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Regulation by EGF Is Maintained in an Overexpressed Chimeric EGFR/*neu* Receptor Tyrosine Kinase

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The effects of a ligand regulated *neu* tyrosine kinase were examined in NIH 3T3 cells. A chimeric construct encoding the human EGF receptor extracellular domain fused to the tyrosine kinase domain of the rat *neu* cDNA was expressed under the transcriptional control of the Moloney murine leukemia virus LTR promoter. This resulted in higher levels of expression of the chimeric receptor than were previously obtained from the SV40 virus early promoter in the same cells. The chimeric receptor showed strict ligand-dependent tyrosine kinase and signal transducing activities for the induction of growth-regulated biochemical activities and DNA synthesis in resting cells. The ligand-activated cells became morphologically transformed and grew in agar in the presence of EGF and TGFβ as efficiently as did the ligand-independent *neu* oncogene-transformed cells. Our results establish similarities between the signal pathways of the EGF receptor and the *neu* tyrosine kinase.

Key words: ligand-dependent transformation, signal transduction, TGF-beta, ornithine decarboxylase, glucose transport

Several recently cloned tyrosine kinase genes have been found to encode previously uncharacterized polypeptides. The need for the definition of the functions for receptor-type tyrosine kinases is especially obvious, because these kinases may turn oncogenic via somatic mutations and can be regulated by ligands, which allows studies of their physiology in complex organisms. Among a dozen receptor tyrosine kinases whose amino acid sequences have been deduced from cDNA, the ligand is known only for five, namely, those encoding receptors for EGF, PDGF-A, PDGF-B, CSF-1, insulin, and

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FCS, fetal calf serum; LTR, long terminal repeat; MuLV, Moloney murine leukemia virus; ODC, ornithine decarboxylase; PTyr, phosphotyrosine; TGF, transforming growth factor.

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IGF-1 [1]. The *neu* oncogene encodes the closest homologue of the EGF receptor [2,3], but in the absence of a known ligand, no function for *neu* has been defined. Only oncogenic activities of *neu* have been characterized [4]. This gene, when activated by a point mutation in the transmembrane domain of the receptor protein, transforms various cell types and causes polyclonal mammary carcinomas in transgenic mice [5]. Amplification of the human homologue of rat *neu* (*erb*B2/HER2) in mammary carcinomas suggest that overexpression of the *erb*B2 oncoprotein may be involved in the mammary carcinogenesis also in humans [6–9].

We have linked the ligand-binding extracellular domain of the EGF receptor to the *neu* tyrosine kinase and used this construct to study the functions of *neu* [10,11]. In the initial study, we used an SV40 virus early promoter for expression of the fusion construct in NIH 3T3 mouse fibroblasts to obtain receptor levels sufficient for cell transformation: generally, $1–5 \times 10^5$ receptors per cell. We now report on expression of the EGFR/*neu* hybrid protein using the Moloney murine leukemia virus (MuLV) LTR promoter, which regularly drives transcription to higher levels than does the SV40 early promoter. Ligand control of the *neu* tyrosine kinase and several steps of possible signal transduction pathways were studied.

MATERIALS AND METHODS

Cells and Transfections

The NIH 3T3 cells (ATCC No. CLR1658) were transfected according to the calcium phosphate precipitation technique [12]. The NN cells expressing only the neomycin-resistance marker gene were used as control cells in most experiments, since all transfections included the marker plasmid pSV2*neo* (ATCC no. 37149). The NEN cell clones [10] express various levels of the chimeric EGFR/*neu* protein: NEN7 (2×10^6 receptors/cell), NEN16 (4×10^5 receptors/cell), NEN37 (4×10^5 receptors/cell), and NEN49 (5×10^5 receptors/cell). The N6 cells express the *neu* proto-oncogene (LTR*neu*N), whereas the NT11 and NT12 cells express the *neu* oncogene differing from the proto-oncogene by a single-point mutation in the transmembrane domain (SV2*neu*NT [13]). The Cl25 cells are NIH 3T3 derivatives having approximately 4×10^5 human EGFR per cell [14].

The cells were grown at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and antibiotics. For selection of the transfectants, neomycin (G418; GIBCO) was added at a concentration of 200–400 μg/ml.

Molecular Clones

Moloney murine leukemia virus LTR from the plasmid pMP-1 [15] was ligated to the PvuII site of pSP72 (Promega Biotec) cloning vector to get the plasmid pLTRP, which was used as a donor for subsequent clonings. The hybrid EGFR/*neu* construct and SV40 polyadenylation sites were taken from pSV2EGFR/*neu* [10] and linked downstream of the promoter in pLTRP to get the expression vector pLTR EGFR/*neu*. Similarly, the proto-oncogene *neu* cDNA and SV40 polyadenylation site from plasmid pSV2*neu*N [13] were transferred to pLTRP to get the expression plasmid pLTR*neu*N.

Metabolic Labeling, Immunoprecipitation, and Immunoblotting

Labeling with [^{35}S]methionine and immunoprecipitation were done as described before [10]. Exponentially growing cells were grown first for 48 h in 10% FCS and then

in 1% FCS for 24 h. Cells were treated with 10% serum, 50 μM Na_2VO_4 or 1.6 nM EGF (Collaborative Research), as detailed in the Results, before their solubilization in the sample buffer. Electrophoresis, blotting, and probing with anti-phosphotyrosine (PTyr) antibodies were done as described [16].

Analysis of DNA Synthesis

Cells were seeded in small plates at 10^5 cells per plate. After overnight incubation in DMEM containing 10% FCS, cells were washed twice and then incubated in medium containing 1% FCS for 24 h. DNA synthesis was stimulated by addition of 10 nM EGF and was monitored 12–24 h later by measurement of bromodeoxyuridine incorporation using a commercial cell proliferation kit (Amersham RPN.20).

Soft-agar Assay

Subconfluent cells were trypsinized and plated at 2×10^4 cells into 24-well plates (Flow Laboratories) in 600 μl DMEM containing 10% FCS and 0.25% agar (Difco) on a bottom layer of 0.5% agar in 1 ml DMEM. The agar contained 3 nM EGF and/or 10 ng/ml TGFβ (R&D Systems Inc.), as detailed in the Results. Colonies of 50 or more cells were counted 14 days later.

Assay of 2-Deoxyglucose Uptake

$1–2 \times 10^5$ cells were grown for 24–48 h, in DMEM supplemented with 10% FCS, then kept in 1% FCS for 48 h prior to the analysis of 2-deoxyglucose (2-DOG) uptake according to Flier et al. [17]. The cells were washed three times with PBS and then incubated in glucose-free DMEM supplemented with 0.1% BSA and 100 nM 2-DOG for 5 min at 37°C. Then, 0.5 μCi [^{3}H]2-DOG (17 Ci/mmol, Sigma) was added, and the uptake was interrupted 5 min later by the addition of ice-cold PBS containing 0.3 mM phloretin (Sigma). The cells were lysed in 0.1 M NaOH and neutralized with 0.1 M HCl prior to measurement of radioactivity by liquid scintillation counting. The 2-DOG uptake was related to the total protein content of the cell lysates, which was measured with the Bio-Rad Protein Assay Kit using BSA as a standard.

Measurement of Ornithine Decarboxylase Activity

About 2×10^5 cells were seeded and grown in DMEM containing 10% FCS for 48 h prior to the transfer of the cells into the low-serum medium (0.5% FCS). After a further 48 h incubation, EGF was added to a final concentration of 5 nM. At the various time points (0, 2, 4, 8, and 24 h), the cells were harvested by scraping and assayed for ornithine decarboxylase (ODC) activity essentially as described earlier [18].

RESULTS

To study the properties of the *neu* tyrosine kinase, a chimeric EGFR/*neu* receptor construct was expressed in NIH 3T3 cells, which have a very low number of endogenous EGFR on their surface [10]. Figure 1 shows the results of anti-phosphotyrosine immunoblotting of NIH 3T3 cells expressing 5×10^5 (clone NEN49), 4×10^5 (clone NEN16), or 2×10^6 (clone NEN7) chimeric EGFR/*neu* receptors/cell. The cells were treated with EGF, FCS, or with sodium orthovanadate, an inhibitor of phosphotyrosine phosphatases (see [19]) before cell lysis, electrophoresis, and immunoblotting. Anti-

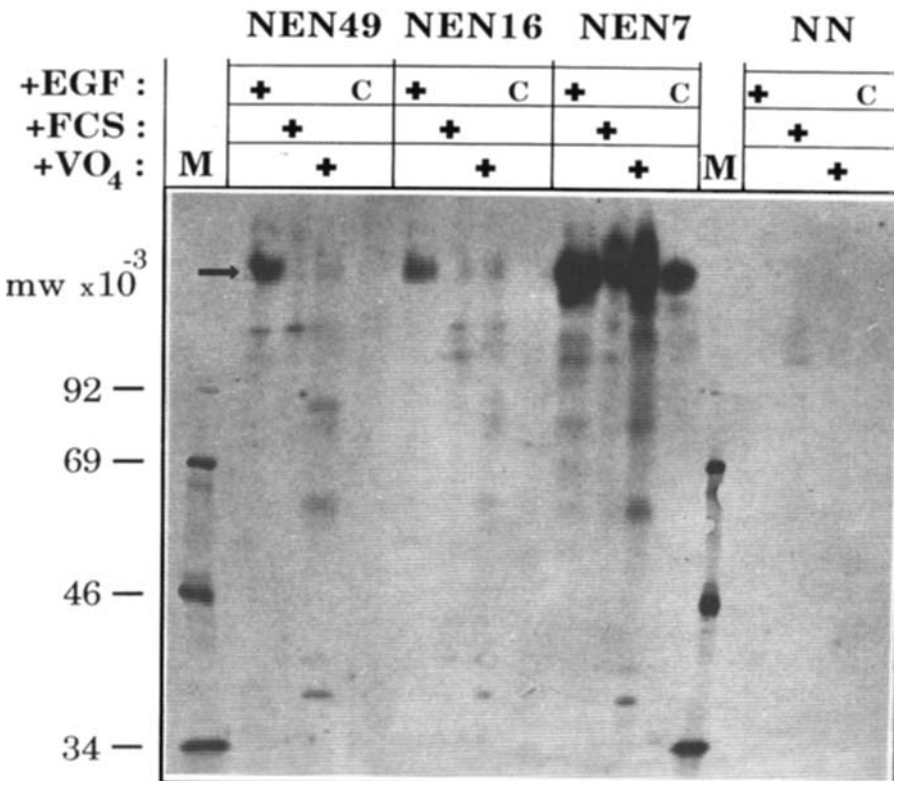

Fig. 1. Detection of tyrosine phosphorylated proteins in EGFR/*neu*-expressing cells. Serum-starved cells were left untreated (C) or treated with either 1.6 nM EGF for 5 min, 10% FSC for 12 h, or Na_2VO_4 for 16 h before solubilization and immunoblotting with anti-Ptyr antibodies [15]. The band corresponding to the mobility of the chimeric EGFR/*neu* protein is marked by an arrow.

phosphotyrosine staining shows that the cells of clones NEN49 and NEN16 contain tyrosine-phosphorylated EGFR/*neu* receptor polypeptides (arrow) only when stimulated with EGF. In contrast, the NEN7 cells also show an increased basal level of tyrosine phosphorylation of the chimeric receptor, and this phosphorylation is further increased in cells exposed to vanadate, serum, or EGF. Thus, the *neu* tyrosine kinase of the NEN7 cells expressing the highest levels of the chimeric receptor is not strictly controlled, but is active also in the absence of ligand. This result is consistent with our earlier observations based on anti-phosphotyrosine immunoprecipitation [10]. However, we can also immunoprecipitate small amounts of the chimeric receptor from a larger amount of unstimulated cells (unpublished data of C. Legraverend and the authors), suggesting that the difference between the stimulated and unstimulated levels of receptor phosphotyrosine is quantitative rather than qualitative.

The retroviral long terminal repeat element from the Moloney murine leukemia virus has been found to direct a high level of expression of genes under its control in various cells. We took advantage of the MuLV LTR vector, which has been used by Schwab et al. [15]. The LTR element and the EGFR/*neu* cDNA including the SV40 polyadenylation signals were ligated to each other in the polylinker of the pSP72 cloning vector as shown in Figure 2. The resulting plasmid was transfected to the NIH 3T3 cells

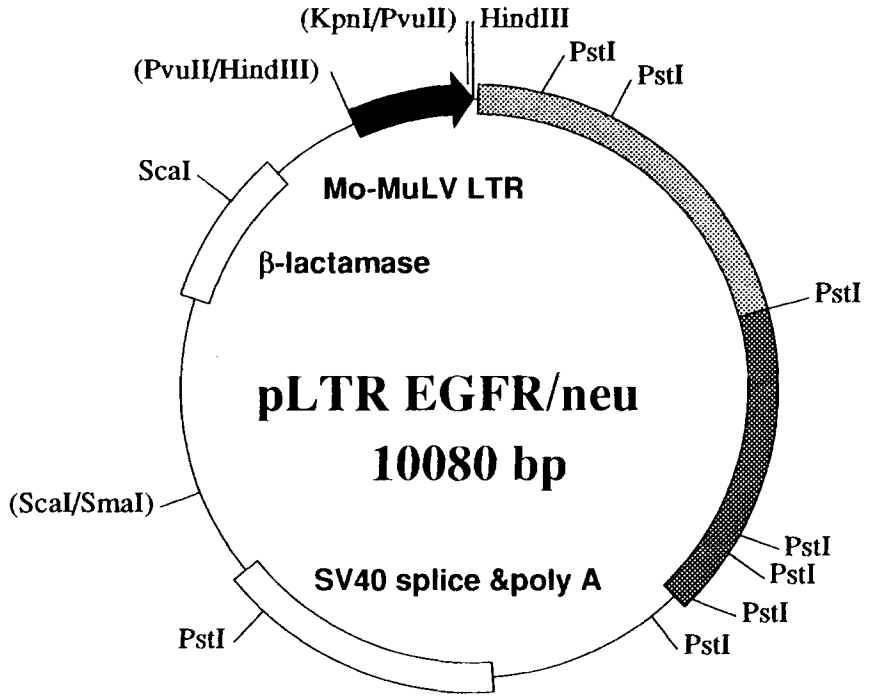

Fig. 2. The structure of expression vector pLTR EGFR/*neu*. The EGFR/*neu* hybrid cDNA (light grey: EGFR-coding region; dark grey: *neu*-coding region) and SV40 splice and polyadenylation sequences were transferred as HindIII-ScaI fragment to pSP72 (Promega Biotec) cloning vector where the MuLV LTR containing fragment (black section) had been placed at the end of the polylinker.

together with pSV2*neo* DNA. Neomycin-resistant clones were screened for expression of the fusion protein by immunofluorescence staining using rabbit antisera raised against a bacterially expressed 142 amino acid peptide derived from the *neu* carboxyl terminus. The amount of metabolically labeled receptor polypeptides in the receptor-positive LTR EN1 and LTR EN2 cell clones are compared in Figure 3 to the amount in the NEN7 cells where expression is driven to exceptionally high levels by the SV40 early promoter [10]. On the basis of the immunoprecipitation result shown in Figure 3, we estimate that the LTR EN1 and LTR EN2 clones express 30 and 70%, respectively, of the amount of receptors present in the NEN7 cells. The figure further shows that essentially no receptor protein is precipitated with antibodies against phosphotyrosine from unstimulated LTR EN1 and LTR EN2 cells. The same was found in a longer exposure, where the unstimulated NEN7 cells showed a weak receptor band in the anti-PTyr precipitates (see also fig. 3 in [10]). On the basis of these results, receptor phosphorylation in the LTR EN1 and LTR EN2 cell clones is more tightly controlled than that in the NEN7 cells, and a significant increase in the PTyr content of the chimeric receptor occurs only in the presence of EGF.

Figure 4 shows the morphology of the LTR EN1 and LTR EN2 cells and control NIH 3T3 cells in the absence and presence of EGF. Similar to our earlier findings, the EGF-treated cells expressing the chimeric construct display highly refractile cell bodies and elongated extensions. These cells also pile up in clumps and are oriented in a

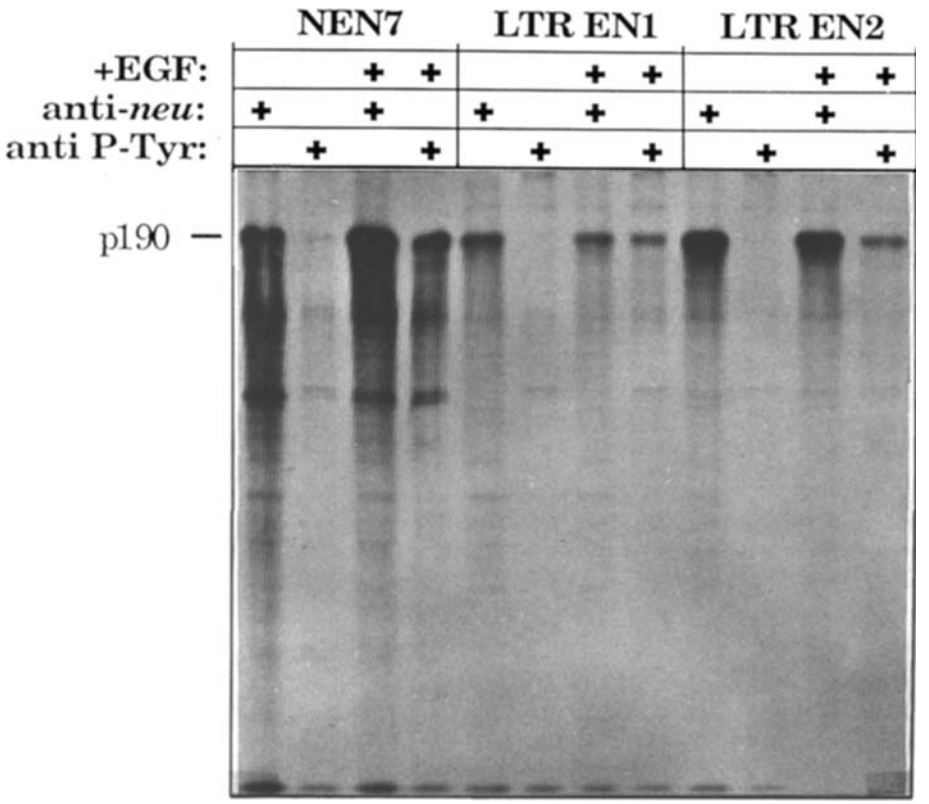

Fig. 3. Analysis of anti-phosphotyrosine (P-Tyr) immunoprecipitates from [^{35}S]methionine-labeled cells. Note that both anti-*neu* and anti-Ptyr antibodies precipitate the 190 kD EGFR/*neu* hybrid protein from the EGF-treated cells. No significant precipitation of p190 is seen in LTR EN cells in the absence of EGF. The p190 band is weakly visible also in the sample of untreated NEN7 cells in this exposure.

crisscross manner characteristic of transformed cells. As can be seen from Figure 5, the addition of EGF also induces DNA synthesis in the LTR EN1 and LTR EN2 cells, which were serum-starved for 24 h before this experiment. A lesser mitogenic response was obtained in the neomycin-resistant cell clone NN, which, similar to the parental NIH 3T3 cells, contains about 3,000 EGFR/cell [20].

Enhanced glucose transport and ornithine decarboxylase (ODC) activities characterize growth factor-stimulated and transformed cells [21,22]. Both parameters were efficiently regulated by EGF in cells expressing the chimeric receptor [11]. Figure 6 shows the time course of ODC enhancement in the LTR EN1 cells after EGF stimulation. It can be seen that the level of ODC activity after 24 h of treatment equals the constitutive activity seen in *neu* oncogene-transformed cells (clone NT12). In contrast, expression of the *neu* proto-oncogene (N6 cells) does not lead to elevated ODC expression (Fig. 6). Similar results were obtained for the glucose transporter activity. These are shown in Table I. Eight hours after stimulation, a three- to fourfold enhancement of glucose transport was observed in the EGF-treated LTR EN1 cells and the NEN37 cells, which express 4×10^5 receptors per cell. These values are very similar to those obtained from the Cl25 clone, which expresses about 3×10^5 EGF receptors per

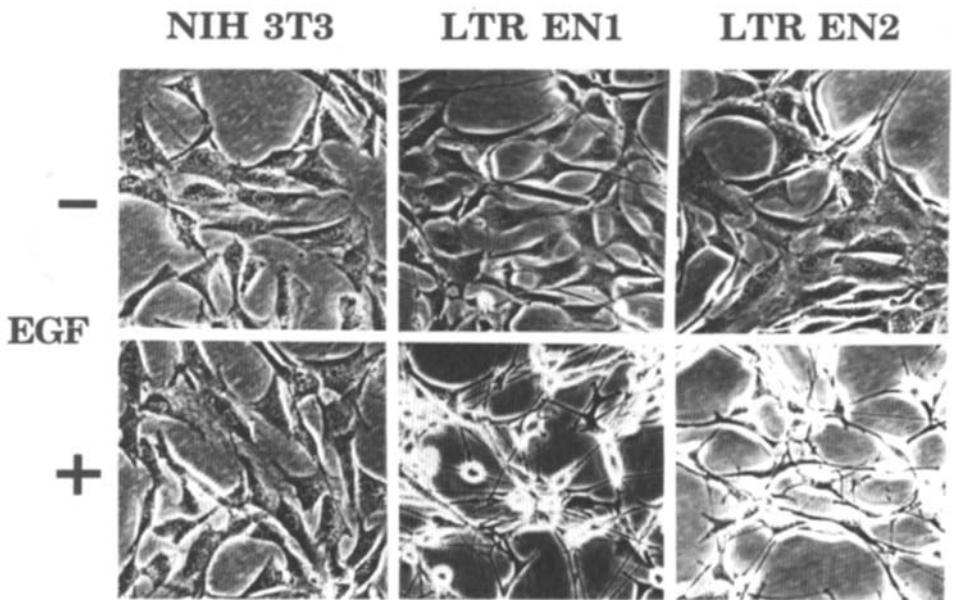

Fig. 4. Cell morphology in the presence and absence of EGF. The cells were grown for 2 days in the absence (−) or presence (+) of 3 nM EGF and photographed in phase-contrast microscopy. Note that EGF-treated LTR EN1 and LTR EN2 cells have highly refractile cell bodies characteristic of a transformed phenotype. The sides of the panels correspond to 280 μm.

cell. As can also be seen from Table I, the neomycin-resistant cell clone NN and the *neu* oncogene transformed NT11 cells had ligand-independent low and high glucose transport activities, respectively. Shown in parentheses for these latter two cells are the mean values obtained 8 h after serum stimulation. Significant stimulation with serum (about fivefold) is obtained only in the NN cells, whereas the glucose transport in the NT11 cells is not enhanced to the same degree (twofold stimulation).

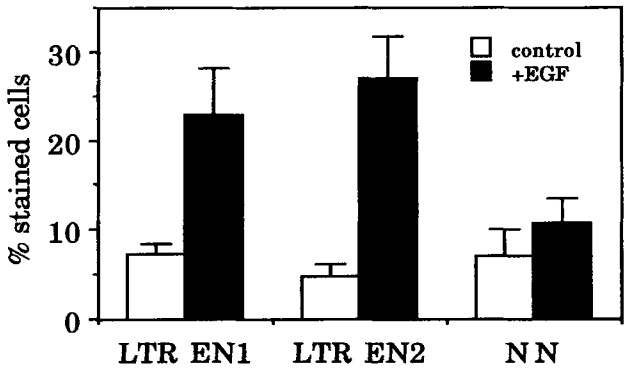

Fig. 5. Effect of EGF on DNA synthesis in the LTR EN cells. DNA synthesis rate was measured as percentage of cells stained with a monoclonal antibody to bromodeoxyuridine incorporated into DNA. Note that there is residual DNA synthesis in this experiment, where the LTR EN cells were first starved for 24 h without serum. However, the rate of DNA synthesis is increased three to fivefold after addition of EGF.

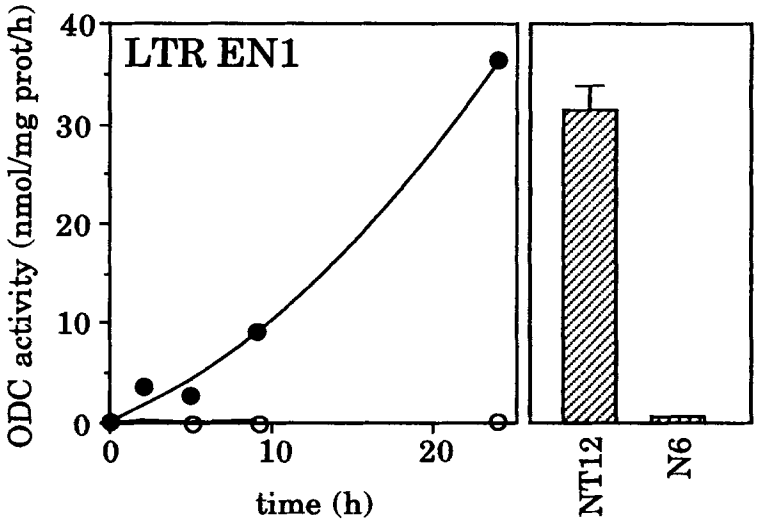

Fig. 6. EGF-dependent ODC activity in serum-starved LTR EN1 cells. Shown are the ODC activities of the LTR EN1 cells measured after various times of treatment with 5 nM EGF. It can be seen that a 24 h treatment with EGF increases the ODC activity to levels (closed circles) seen in the NT12 cells (column in the right-hand panel). The mean values of ODC activity in untreated LTR EN1 cells (open circles) and N6 cells (right-hand panel) were low irrespective of EGF addition.

TABLE I. Effects of EGF on 2-Deoxyglucose Uptake (cpm/min/μg Protein)*

	−EGF	+EGF
LTR EN1	9.0	30.1
NEN37	6.5	18.8
CL25	8.9	23.7
NN	5.9	5.7 (29.0)[a]
NT11	10.0	16.7 (20.3)[a]

*About 80% confluent cultures of cells were incubated in DMEM supplemented with 1% FCS for 48 h. EGF was added to a final concentration of 10 nM. After 8 h, the uptake of 2-DOG was measured as described in Materials and Methods. Mean values of at least two separate experiments are given.
[a]Values in parentheses are results from a parallel experiment where 10% dialyzed FCS was used instead of EGF.

Our earlier studies showed that the EGFR/*neu*-expressing cells grow in agar only in the presence of EGF [10]. The same applies also to the LTR EN1 and LTR EN2 clones (data not shown). However, as the number of clones obtained was relatively small in comparison with *ras* oncogene-expressing cells [10], we have subsequently carried out soft agar experiments in the presence of TGFβ. As can be seen from Figure 7, TGFβ in combination with EGF substantially increased the ability of EGFR/*neu* expressing cells to grow in soft agar, as is also known for the EGFR-expressing cells (for comparison, see Cl25 in Fig. 7). The number of soft agar colonies is also very similar in EGFR/*neu* expressing cell cultures supplemented with EGF and TGFβ and in *neu* oncogene-transformed cultures. However, as can also be seen from Figure 7, neither growth factor alone or in combination could stimulate the growth of NN or N6 cells in soft agar.

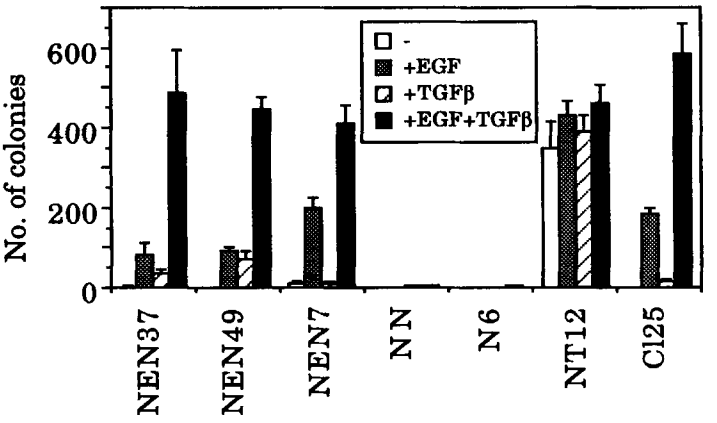

Fig. 7. Effects of EGF and TGFβ on soft agar growth. The cells were suspended and grown in soft agar for 14 days. Note that the addition of TGFβ enhances the effect of EGF. Mean values of at least three separate experiments done in duplicates are given.

DISCUSSION

In this paper, we have further characterized the functions of EGFR/*neu* hybrid protein. Most importantly, the use of the efficient MuLV LTR promoter allowed us to test the possibility that a high number of receptors leads to a deregulation of the hybrid EGFR/*neu* tyrosine kinase. This possibility was put forward by the observation that cells of the clone NEN7, which, under the control of the SV40 early promoter, express an unusually high number of receptors (about 2×10^6 receptors/cell), did not completely control the tyrosine kinase of their hybrid protein [10]. However, in the two MuLV LTR-driven clones, which expressed almost as many EGFR/*neu* hybrid receptors as cells of the clone NEN7, no receptor phosphotyrosine was detected in the absence of EGF.

We consider it likely that the aberrant behaviour of the NEN7 cells results from clonal variation and is not a property of moderately overexpressed EGFR/*neu* construct. On the other hand, the basal activity of the *neu* tyrosine kinase may be critically dependent on a threshold level of its expression, which may vary among different cell types. Sensitive techniques allowed Stern et al. [23] and Bargman and Weinberg [24] to demonstrate small amounts of phosphotyrosine in the experimentally amplified, normal *neu* protein in the G8 cells [25]. We can reproduce such findings using a substantially larger number of cells for immunoprecipitation (C. Legraverend and the authors, unpublished observations), but have here taken advantage of conditions where only an increase of receptor phosphotyrosine over the level seen in the G8 cells is detected.

We also examined the hexose transport and ornithine decarboxylase activities as measures of the function of the overexpressed receptor chimera and compared the activities to those in EGFR-expressing cells. About a threefold ligand-induced elevation of glucose transport was obtained in cells expressing either EGFR or two different cell surface concentrations of the chimeric receptor. Constitutively elevated levels of glucose transport corresponding to the induced levels in EGFR/*neu*-expressing cells were found in the *neu* oncogene-transformed cells and have commonly been seen in various transformation models [17,21] and after growth factor treatment of sensitive cells [26].

Accordingly, EGF stimulation of the glucose transport of the *neu* oncogene or proto-oncogene-expressing cells was enhanced less than twofold. ODC activity, on the other hand, is increased in general over tenfold in various transformed cells [22,27]. The activity of ODC in the LTR EN1 cells was enhanced well over 100-fold during 24 h after EGF stimulation. This was in concordance with the activity obtained in the NT12 cells as well as in stimulated in the NEN37 cells, which express smaller numbers of the chimeric receptors on their surface [11].

Our experiments to further characterize conditions of ancorage-independent growth of EGFR/*neu*-expressing cells showed that the ability of these cells to grow in soft agar is significantly improved if TGFβ is supplied in addition to EGF. The ability of TGFβ alone to maintain agar growth is low, suggesting that the two growth factors have synergistic effects, as in EGFR-overexpressing cells. In fact, the original description of TGFβ included its synergistic action with EGF to cause ancorage-independent growth of normal rat kidney fibroblasts in soft agar [28,29]. Thus, our results demonstrate similarities between the effects of the EGFR and *neu* tyrosine kinases.

These results show that the chimeric receptor establishes an EGF-dependent signal transduction pathway that we cannot yet distinguish from signal transduction by the EGF receptor. However, even minor differences could prove potentially significant in order to understand why there are two so closely related genes, EGFR (*erb*B/HER1) and *neu* (*erb*B2/HER2), and corresponding receptors in mammalian cells and tissues. A fundamental question is whether the signals induced by EGF and the *neu* ligand and transduced by the EGFR and the *neu* receptor converge to a common pathway that induces indistinguishable genomic responses or whether a cell expressing both receptors can distinguish between the two stimuli. Also, the patterns of expression of these genes differ and *neu* protein has been found in many nonproliferating cells in vivo [30]. Thus, there may be cell differentiation—rather than proliferation-associated functions—that distinguish between signals from the two receptors [30].

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