorylated in tyrosine after EGF stimulation are the EGFR/*neu* and *neu* polypeptides migrating in the region of 190,000 m.w. and the EGFR of 175 kD molecular weight. Although the addition of EGF induced an increase in the labeling of the EGFR/*neu* and EGFR polypeptides (on average eight- and five-fold, respectively, from three separate experiments), it had relatively little effect on the phosphorylation of the *neu* oncoprotein (on an average 1.5-fold increased phosphorylation in the NT12 cells). At this level of sensitivity, the NIH 3T3 cells yielded no radiolabeled polypeptides, and only faint, constitutive signals were obtained from the N6 cells expressing the normal *neu* protein.

In addition to the major tyrosine phosphorylated receptor polypeptides, the receptor-expressing cells also displayed other labeled polypeptide bands. The most prominent of these with molecular weights of 145 and 124 kD were specific to the *neu*-transformed NT12 cells (see asterisks between the corresponding lanes in Fig. 1). Other, slightly less intense bands at 105, 84, 70,

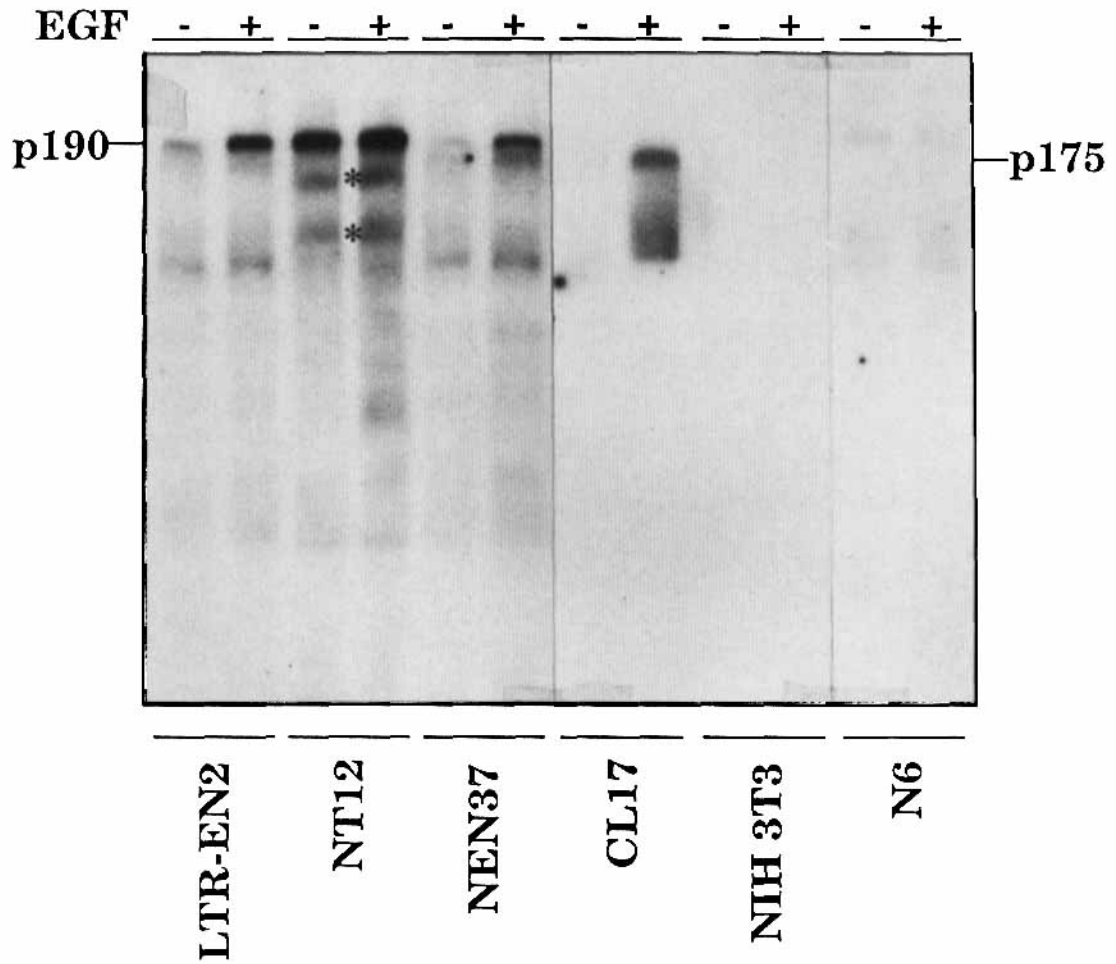

Fig. 1. Comparison of tyrosine phosphorylation in starved and EGF-stimulated receptor expressing cells by Western blotting using P-tyr antibodies. Serum-starved cells were stimulated for 30 min with 3 nM EGF after a 15 min 300 μM sodium orthovanadate preincubation. Cultures were extracted in boiling electrophoresis sample buffer and 300 μg of cell lysate protein was used for analysis. After SDS-PAGE, tyrosyl phosphorylated proteins were identified by immunoblotting with anti-P-tyr antibodies and [^{125}I]protein A. LTR-EN2 cells express LTR-driven EGFR/*neu* chimeric receptors, NEN37 cells express EGFR/*neu* chimeric receptors under the SV40 promoter, NT12 cells express *neu* oncogene and N6 cells express *neu* protooncogene. Tyrosine-phosphorylated polypeptides specific for the NT12 cells are marked with asterisks. As expected, no labeled bands were detected in NIH 3T3 cells.

and 50 kD were shared between the LTR-EN2 and NEN37 cells, which express EGFR/*neu* chimeric receptors under the LTR and the SV40 promoter, respectively, and NT12 cells.

Turnover of the *neu* Receptor and Oncoprotein in NIH 3T3 Cells

To compare the differential degradation of the *neu* protein and the *neu* oncoprotein in the transfected NIH 3T3 cells we labeled *neu* oncogene-transformed NT12 and *neu* protooncogene-expressing N6 cells metabolically with [^{35}S]methionine to isotopic equilibrium and then incubated the cells in media containing nonradioactive methionine for various periods of time, as indicated in Figure 2. As can be seen from the autoradiogram of immunoprecipitated *neu* polypeptides (Fig. 2A) and from the corresponding scanning results plotted in Figure 2B, there is a rapid decay of *neu* oncoprotein, with a half-life of about 1.5 hr (open circles). By contrast, little receptor degradation is evident in the *neu* protooncogene-expressing cells (closed triangles). This is consistent with the results previously published by Stern et al. [45]. We have shown earlier that the half-life of the EGFR/*neu* receptor is longer than 4 hr, and after EGF treatment it decreases to about 50 min [18]. Thus the level of tyrosine phosphorylation of the receptors (see Fig. 1) correlates inversely with their half-lives in the NIH 3T3 cells.

TPA Inhibition of EGF/*neu*-Dependent Tyrosine Phosphorylation

Our recombinant EGFR/*neu* polypeptide retains a threonine residue for potential phosphorylation by protein kinase C [16]. We have earlier shown that TPA prevents the EGF induced DNA synthesis via this chimeric receptor [18]. To determine whether the phosphorylation by EGF of the chimeric receptor was inhibited by the tumor promoter TPA, as is the case in the EGFR [46,47], we monitored the EGF-stimulated tyrosine phosphorylation of the chimeric receptor in the NEN37 cells, which were stimulated with EGF for 5 min with or without a 30 min pretreatment with 100 nM TPA (or with 1 μM okadaic acid). Both TPA and okadaic acid blocked most of the enhancement of P-tyr receptor signal induced by EGF in cells expressing the EGFR/*neu* chimeric receptors (NEN37 cells in Fig. 3). Similar results were obtained in the LTR-EN2 cells, which express higher levels of the same receptors (data not shown). The amount of phosphotyrosine in the *neu* oncoprotein was also about 50% decreased by the TPA treatment, but it was not significantly affected by either EGF or okadaic acid in the NT12 cells.

Prolonged Treatment With TPA Inhibits the Induction of Immediate Early mRNAs by Ligand-Activated *neu*

We have shown earlier that EGF efficiently induces the expression of growth factor-regulated genes in cells expressing the chimeric EGFR/*neu* receptor [20–22]. Poly(A)$^+$ RNA from quiescent cells stimulated with EGF was labeled into radioactive complementary DNA and hybridized to dot filters containing each of the cDNA clones representing 78 serum-inducible genes [37]. Figure 4A shows that the expression of the *Krox*20 transcription factor, T-factor, and B10 mRNAs are stimulated by EGF. Results shown in Figure 4 also indicate that a 24 hr treatment with TPA, known to exhaust protein kinase C activity [48], prevents the induction of these mRNAs by EGF. These autoradiograms were quantitated with a densitometer. As a result, Figure 4B shows an estimate of the level of steady-state mRNAs of ten different genes in NEN37 cells stimulated with EGF with and without TPA pretreatment, compared with unstimulated cells. The GAPDH mRNA represents a constitutively expressed gene used as a control. Taken together, these results imply that TPA almost completely blocks this genomic response, which presumably occurs at least part through a protein kinase C-mediated mechanism.

neu Oncoprotein Prevents Growth Factor Induction of Serum-Responsive Genes

Although the EGF-activated *neu* tyrosine kinase was capable of inducing several of the serum-responsive mRNAs in the NEN37 cells, these immediate early mRNAs were not elevated in *neu* oncogene-expressing cells. We also analyzed their induction by platelet-derived growth factor (PDGF) and serum in the *neu*-transformed cells because these growth factors are strong inducers of mitogenesis and gene activation in NIH 3T3 cells. Serum-starved control HB cells expressing EGFR/*neu* chimeric receptors and *neu* oncogene-transformed LTR-NT7 cells were stimulated with 10 ng/ml PDGF or 20% serum. Polyadenylated RNA was isolated and analyzed by Northern blotting and hybridization with cDNA probes for the tran-

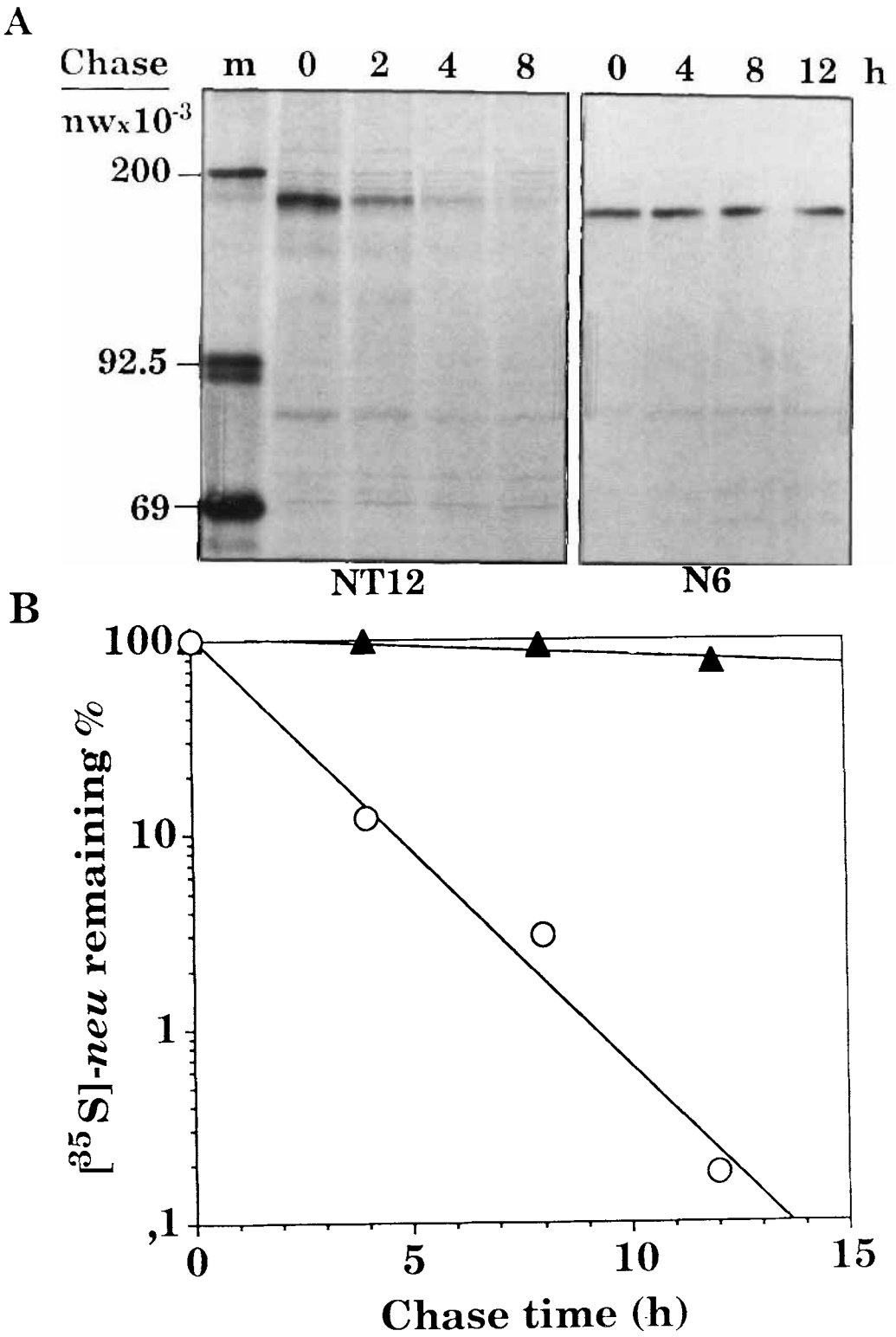

Fig. 2. Half-lives of the *neu* protein and oncoprotein in NIH 3T3 cells. The N6 and NT12 cells expressing the *neu* protooncogene and oncogene, respectively, were labeled to isotopic equilibrium with [^{35}S]methionine and transferred to a medium containing excess nonradioactive methionine for the indicated times of chase. Lysed cells were immunoprecipitated with the anti-*neu* antiserum and the immunoprecipitates were analyzed by SDS-PAGE (**A**). Radioactivity in the p185^{neu} polypeptide band was quantitated by densitometric scanning from three independent experiments and the mean values were plotted against chase time (**B**). Closed triangles, normal *neu* protein; open circles, *neu* oncoprotein.

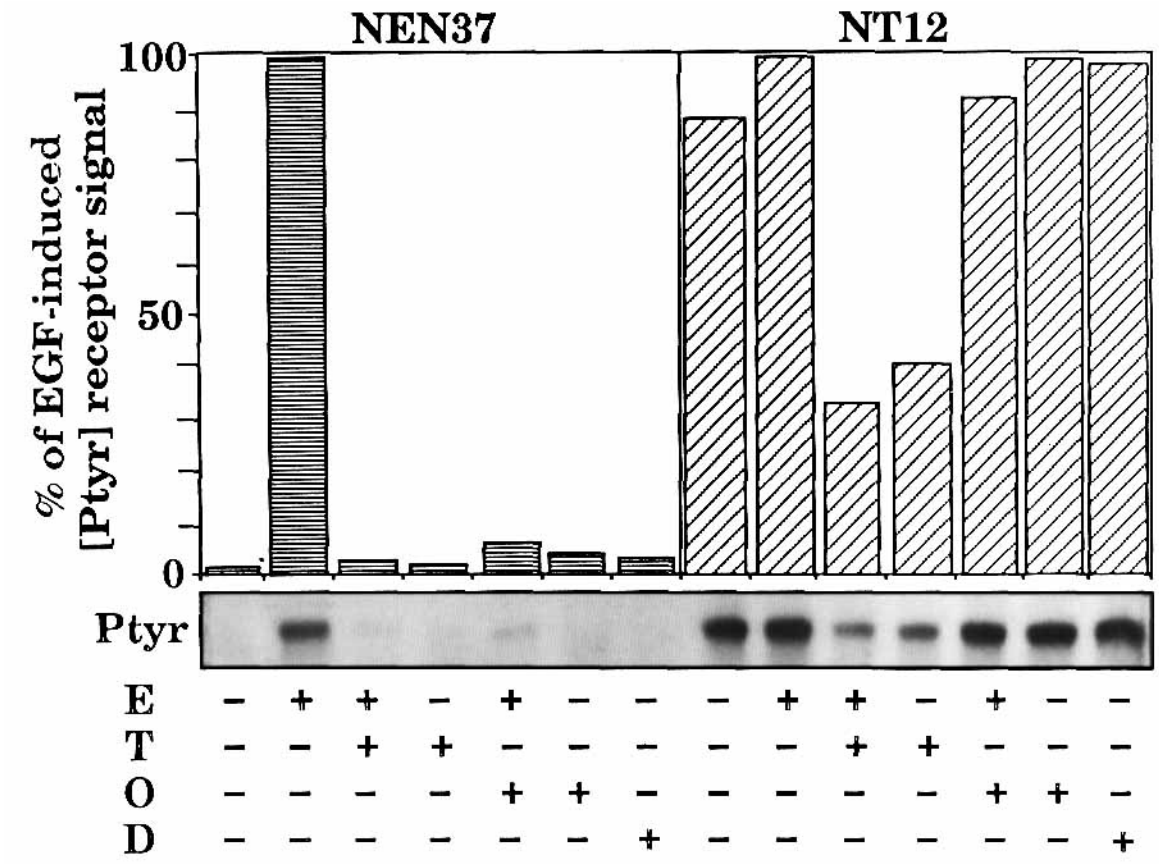

Fig. 3. Effect of TPA and okadaic acid on the tyrosine phosphorylation of the EGFR/*neu* receptor and the *neu* oncoprotein. The cells were plated at a density of 5 × 10^5/5 cm plate and serum-starved for 24 hr before treatment with 100 nM TPA (T), 1 μM okadaic acid (O) or DMSO (D), used as a solvent for the tumor promoters. EGF (E) (3 nM) was added to the cultures 30 min after the addition of TPA or okadaic acid and the cells were lysed 5 min later. Immunodetection of phosphotyrosine-containing EGFR/*neu* receptor and *neu* oncoproteins was performed as described in Materials and Methods. The bars show relative signal intensities from 300 μg cell protein in the different lanes.

scription factors N10 and *jun*B [38,49]. In the HB cells, N10 mRNA was maximally about ninefold induced at 1 hr after the addition of PDGF, whereas in the *neu*-transformed LTR-NT7 cells there was an almost total loss of PDGF and serum responses for N10 (about 1.5-fold maximal induction) (Fig. 5). PDGF enhanced *jun*B mRNA about threefold and 1.6-fold in the HB and LTR-NT7 cells, respectively (Fig. 5B, bottom panel). Thus most of the response of these transcription factor mRNAs to PDGF was lost in the *neu*-transformed cells.

Expression of the c-*jun* Protein in Normal and *neu* Oncogene-Expressing Cells

Both TPA and the *neu* oncoprotein were capable of blocking the induction of immediate early mRNAs in the transfected cells. Earlier studies have shown that TPA-treated as well as *neu* and c-Ha-*ras* oncogene-transformed cells have an elevated AP-1 transcription factor activity [20,22,50]. The *jun* and *fos* proteins are major components of this factor, which binds to a TPA-responsive enhancer element (TRE), a consensus sequence in a variety of enhancers [51]. However, our earlier studies indicated that the biosynthesis of c-*jun* or c-*fos* proteins was not markedly enhanced above the control level when they were immunoprecipitated from cell lysates metabolically labeled for 45 min [20]. Since the *jun*/AP-1 protein has a short biological half-life [52,53], it was possible that a difference of protein turnover could cause major differences in the steady-state protein levels. In this study, we therefore examined the biosynthesis of c-*jun* protein ($p39^{jun}$) from cell lysates metabolically labeled for 3 hr in the presence or absence of 10 nM EGF. Figure 6A shows that the amount of c-*jun*/AP-1 protein in serum-starved *neu* oncogene-transformed NT11 cells was about fourfold elevated compared with serum-starved EGFR/*neu* receptor-expressing NEN37 cells. In

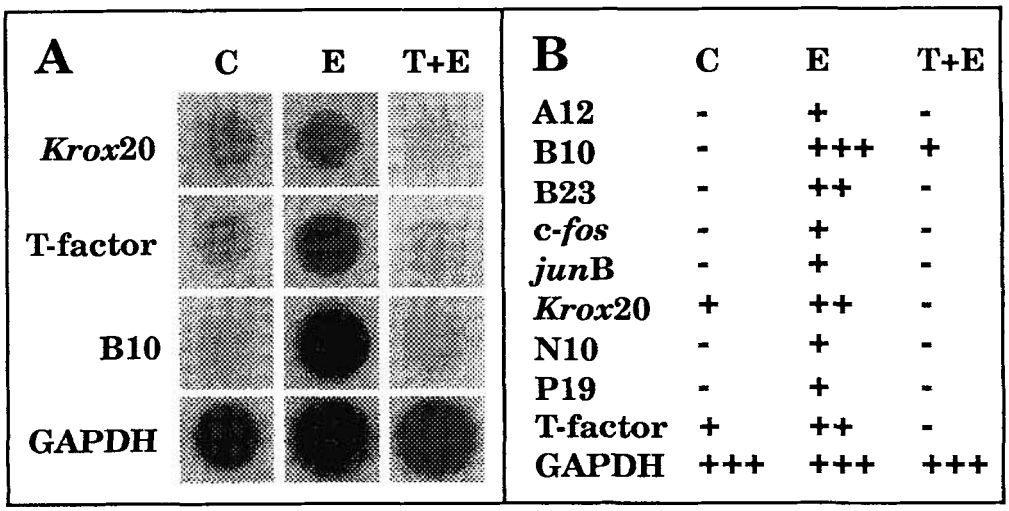

Fig. 4. A, B: TPA inhibition of EGF-inducible genes. Quiescent NEN37 cells were stimulated with EGF for 30 min (E) with or without a 24 hr TPA pretreatment (T). Isolated mRNA was labeled into complementary DNA and hybridized to Gene Screen Plus filters containing cDNA clones of serum-inducible genes [24]. C; hybridization with labeled cDNA from untreated cells.

serum-starved *neu* and c-Ha-*ras* oncogene-expressing NEN37 derivatives (LTR-NT7 and EJ10) the c-*jun*/AP-1 synthesis was also about threefold elevated compared with normal NEN37 cells. Figure 6A further shows that, although the EGF-treatment enhanced the metabolic labeling of c-*jun*/AP-1 protein about sixfold in NEN37 cells, the corresponding increase was only 1.5-fold both in *neu* and c-Ha-*ras* oncogene-expressing LTR-NT7 and EJ10 cells.

To measure the half-life of the c-*jun* protein we incubated the NEN37 and NT12 cells for 6 hr with radioactive methionine in order to label the c-*jun* protein to isotopic equilibrium and subsequently chased the label with nonradioactive methionine for the time periods indicated in Figure 6B,C. Scanning densitometry of immunoprecipitated c-*jun* polypeptides showed that the half-life of the c-*jun* protein in the NEN37 cells was less than 2 hr and in the NT12 cells the half-life was longer than 4 hr (Fig. 6C). This difference in polypeptide turnover could thus explain part of the elevated levels of the c-*jun* protein in the *neu*-transformed cells.

DISCUSSION

Delineation of extracellular and intracellular signals that stimulate cells to proliferate in an uncontrolled manner might give insights into the multiple pathways that normally control cell growth. In the present study, we wanted to examine how the *neu* oncogene interferes with the activation of gene expression through growth factor receptors. For comparison of gene regulation in *neu*-transformed and *neu* ligand-stimulated cells, we exploited cells bearing a chimeric EGFR/*neu* receptor, which we have previously characterized in detail [16–19]. This receptor is capable of delivering a signal for the activation of immediate early growth factor-responsive genes, such as c-*jun*, *jun*B, and c-*fos* as well as delayed early genes such as the glucose transporter and ornithine decarboxylase [20].

Our data confirm earlier reports showing that the *neu* oncoprotein is a constitutively active tyrosine kinase, which has an accelerated turnover rate [45,54,55]. Kokai and coworkers [56] as well as Stern et al. [9] have demonstrated that EGFR mediates phosphorylation of $p185^{neu}$ at tyrosine as well as serine/threonine residues in Rat-1 cells without a concomitant activation of the *neu* tyrosine kinase. Stern et al. [45], Di Marco et al. [6], and Epstein et al. [57] have shown that normal $p185^{neu}$ contains phosphotyrosine when expressed above a treshold level. However, at modest expression levels, the amount of phosphotyrosine is small in comparison with the *neu* oncoprotein [5,54,58], as is evident from our Western blotting results. Our results also show that the endogenous mouse EGFR of NIH 3T3 cells (about $5–10 \times 10^3$ receptors/cell) is not detected in these conditions [18,20]. The low number of EGFRs may also explain why *neu* phosphorylation in trans by the EGFR was not observed.

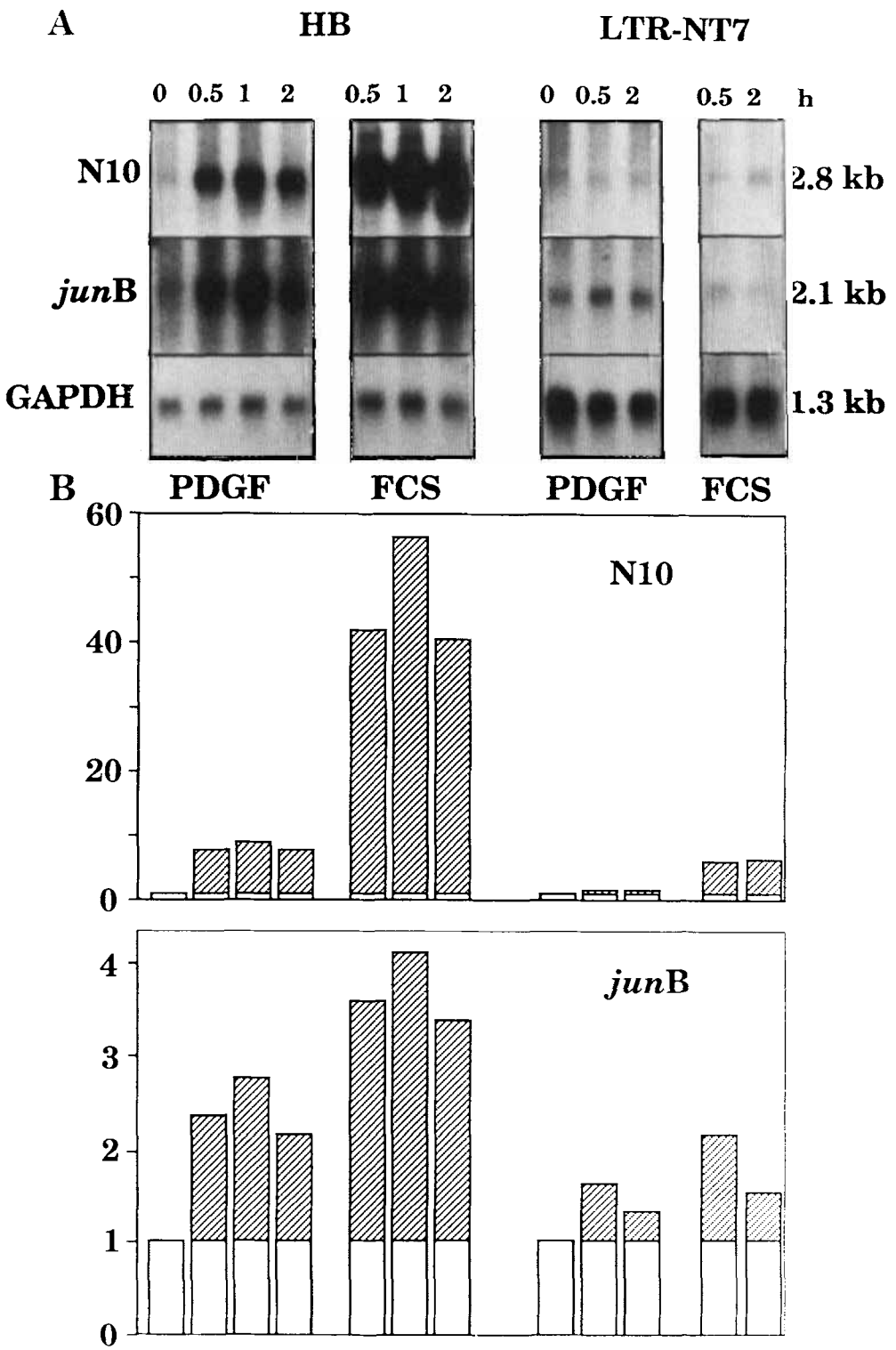

Fig. 5. PDGF and serum inducibility of N10 and *junB* mRNAs in control cells (HB) and cells expressing the *neu* oncogene (LTR-NT7). The cells were serum-starved overnight and stimulated with 10 ng/ml PDGF or 20% serum for the indicated periods of time. Polyadenylated RNA was analyzed by Northern blotting and hybridization with the N10 and *junB* cDNA probes and as a control for the amount of RNA loaded with the invariant GAPDH probe. The hybridization results are shown in **A**, and the columns in **B** show the quantitation of the corresponding signals. The shaded portions of columns indicate the -fold of induction above the basal level (normalized to 1). The scanned values were corrected against the GAPDH hybridization signals from the same blots. Note the loss of PDGF and serum responses for N10 mRNA and the partial inhibition of *junB* mRNA induction.

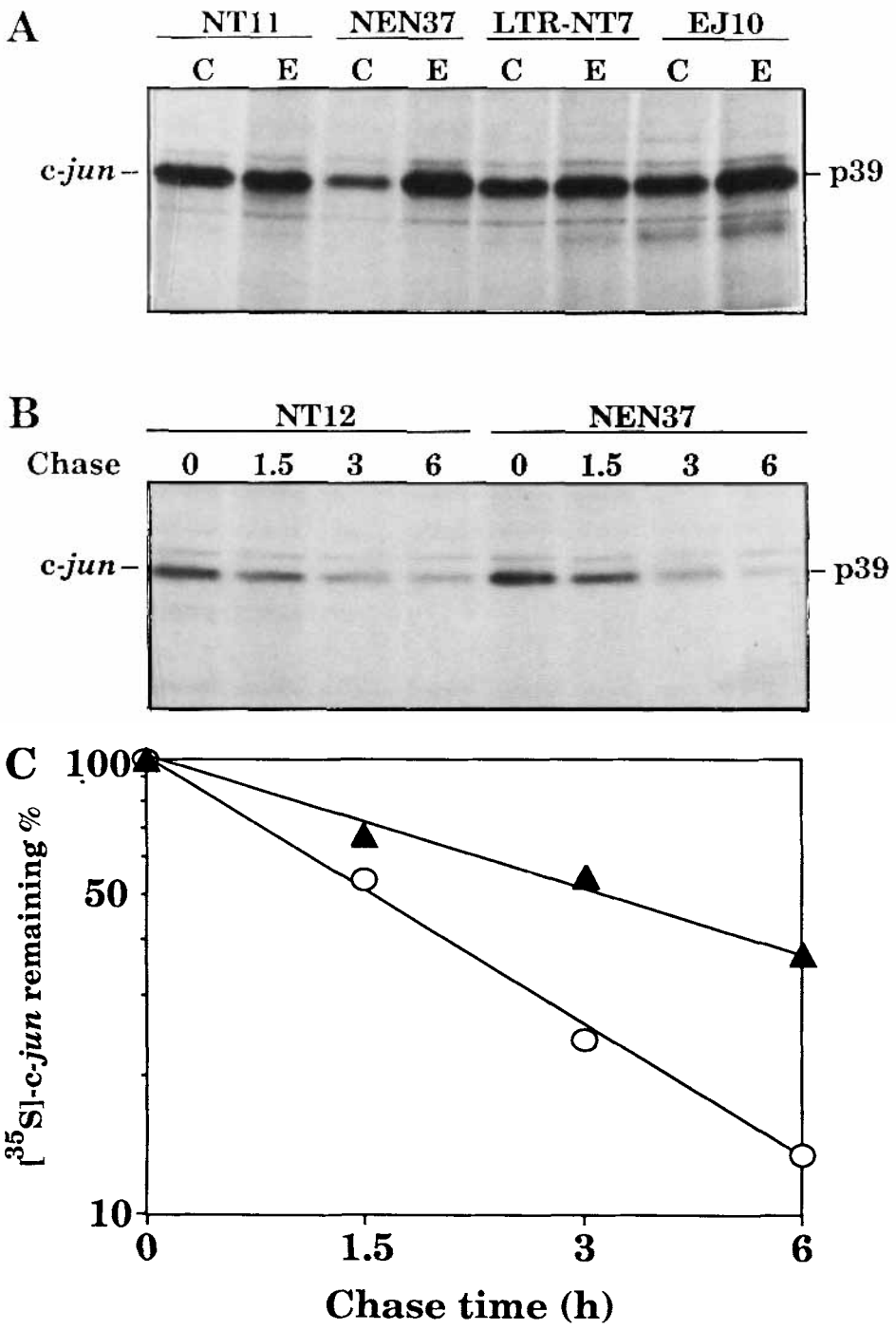

Fig. 6. **A:** Immunoprecipitation of *jun* protein ($p39^{jun}$) from serum-starved (C) and EGF-treated (E) cells. Serum-starved NT11 cells expressing the *neu* oncogene, NEN37 cells expressing the chimeric EGFR/*neu* protein, and derivatives of the latter expressing the *neu* oncogene (LTR-NT7) or the c-Ha-*ras* oncogene (EJ10) were treated with 10 nM EGF and labeled with [^{35}S]methionine for 3 hr. Cell lysates were immunoprecipitated with the rabbit anti-*jun* peptide antibodies and analyzed via 10% SDS-PAGE. **B,C:** Turnover of *jun*-protein in *neu* oncogene-transformed and normal cells. Serum-starved NT12 and NEN37 cells expressing the *neu* oncoprotein and the EGFR/*neu* receptors, respectively, were labeled with [^{35}S]methionine for 6 hr and then incubated for the indicated times of chase in medium containing excess nonradioactive methionine. Lysed cells were immunoprecipitated with the anti-*jun* antibodies, and the immunoprecipitates were analyzed by 10% SDS-PAGE (B). Radioactivity in the p39 polypeptide band was quantitated by densitometric scanning from two independent experiments, and the mean values were plotted against chase time (C). Closed triangles, NT12 cells; open circles, NEN37 cells.

Several other phosphotyrosine-containing polypeptides were observed in cells expressing the recombinant receptors. Some of these may represent substrates for the *neu* tyrosine kinase, and their enhanced phosphorylation in the *neu* receptor-expressing cells in comparison with EGFR expressing cells may indicate differences in substrate specificities between the two receptors. Interestingly, the molecular weights of the major P-tyr containing polypeptides of 145,000 and 124,000, specific for NT12 cells are very similar to the molecular weights of the recently discovered physiologically interesting substrates for the PDGFR and EGFR. These include phospholipase Cγ (M_r 145,000 [59]) and the GTPase activating protein (GAP; M_r 124,000 [60]) [61,62]. Also, the M_r 84,000 phosphotyrosyl polypeptide, which is detected in the NT12 cells and whose phosphorylation is increased in EGF-treated cells expressing the EGFR/*neu* receptor migrates with the mobility of the PI kinase, which is a known substrate for the PDGFR [63]. The definitive identification of the polypeptides phosphorylated in our transfected cells will require further work using specific antibodies.

Receptor down-regulation and degradation have been reported to be typical for ligand-activated growth factor receptors having tyrosine kinase activity [64,65]. The half-lives were determined by equilibrium metabolic labeling and pulse-chase experiments. In previous studies the half-life for the *neu* protooncogene-encoded protein has been estimated to be about 7 hr in DHFR-G8 cells and for the *neu* oncogene about 1.5 hr in the transformed B104-1-1 cells [45]. Our results are consistent with these observations, showing a prolonged half-life for the normal *neu* protein and only about 1.5 hr for the *neu* oncoprotein.

Phorbol ester tumor promoters have been reported to block tyrosine-specific phosphorylation of the EGFR via activation of protein kinase C, which phosphorylates a target threonine residue (Thr 654) in the juxtamembrane region of the EGFR [46]. Phorbol ester treatment also leads to abolition of high-affinity binding sites of EGFR [66] and chimeric HER1-2 receptors [67]. In our experiments TPA pretreatment blocked the *neu*-specific kinase activity stimulated by EGF. However, tyrosyl phosphorylated *neu* oncoprotein was about 50% decreased in TPA-treated cells, although earlier studies of Dobashi et al. [68] using immunoprecipitation of *neu* protein from ^{32}P-labeled cells and immunocomplex kinase reactions showed that TPA affects only the normal *neu* protooncogene-encoded protein but not the oncoprotein. Our own results are likely to represent better the situation in intact cells, in that the treated cells were directly lysed in denaturing conditions for analysis. Interestingly, in our experiments the phosphatase inhibitor okadaic acid did not affect phosphotyrosine in the *neu* oncoprotein. However, okadaic acid was as effective as TPA in preventing EGF-induced tyrosyl phosphorylation of the EGFR/*neu* receptor. Therefore, the activities of the ligand-dependent *neu* tyrosine kinase and the *neu* oncoprotein tyrosine kinase are differentially inhibited by TPA and okadaic acid, which act by different routes [24,25].

Besides the inhibition of receptor tyrosyl phosphorylation upon EGF binding, long-term TPA treatment known to down-regulate protein kinase C [48] also prevented the induction of several rapidly activated mRNAs in the EGF-treated cells. Hybridization of reverse transcriptase-labeled cellular mRNA to cDNAs representing serum-inducible genes showed that EGF stimulation of serum-starved NEN37 cells expressing EGFR/*neu* chimeric receptors resulted in a sequential activation of various mRNAs including the *jun*B and N10 mRNAs. In the cells which had been exposed to TPA most of these mRNAs no longer responded in a detectable manner.

We were also interested in comparing the induction of *jun*B mRNA and several other serum-stimulated mRNAs in response to growth factor stimulation in *neu* oncogene-transformed cells. However, because the *neu* oncoprotein kinase activity did not effectively respond to EGF or TPA, we instead used PDGF and serum, which are strong inducers of serum-regulated immediate early mRNAs [37,38]. Strikingly, our findings indicate that the *neu* oncoprotein blocks the signals needed for immediate early mRNA induction. There was a loss of mRNA induction by PDGF and serum in *neu* oncogene-expressing cells. For example, although there was a ninefold induction of the N10 mRNA after PDGF treatment of the HB cells, the N10 mRNAs was barely detectable in PDGF-treated LTR-NT7 cells.

Subsequently, we studied the biosynthesis of the proteins encoded by genes whose expression was significantly increased by the ligand-activation of *neu* tyrosine kinase and blocked by the

neu oncoprotein. The expression of the *jun* and *fos* oncogene-encoded polypeptides was analyzed by immunoprecipitation of serum-starved and EGF-stimulated cells expressing the *neu* oncogene. As expected on basis of the results of mRNA analysis of the *neu* oncogene-transformed cells, the biosynthesis of the *jun* protein was only slightly elevated in the *neu* oncogene-expressing cells labeled for 45 min [20]. Serum-starved NT11 and c-Ha-*ras* oncogene-transformed cells, which were labeled for longer time periods (e.g., 3–6 hr) showed, however, clearly elevated amounts of *jun* protein compared to the similarly treated NIH 3T3 cells or NEN37 cells. EGF treatment enhanced only slightly the synthesis of the *jun* and *fos* proteins in NIH 3T3 and NT11 cells, whereas the synthesis of these proteins was highly increased on EGF treatment of the NEN37 cells. These data indicated that the half-life of the *jun* protein may be altered in oncogene-transformed cells. This was confirmed in pulse-labeling experiments. The reported half-lives of *jun* protein vary greatly from 1.5–6 hr in different cells [52,53]. There was a detectable accumulation of the *jun* protein due to its prolonged half-life in c-Ha-*ras* and *neu* oncogene-transformed cells (over 4 hr) compared with the corresponding protein precipitated from the EGFR/*neu* expressing NEN37 cells (half-life less than 2 hr). The *jun* and *fos* proteins are components of the activator protein-1 (AP-1) complex, which binds to TRE element present in promoter regions of various TPA-inducible genes [51,69,70]. Interestingly, many of these TPA-inducible genes are also activated by the c-Ha-*ras* oncogene apparently through the TRE element [71]. Our own studies have indicated that the *neu*-transformed cells also have an increased AP-1 activity [20].

Although comparisons of gene expression between mitogen-stimulated normal cells and transformed cells must be interpreted with caution, our experiments suggest that the down-regulation of the immediate early mRNA response in the *neu* oncogene-expressing cells concerns both the PDGF receptor tyrosine kinase-mediated signals and the more generalized activation signals obtained by serum stimulation. This down-regulated response is not the result of a lack of the growth factor receptors from the cell surface as shown by radioactive ligand binding assays (L.L., Monica Nister et al., unpublished data), nor can the inhibition be explained solely on the basis of differences in the cell cycle parameters. A more likely explanation is that certain transcription factor complexes are modified in the oncogene-expressing cells. This could be reflected, e.g., as an increased half-life of the *jun* proteins, and it could also result in deregulation of the promoter functions of the immediate early genes, which are known to be affected by complex feedback mechanisms involving the AP-1 complex. The effects of TPA and oncogene expression could thus mimic each other and cause the transcriptional shut-off of a large part of this set of genes, while allowing the expression of others, such as GT and ODC, which characterize the transformed phenotype, including *neu*-transformed cells. Delineation of the responsible controlling elements will characterize the transformed phenotype at the level of transcriptional deregulation of cellular genes.

ACKNOWLEDGMENTS

We thank Drs. Daniel Nathans and Robert Weinberg for molecular clones; Drs. Hirota Fujiki and Takashi Sugimura for okadaic acid; Dr. Catherine Legraverend for help in some of the experiments; and Elina Roimaa, Raili Taavela, and Tapio Tainola for excellent technical help. This work received financial support from the Finnish Cancer Organizations, Academy of Finland, the Sigrid Juselius Foundation, Orion Corporation Research Foundation, and Research Science Foundation of Farmos.

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