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A single autophosphorylation site confers oncogenicity to the Neu/ErbB-2 receptor and enables coupling to the MAP kinase pathway

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The transforming potential of the Neu/ErbB-2 receptor tyrosine kinase undergoes inactivation by deletion of the non-catalytic C-terminal tail, which contains five autophosphorylation sites. To determine which site is essential for oncogenicity, we tailed the C-terminally-deleted mutant with individual autophosphorylation sites. Complete restoration of the transforming action *in vitro* and *in vivo* was conferred by a stretch of 12 amino acids that contained the most C-terminal tyrosine autophosphorylation site (Y1253). Reconstitution of transformation was specific to this amino acid sequence because none of the other autophosphorylation sites, when grafted individually, caused transformation, and replacement of the tyrosine with a phenylalanine residue significantly reduced the oncogenic potential of both the full-length and the tailed proteins. When present alone the most C-terminal sequence enabled coupling to a biochemical pathway that includes Ras, MAP kinase and transactivation of Jun. These results indicate that the multiplicity of autophosphorylation sites on a receptor tyrosine kinase is not essential for transformability, and implicate the MAP kinase pathway in transduction of the oncogenic signal of Neu/ErbB-2.

Key words: growth factor receptor/*jun*/oncogene/signal transduction/Src homology 2 domain/tyrosine kinase

Introduction

The autonomous regulation of growth that characterizes unicellular organisms is replaced in higher eukaryotes by a complex control machinery that utilizes intercellular communication networks. Sustained activation of this pathway appears to underlie the continuous growth of cancer cells. An exemplification of this process is provided by the Neu protein. This transmembrane glycoprotein normally functions as a co-receptor for growth-regulatory molecules, including neuregulins (Peles and Yarden, 1993). Upon ligand binding, the catalytic activity of the cytoplasmic portion, a tyrosine-specific protein kinase, undergoes activation and subsequently phosphorylates itself and interacts with a group of cytoplasmic signaling proteins, that includes phospholipase Cγ (PLCγ), Ras-GTPase activating protein (Ras-GAP) and phosphatidylinositol 3′-kinase (PI3K) (Fazioli *et al.*, 1991; Peles *et al.*, 1991, 1992). Oncogenic activation of Neu appears to utilize the same biochemical pathway, but the ligand-induced initiation event is replaced by various genetic modifications. One modification involves a carcinogen-induced point mutation that replaces a valine residue within the single transmembrane domain with a glutamic acid (Bargmann *et al.*, 1986), and results in the formation of neurogenic tumors in rodents. An alternative genetic mechanism, which involves overexpression of the wild-type protein, is believed to contribute to the virulent phenotype of a subset of human adenocarcinomas (Slamon *et al.*, 1987).

Apparently, both mutation and overexpression of Neu result in stabilization of receptor dimers (Sternberg and Gullick, 1989; Weiner *et al.*, 1989; Ben-Levy *et al.*, 1992; Stancovski *et al.*, 1992), which are essential for maintaining the tyrosine kinase in its active state. As a result, Neu undergoes autophosphorylation on five tyrosine residues that are located on the non-catalytic C-terminus of the protein (Hazan *et al.*, 1990; Segatto *et al.*, 1990; Akiyama *et al.*, 1991). The autophosphorylated tyrosine residues function as docking sites for proteins that contain a structural motif, called Src homology-2 domain (SH2) (Gish and Pawson, 1992). It has been shown in many tyrosine kinase molecules that the amino acid sequence surrounding each phosphotyrosine residue determines the identity of the SH2-containing protein that will become associated with the phosphorylated docking site (Songyang *et al.*, 1993). However, no direct identification of the SH2-containing ligands of individual autophosphorylation sites of Neu has been reported. Nevertheless, replacement of three or five tyrosine residues, within autophosphorylation sites of Neu, with phenylalanine residues, resulted in a proportional reduction in the ability to phosphorylate PLCγ and to induce a mitogenic effect (Segatto *et al.*, 1992). The effect of mutagenesis of individual autophosphorylation sites is less clear. Whereas in the wild-type human Neu protein abolishment of the C-terminal tyrosine autophosphorylation site had no effect on transforming potential (Segatto *et al.*, 1990), the same mutation completely abolished transformation by the Glu659 version of Neu (Akiyama *et al.*, 1991). Likewise, deletion of the C-terminus of the latter mutant dramatically elevated the transforming potential (Akiyama *et al.*, 1991).

The present study addressed the identification of the effector portion of Neu. Presumably, the hypothetical effector site enables the tyrosine kinase to interact specifically with the biochemical pathway(s) that is critical for the generation of the transformed phenotype. The experimental strategy that we undertook was based on the observation that deletion of the whole C-terminal tail of Neu abrogated its oncogenic function. It was, therefore, expected that grafting individual docking sites for signaling

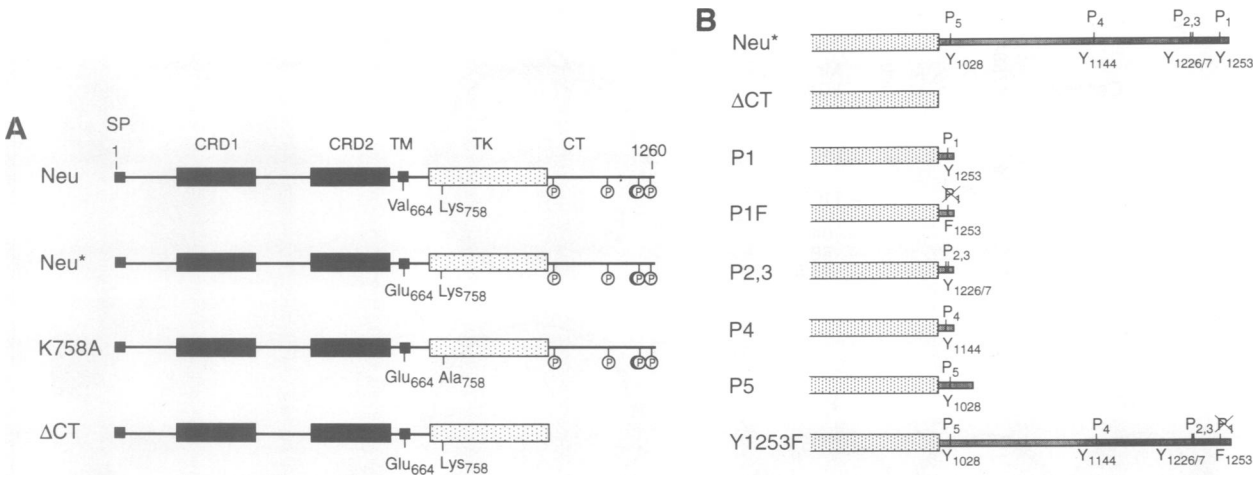

Fig. 1. Schematic representation of mutant Neu proteins. (**A**) The domain structure of the normal Neu protein is represented by boxes that correspond to the signal peptide (SP), cysteine-rich domains (CRDs), transmembrane domain (TM) and the tyrosine kinase (TK) sequence. The C-terminal tail (CT) is shown with the five known autophosphorylation sites. The transforming Neu* protein differs only in one amino acid: it contains a glutamic acid at the transmembrane domain (residue 664) instead of a valine. The kinase-defective mutant (K758A), in which the lysine residue within the nucleotide binding site was replaced by an alanine, and the C-terminally deleted protein (ΔCT) are derivatives of the oncogenic version of Neu. (**B**) C-terminal mutants. The distal part of the tyrosine kinase domain is represented by a stippled box. Various portions of the non-catalytic C-terminus of Neu were linked to the tyrosine kinase domain. The identities of the individual tyrosine autophosphorylation sites that were included in each portion of the C-terminus are indicated by the letter P above the structure and by the letter Y and the corresponding residue number in the full-length Neu* protein. Mutants in which the most C-terminal phosphorylation site was abolished by replacement with phenylalanine are marked with the letter F. Note that all mutants were derived from the oncogenic version of Neu (V664E).

molecules, together with their autophosphorylated tyrosine residues, into the C-terminally truncated receptor would enable reconstitution of the transforming potential. This 'add-back' approach revealed that the most C-terminal autophosphorylation site of Neu is the only site that can fully restore oncogenesis. We further present evidence supporting the possibility that coupling to Ras and MAP kinase, and transactivation of transcriptional activity of c-Jun, are included in the underlying biochemical pathway.

Results

The C-terminal autophosphorylation site of Neu confers full oncogenic potential when present alone

The rat transforming protein, Neu, offers the advantage of being a constitutively active receptor tyrosine kinase that is permanently coupled to signaling pathways responsible for transformation (Bargmann and Weinberg, 1988; Yarden, 1990). We therefore assumed that it would be possible to map the effector domain of this receptor by introducing mutations in the non-catalytic C-terminal portion of the cytoplasmic domain. As a first step towards this aim, a Neu* protein whose whole C-terminal domain was deleted (ΔCT, Figure 1A), was constructed and expressed in murine fibroblasts. As control we used two full-length Neu proteins. These are the transforming version of the receptor, which contains a glutamic acid in the transmembrane domain (named V664E or Neu*), and a kinase-defective mutant of this protein (Peles *et al.*, 1991), whose nucleotide-binding lysine residue was replaced by an alanine (named K758A, Figure 1A). Expression levels of the three proteins, in drug-selected clones of transfected NIH-3T3 fibroblasts, were determined by means of biosynthetic labeling (Figure 2A) or by Western blotting (data not shown) and found to be comparable. The transforming potential of the ΔCT mutant was then compared with that of the oncogenic V664E protein and that of the kinase-defective K758A mutant. Four different transformation assays were used: focus formation in monolayers of (i) Rat-1 fibroblasts (Figure 2B and Table I) or (ii) mouse NIH-3T3 fibroblasts (Table I), (iii) a colony formation assay of clonal cell lines that stably expressed the mutant proteins (Figure 2C), and (iv) analysis of the ability of these cell lines to form tumors in athymic mice (Figure 3). In all of these cell transformation tests the ΔCT mutant was non-transforming, as was the kinase-defective Neu protein. As expected, the non-transforming C-terminally deleted mutant of Neu* displayed no tyrosine autophosphorylation in living cells or in an *in vitro* kinase assay (Figure 4) due to the absence of all of the tyrosine autophosphorylation sites.

We assumed that the completely inactive ΔCT mutant could be functionally reconstituted as a transforming protein by grafting specific individual autophosphorylation sites into its C-terminus. To this end we constructed a modified ΔCT mutant that was tailed with the most C-terminal 12 amino acids of Neu (Figure 1B), including the major phosphorylation site at tyrosine residue 1253 (Hazan *et al.*, 1990). The length of the amino acid sequence that flanked this tyrosine residue at both sides is apparently sufficient to establish a functional docking site for SH2-containing proteins (Songyang *et al.*, 1993). As a control of the single autophosphorylation site-containing protein (named P1), we constructed a mutant in which the terminal tyrosine was replaced by a phenylalanine (Figure 1B). The latter mutant, named P1F, was therefore devoid of any autophosphorylation site. Indeed, stable expression of the P1F protein at high levels indicated that it underwent neither tyrosine phosphorylation in living cells (Figure 4A), nor autophosphorylation *in vitro* (Figure 4B).

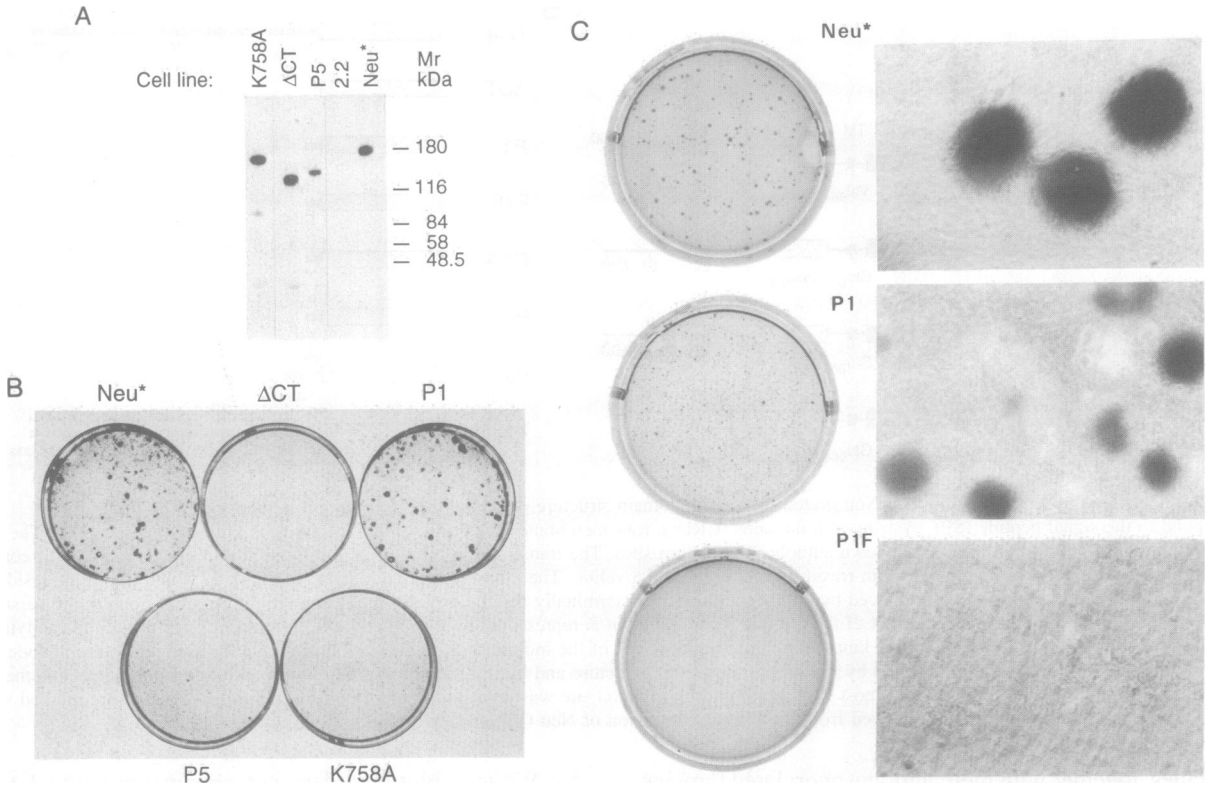

Fig. 2. *In vitro* transformation assays of Neu mutants. (**A**) The level of expression of the various mutant proteins was determined in stable clones of drug-selected NIH-3T3 fibroblasts by immunoprecipitation of biosynthetically [^{35}S]methionine-labeled proteins. For biosynthetic labeling, 10^6 cells that had been selected for expression of the full-length transforming Neu (Neu*), a kinase-defective mutant (K758A), a C-terminal deletion mutant (ΔCT), or a construct that contained only the most internal phosphorylation site (P5), were incubated for 12 h with [^{35}S]methionine (50 μCi/ml). The Neu proteins were immunoprecipitated with a mixture of monoclonal antibodies to rat Neu (Yarden, 1990). Control untransfected cells (labeled 2.2) were used in both analyses. (**B**) Focus formation assay. Sparse monolayers of Rat-1 fibroblasts in 10 cm dishes were transfected with 10 μg of supercoiled plasmid DNA of the indicated constructs. Three weeks after transfection the cells were stained with Giemsa and photographs were taken. (**C**) Soft agar colony formation assay. Stable clones of drug-selected murine fibroblasts that expressed the indicated Neu mutants were seeded at a density of 10^4 cells per 3.5 cm dish in 0.3% agar. The cultures were maintained for 3 weeks in a humidified incubator at 37°C and fed with fresh medium every 4 days. The higher magnification photographs demonstrate the size differences that were displayed by the P1 colonies as compared with the full-length oncogenic Neu.

By contrast, the P1 protein underwent tyrosine phosphorylation both *in vitro* (Figure 4B) and in living cells (Figure 4A).

Transformation assays performed in either Rat-1 or NIH-3T3 fibroblasts indicated that tailing the C-terminus of the tyrosine kinase domain of Neu* with a single autophosphorylation site (Y1253) fully restored the transforming potential. Thus, murine fibroblasts that expressed the P1 mutant protein developed tumors in athymic mice at a rate that was not significantly different from the rate of tumorigenic growth of Neu*-transformed cells (Figure 3). In addition, the P1-expressing cells formed foci on a monolayer of murine (Table I) or rat fibroblasts (Figure 2B and Table I) and grew as colonies in soft agar (Figure 2C). By contrast, P1F-expressing cells formed virtually no colonies in soft agar (Figure 2C), and they developed tumors in athymic mice significantly more slowly than the P1 mutant (Figure 3).

Table I. Transforming efficiencies of mutant *neu* genes

	NIH-3T3	Rat-1
Neu* (V664E)	1.0	+
Kinase-defective Neu* (K758A)	0	−
ΔCT	0	−
P1	1.35	+
P2,3	0.03	−
P4	0.15	−
P5	0.02	−

The indicated mutants of the *neu* cDNA were cloned into an expression vector downstream of the SV40 early promoter. The corresponding plasmids were transfected into NIH-3T3 fibroblasts, or Rat-1 fibroblasts and their transforming activities were examined by the number of foci that were induced. These displayed only limited variation in Rat-1 cells and were, therefore, represented by either + or −. In the case of NIH-3T3 cells the numbers were normalized by comparison with the transforming efficiency of the full-length *neu**. One nanogram of the latter cDNA induced 0.16 foci. The experiment was repeated twice with NIH-3T3 cells and three times with Rat-1 fibroblasts and yielded essentially the same relative transforming efficiencies.

The transforming activity of the C-terminal tyrosine is partially redundant in the presence of other autophosphorylation sites

The full oncogenic potential of P1 raised the possibility that the most C-terminally located autophosphorylation site (Y1253) is the major sequence that confers oncogenicity to Neu, and in its absence the transforming function would be impaired. To address this question we introduced a

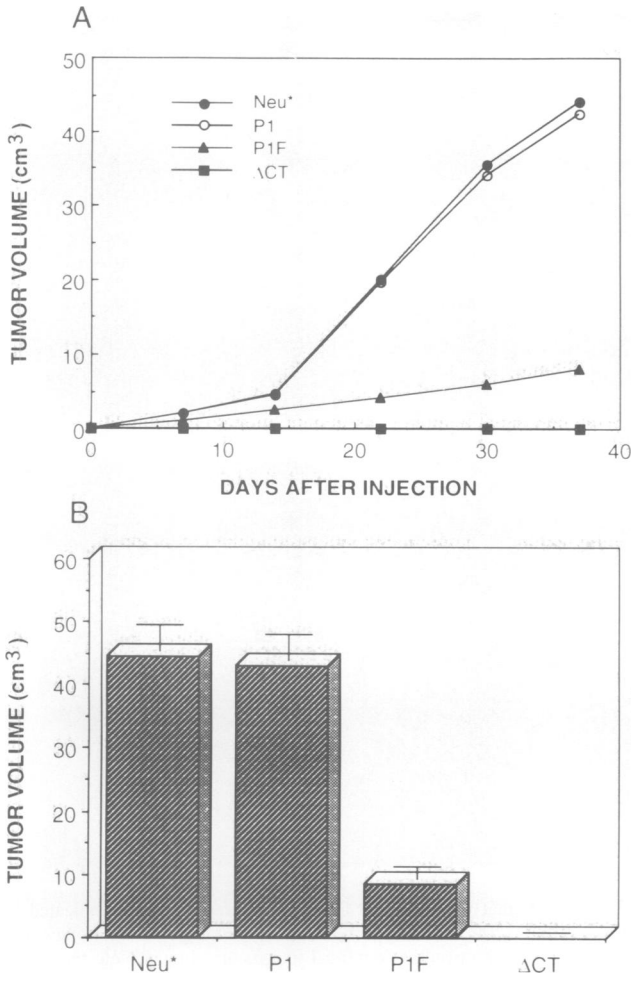

Fig. 3. Tumor formation assay of Neu* mutants. 5×10^6 murine fibroblasts that stably expressed the indicated mutants of Neu* were injected subcutaneously into groups of seven male athymic mice. Tumor volumes were determined at the indicated time intervals; their averages are presented in **A**. The histogram shown in **B** depicts tumor volumes + SD (bars) of the various groups of seven mice at day 37 after injection. The experiment was repeated three times.

tyrosine to phenylalanine mutation of this site in the full-length transforming receptor. The resultant protein, named Y1253F (Figure 1B), was expressed at high levels in murine fibroblasts (Figure 4C) and displayed relatively high tyrosine autophosphorylation in living cells (Figure 4C). This indicated that the absence of the terminal tyrosine did not significantly reduce the level of autophosphorylation of the Neu* protein. However, despite comparable autophosphorylation, the transforming activity of the phenylalanine 1253 mutant in NIH-3T3 fibroblasts was only 17% ± 1% of the transforming efficiency of the oncogenic version of Neu (determined in two independent experiments). Yet, this mutant was clearly more transforming than ΔCT or K758A proteins because both proteins displayed zero transformability (Table I). Hence, we concluded that the terminal autophosphorylation site is the major, but not the only, structural determinant of the non-catalytic C-terminus of Neu, that is essential for the oncogenic potential.

Site-specificity of the transforming function of Neu

The partial functional redundancy of the terminal autophosphorylation site raised the possibility that other sites may

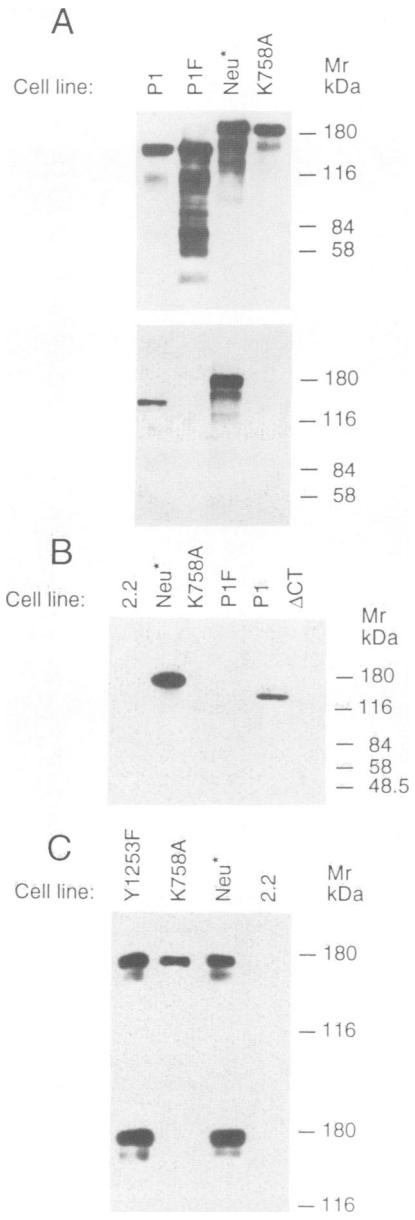

Fig. 4. Tyrosine phosphorylation of various Neu mutants in living cells and *in vitro*. The Neu protein was immunoprecipitated with a mixture of anti-Neu monoclonal antibodies from whole lysates prepared from 10^6 cells expressing the indicated mutants or from untransfected parental cells (2.2). The immunoprecipitates were analyzed by gel electrophoresis and Western blotting with either rabbit anti-Neu antibodies (upper panels in **A** and **C**), or with antibodies to phosphotyrosine (lower panels in A and C). Alternatively, the immunocomplexes were incubated with 10 mM $MnCl_2$ and [γ-^{32}P]ATP and subjected to phosphorylation *in vitro* (**B**). The resulting autoradiograms are shown and the locations of molecular weight marker proteins are indicated.

confer transforming activity to a C-terminally-deleted Neu*. To address this possibility we generated two additional constructs, P2,3 and P4, that contained individual 12 amino acid autophosphorylation sites directly linked to the C-terminal tail of the tyrosine kinase (Figure 1B). A third mutant, named P5, contained the most N-terminal autophosphorylation site, namely Tyr1028 of the full-length Neu protein. This mutant, however, was generated by premature termination and it carried a tail of 43 amino

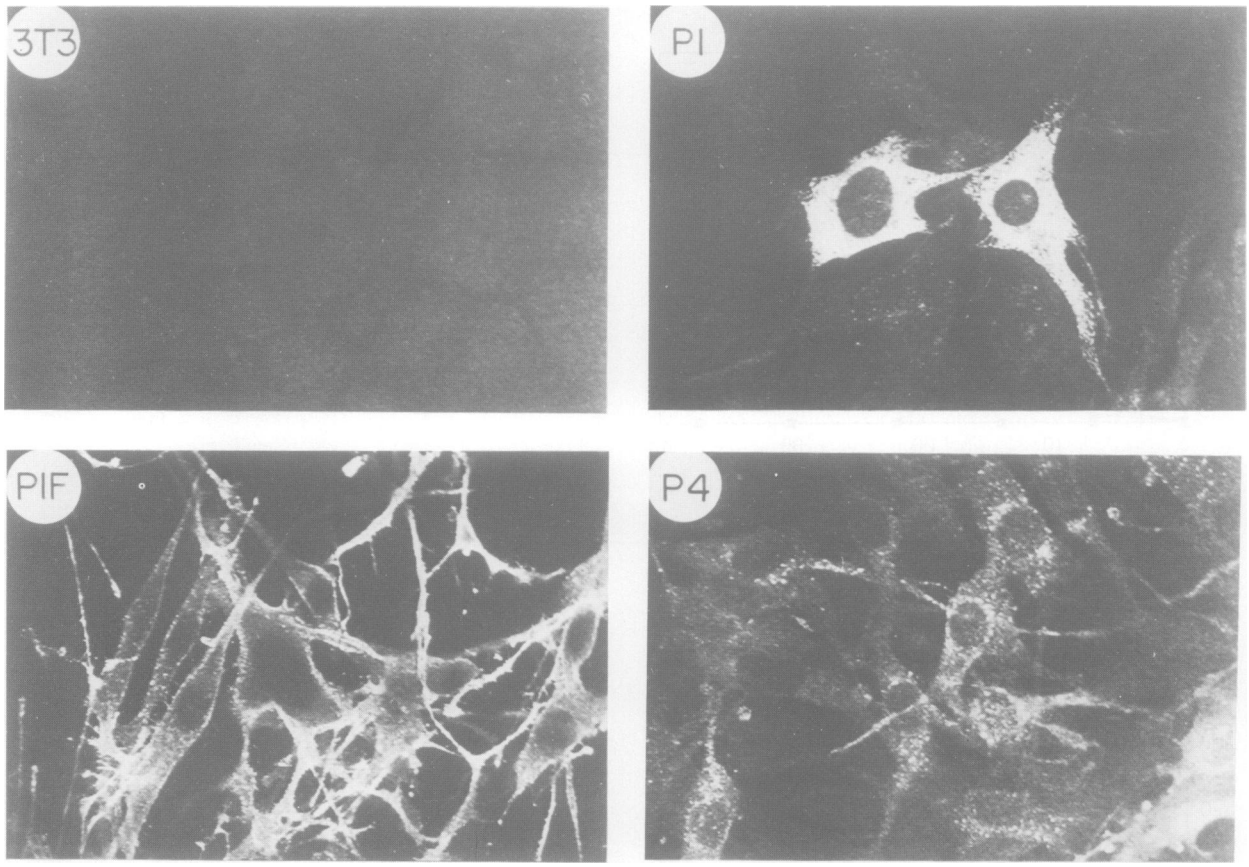

Fig. 5. Immunocytochemical localization of mutant Neu* proteins. Mouse 3T3 fibroblasts that were selected for stable expression of the indicated Neu* mutants, or the untransfected cell line (labeled 3T3), were grown on glass coverslips. The monolayers were stained with mouse antibodies to Neu (Ab-4) which were then visualized by using a secondary fluorescent donkey antibody. The resulting confocal images are shown. Note the plasma membrane staining of P1F and cytoplasmic staining of other mutants.

acids distal to the tyrosine kinase domain (Figure 1B). Immunoprecipitation of the various mutant Neu proteins from cells that had been metabolically labeled with [^{32}P]-orthophosphate, indicated that in contrast to P1F, which had no detectable phosphotyrosine, P1, P2,3 and P4 were found to have phosphotyrosine contents of 5, 12 and 1.4%, respectively, upon phosphoamino acid analysis of the immunoprecipitates. Immunocytochemical localization of these mutant proteins, expressed in murine fibroblasts, revealed that P1 (Figure 5), P2,3 (data not shown) and P4 (Figure 5) shared with the full-length transforming Neu a primarily cytoplasmic localization, which was probably due to the high rate of endocytosis of Glu664-containing proteins (Yarden, 1990). In contrast, the non-phosphorylatable mutant P1F was localized mostly to the plasma membrane (Figure 5).

Focus formation assays performed on monolayers of Rat-1 fibroblasts showed that none of the single autophosphorylation site-containing proteins, namely P2,3, P4 and P5, was able to cause transformation (Table I and Figure 2B). However, this assay may not have been sensitive enough to detect residual transforming activities. We therefore used a more sensitive transformation assay that involves transfection of NIH-3T3 cells. The results of this focus formation assay are summarized in Table I. Evidently, none of the single autophosphorylation site receptors displayed transforming activity comparable with that of the P1 protein. Nevertheless, the transforming activity of each of the constructs, which contained a single autophosphorylation site, was reproducibly higher than the transforming efficiency displayed by a protein that was devoid of all of the phosphorylation sites (ΔCT), or a kinase-defective Neu (K758A). Taken together, the results of the transformation assays indicated that the oncogenic function of Neu* is specifically associated with the most C-terminal autophosphorylation site. However, other phosphorylation sites can partially mediate this function and, therefore, may account for the residual transforming ability of Y1253F.

Transformability of the most C-terminal autophosphorylation site is mediated by Ras

Ras proteins play a pivotal role in the control of cellular growth and they are required for transformation by many, but not all, oncogenes (Downward, 1992). We, therefore, examined the possibility that the P1 autophosphorylation site enabled coupling of Neu to Ras. This was analyzed by microinjection of a monoclonal anti-Ras antibody, named Y13-259, that can neutralize natural Ras proteins in murine fibroblasts (Furth *et al.*, 1982). Injection of the anti-Ras antibody, unlike control rat immunoglobulins, into P1-expressing NIH-3T3 cells induced phenotypic reversal in most cells (Figure 6). This was evident by disappearance of the spindle-shaped morphology and the bright refractile cell borders which characterize *neu*-transformed cells. In order to determine the effect of

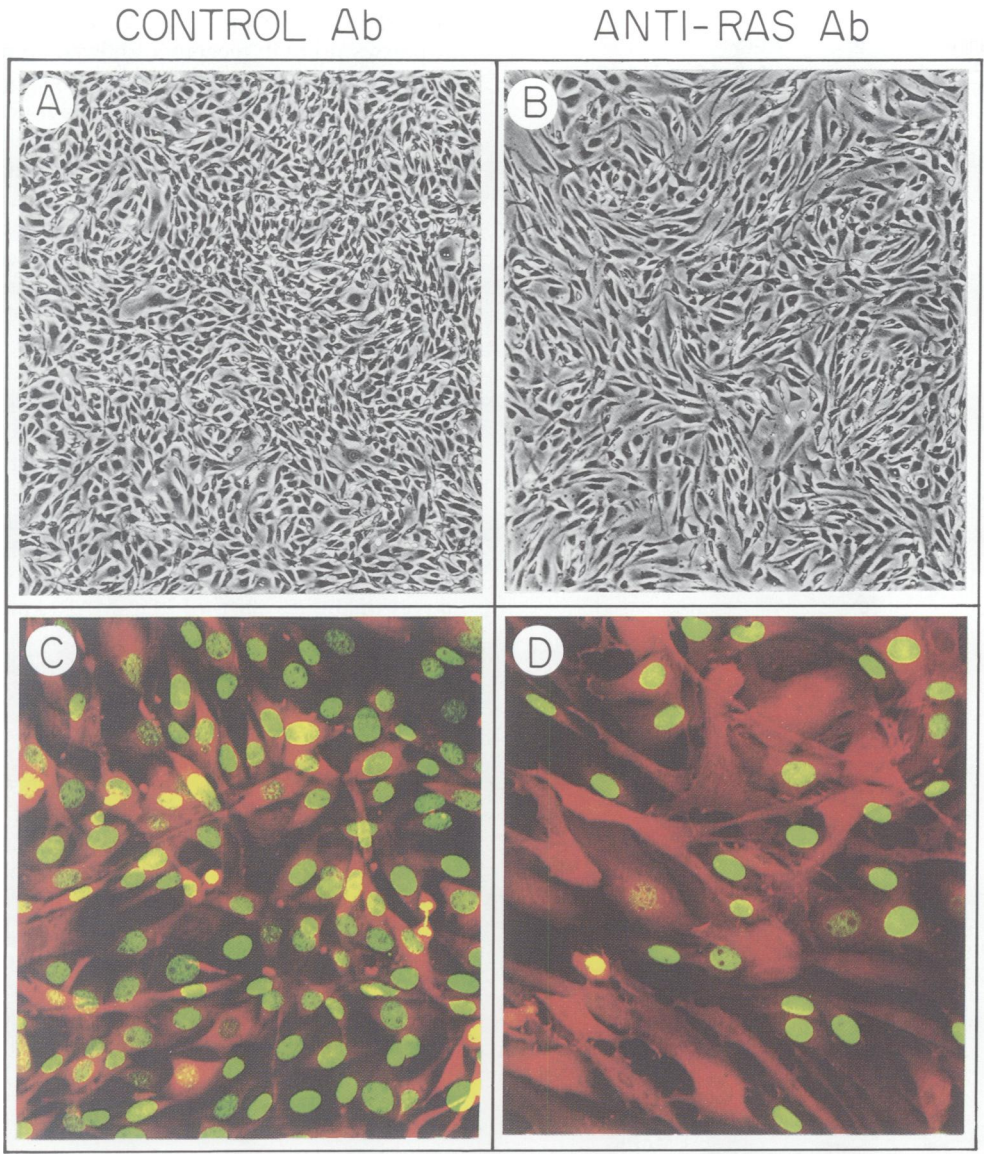

Fig. 6. Biological effects of microinjected anti-Ras antibodies on P1-expressing cells. 3T3 fibroblasts that were selected for overexpression of the P1 mutant of Neu* underwent microinjection with either purified rat immunoglobulin-G (7 mg/ml, **A** and **C**), or with Y13-259 rat monoclonal anti-Ras antibody (**B** and **D**). An attempt was made to inject all cells within the areas photographed. The monolayers were then incubated for 24 h in the presence of BrdU (10 μg/ml). Shown are photomicrographs that were obtained by using phase microscopy (A and B), or fluorescent micrographs (C and D) that were obtained by co-staining with antibodies to rat immunoglobulins (red), and anti-BrdU antibodies (green).

inhibition of Ras action on DNA synthesis in P1-expressing cells, we first injected the cells with anti-Ras antibodies, and then labeled nuclei of S-phase cells with BrdU. This was followed by double immunostaining that permitted visualization of both labeled DNA and the microinjected anti-Ras antibody. Unlike control antibodies, which did not significantly affect DNA synthesis (Figure 6C), anti-Ras antibodies inhibited DNA synthesis in ~80% of the injected cells (Figure 6D). Taken together, these results indicate that Ras is an essential component of the signaling pathway that is utilized by the most terminal autophosphorylation site of Neu.

Activation of MAP kinase correlates with the transforming potential of mutant Neu proteins

Ras activation is expected to trigger a protein kinase cascade that involves the Raf-1 protein kinase and eventually MAP kinase activation (Howe *et al.*, 1992). Since coupling of Neu to MAP kinase has not been shown previously, we first addressed this possibility by using a ligand-stimulatable chimeric Neu protein. This protein, termed NEC, included the extracellular domain of the EGF receptor and the cytoplasmic portion of Neu (Ben-Levy *et al.*, 1992; Peles *et al.*, 1991, 1992). Murine fibroblasts that expressed the chimera (NEC cells) were incubated in the presence or absence of EGF and the activity of their MAP kinase protein was measured by using a Western blot 'gel shift' assay (Leevers and Marshall, 1992), and also by employing an *in vitro* phosphorylation assay of the exogenous substrate myelin basic protein (MBP). The results of this experiment clearly indicated that MAP kinase was activated: this was evident from the decrease in its electrophoretic mobility and by the increase in its ability to phosphorylate MBP

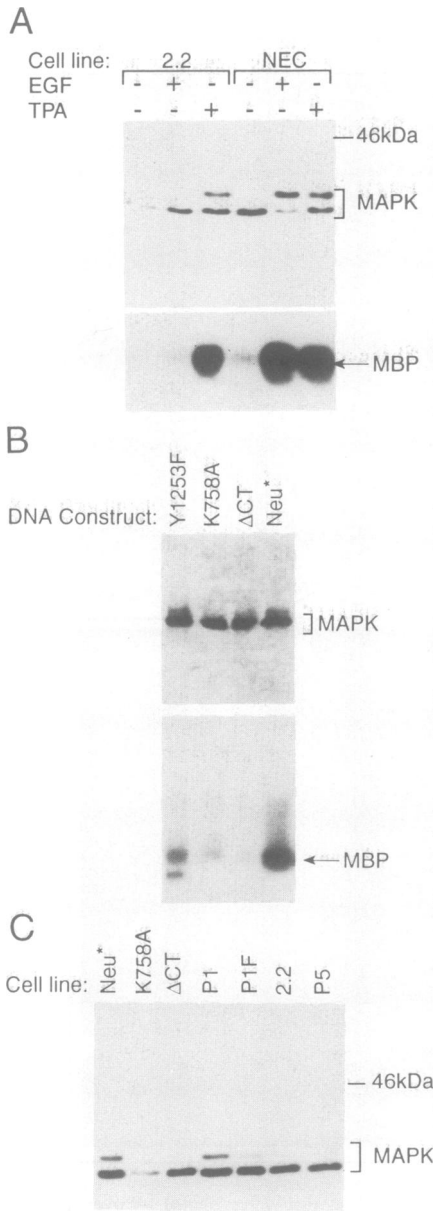

Fig. 7. Interactions with MAP kinase. (**A**) MAP kinase activation by ligand-stimulated Neu tyrosine kinase. Murine fibroblasts that expressed a chimeric Neu−EGF receptor tyrosine kinase (NEC), or the parental untransfected cells (2.2), were incubated with either EGF (20 ng/ml), TPA (10 nM), or left untreated. Following 20 min of incubation at 37°C cell lysates were prepared and subjected to gel electrophoresis and Western blotting with an anti-MAP kinase antibody (upper panel). Alternatively, MAP kinase was first immunoprecipitated from whole cell lysates and then subjected to MBP kinase assay *in vitro*. The resulting autoradiogram is shown (lower panel). Note the gel mobility shift of the MAP kinase protein in some lanes. (**B**) MAP kinase activation assays of COS-1 cells expressing various Neu mutant proteins. Monolayers of COS-1 cells (5×10^6 cells/10 cm plate) were co-transfected with plasmids that encode the indicated Neu* mutant together with a plasmid encoding a MAP kinase/Erk-2-Myc fusion protein. MAP kinase activation was determined by Western blotting (upper panel) with an antibody to a Myc epitope or by performing MBP kinase assay in immunoprecipitates of the MAP kinase/Erk-2-tag fusion protein (lower panel). (**C**) MAP kinase activation assays in NIH-3T3 cells expressing various Neu* mutant proteins. Clonal cell lines that were selected for stable expression of the indicated mutants of Neu* were seeded at 3×10^5 cells/10 cm plate, grown for 24 h and then starved for 12 h in the absence of serum. Cell lysates were then prepared and subjected to Western blot analysis with antibodies to MAP kinase. The location of MAP kinase is indicated.

(Figure 7A). The untransfected control cell line, which expresses no EGF receptors, showed no response to EGF, but its MAP kinase underwent activation by the protein kinase C ligand 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA, Figure 7A).

In order to examine the proposition that the oncogenic mutation of Neu also leads to MAP kinase activation, we co-transfected COS-1 cells with the transforming version of Neu, together with an expression vector that directed synthesis of a $p44/42^{mapk/erk}$ fusion protein containing a Myc-derived peptide tag. As is evident from Figure 7B, expression of the transforming version of Neu led to a gel-mobility shift of p44/42 and stimulation of MAP kinase activity in analogy to ligand activation. The dependency of MAP kinase activation on specific phosphorylation sites was then examined in COS-1 cells (Figure 7B) or in murine cell lines that stably expressed the various mutant proteins (Figure 7C). As expected, Neu proteins, that were either devoid of all of the autophosphorylation sites (ΔCT) or catalytically inactive (K758A), were unable to induce a gel mobility shift of the MAP kinase protein and to activate its catalytic function *in vitro* (Figure 7B). Interestingly, however, the partially transforming Y1253F mutant was able to induce a MAP kinase mobility shift, but it failed to stimulate MAP kinase activity towards MBP to the extent that was achieved by the oncogenic Neu (Figure 7B). This may be related to the 6-fold reduction in the transforming potential of the Y1253F protein. Out of the C-terminal mutants that were examined in NIH-3T3 cells, only P1 induced a mobility shift that was comparable with the effect of the transforming Neu. A partial effect was observed with the P1F protein, but the P5 protein was inactive. This pattern of relative ability to affect MAP kinase correlated with the potential of the corresponding mutant proteins to induce cellular transformation (Figure 3 and Table I), thus supporting the possibility that the oncogenic action of Neu is mediated by MAP kinase.

Activation of c-Jun by C-terminal mutants of Neu

MAP kinase activation by a variety of mitogens induces re-entry into the cell cycle by phosphorylation of both the ternary complex factor (Gille *et al.*, 1992) and c-Jun (Pulverer *et al.*, 1991; Smeal *et al.*, 1992), possibly via translocation of the kinase to the nucleus (Chen *et al.*, 1992). c-Jun is a component of the AP-1 transcription complex and its phosphorylation on serine residues, within the activation domain, increases its transactivation potential (Hunter and Karin, 1992). Several oncoproteins, including v-Sis, v-Src, Ha-Ras and Raf-1, mediate stimulation of c-Jun activity by such phosphorylation (Smeal *et al.*, 1992). This raised the possibility that the oncogenic Neu protein similarly mediates a signaling cascade that culminates in enhancement of transactivation of c-Jun. In order to examine this possibility and to correlate it with the oncogenic action of C-terminally modified Neu* proteins, we co-transfected COS-7 cells with three expression vectors. These were (i) a c-Jun expression vector containing a retroviral promoter, (ii) a plasmid that directed the synthesis of Neu or its various mutants, and (iii) a promoter−reporter vector in which the AP-1-containing promoter of the collagenase gene drives expression of the luciferase gene (Devary *et al.*, 1992). This experimental

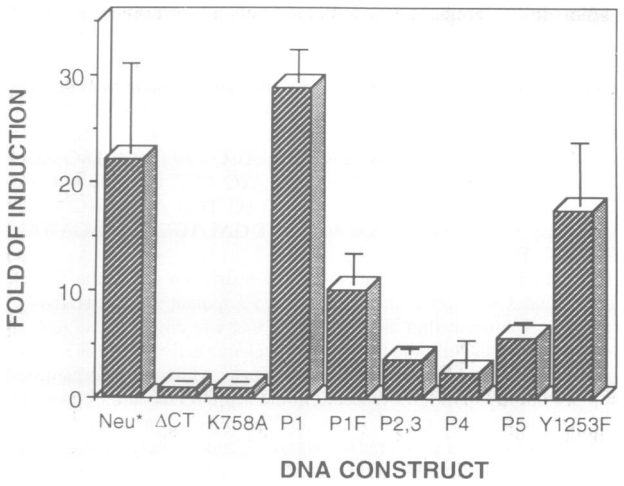

Fig. 8. The effect of various Neu* mutant proteins on transactivation of c-Jun. COS-7 monolayers (4×10^5 cells/6 cm plate) were transfected with a mixture of three plasmids (1 μg DNA each). These were an expression vector that encodes the indicated mutant of the Neu* protein, a c-Jun expression vector and a collagenase–luciferase plasmid. Luciferase activity was measured 36 h after transfection. The averages + SD (bars) of four independent transfection experiments are shown in the histogram.

system allows quantitative determination of c-Jun transactivation by using a luciferase enzymatic assay.

Figure 8 depicts the results of this experiment. Evidently, expression of the oncogenic Neu protein resulted in 20- to 30-fold stimulation of the transactivation function of c-Jun, but the non-transforming proteins K758A and ΔCT displayed no significant activity in this assay. Importantly, the fully transforming P1 protein displayed activity comparable with that of Neu*, and intermediate levels of activation were induced by all other C-terminal mutants of Neu*. Remarkably, P1F and Y1253F displayed higher activity than the other mutants. The pattern of relative activities of the various mutants in COS cells was, in general, similar to their ability to transform fibroblasts (Table I and Figure 3). The overall correlation between c-Jun transcriptional activity and oncogenicity, together with the different abilities of the C-terminally mutated receptors to activate MAP kinase, suggest that the most C-terminal autophosphorylation site may confer oncogenicity to Neu through coupling of the tyrosine kinase to a biochemical pathway that includes Ras, MAP kinase and c-Jun.

Discussion

Multiplicity of tyrosine autophosphorylation sites is shared by all receptor tyrosine kinases and thought to underlie the pleiotropic cellular response to growth factors through a mechanism that involves binding of various SH2-containing proteins to specific autophosphorylation sites (Pawson and Schlessinger, 1993). Previous attempts to address the necessity of specific autophosphorylation sites for the oncogenic function of Neu (Akiyama *et al.*, 1991; Segatto *et al.*, 1992), or for the mitogenic action of the related receptor for EGF (Honegger *et al.*, 1988; Sorkin *et al.*, 1992) made use of individually mutated autophosphorylation sites in the context of full-length or partially truncated receptors. This approach may be complicated by potential compensatory roles played by adjacent autophosphorylation sites, and perhaps also by the exposure of cryptic sites in the mutated proteins (Walton *et al.*, 1990). Here we undertook an alternative approach that has not been used before, and may overcome such potential limitations. The approach was based on the construction of receptor mutants whose C-terminal tails were replaced by very short sequences, that contained individual autophosphorylation sites and, therefore, may qualify as docking sites for specific signaling molecules. The advantage of this strategy for the determination of site-specificity is demonstrated by the remarkably higher transforming potential of the P1 protein in comparison with other single-site receptors. The identification of this site as the most critical for oncogenicity is consistent with the observation that mutagenesis of the corresponding tyrosine (Y1253) in the context of the holoreceptor reduced the transforming potential of Neu* by 6-fold. In addition, this observation is in agreement with the complete inhibition of the transforming action of the analogous Y1248F mutant of human Neu* (Akiyama *et al.*, 1991).

Although the most C-terminal autophosphorylation site was sufficient to induce tumorigenesis in the absence of any other autophosphorylation site, the residual transforming activity of Y1253F implies that oncogenesis is not exclusively associated with the terminal site. This conclusion is supported by the observation that P2,3, P4 and P5 proteins displayed very low, but reproducible, transforming activity that was completely absent in the ΔCT and K758A mutants (Table I). One possible explanation for the residual transforming activity of P2,3, P4 and P5 proteins is that phosphorylation sites, other than Tyr1253, can functionally replace it by providing weak or cooperative coupling to the same biochemical pathway that is used by P1. Alternatively, distinct downstream mediators, which involve specific autophosphorylation sites, may independently cause transformation. For example, the receptor for the platelet derived growth factor (PDGF) relays a mitogenic signal through either PLCγ or PI3K (Valius and Kazlauskas, 1993).

The partial *in vivo* tumorigenic activity of P1F (Figure 3) is surprising because this mutant contained no autophosphorylation site and it underwent no phosphorylation either *in vitro* or in living cells (Figure 4). This limited transforming potential may be mediated by a relatively inefficient coupling mechanism to the MAP kinase (Figure 7C) and c-Jun (Figure 8) pathway. For example, the fact that P1F is confined to the plasma membrane and undergoes only limited endocytosis (Figure 5), may enable this mutant to interact constitutively with related ErbB proteins, and thereby utilize in part their signaling pathways.

The identity of the signaling molecule that directly interacts with the P1 site remains unknown. Our attempts to identify such an *in vivo* substrate have failed, presumably because its complex with the constitutively internalizing Neu* is transient. However, the dependency of P1 transformability on Ras (Figure 6) suggests the involvement of one of the Ras-interacting SH2-containing proteins, namely Ras-GAP, GRB2/Sem-5 or SHC. On the other hand, inspection of the amino acid sequence of the most C-terminal autophosphoryltion site of Neu (Tyr1253-Leu-Gly-Leu) suggests that it may function as a docking site for the phosphatidylinositol-specific phospholipase

(PLCγ). This is based on the sequence specificity of the N-terminal SH2 domain of PLCγ (Songyang *et al.*, 1993), as well as on sequence homology with one of the PLCγ-specific sites in the receptor for EGF (Tyr1173-Leu-Arg-Val) (Rotin *et al.*, 1992), and the corresponding site in the receptor for fibroblast growth factors (Tyr766-Leu-Asp-Leu) (Mohammadi *et al.*, 1992; Peters *et al.*, 1992). Indeed, a synthetic phosphopeptide corresponding to the amino acid sequence of P1 was able to inhibit the association between Neu and recombinant SH2 domains of PLCγ *in vitro* (our unpublished results). The relevance of this observation to living cells is unclear. However, it could provide an alternative pathway for activation of MAP kinase through stimulation of protein kinase C, which in turn can directly phosphorylate Raf-1, a MAP kinase kinase kinase (Kyriakis *et al.*, 1992; Sozeri *et al.*, 1992; Kolch *et al.*, 1993).

Because a single autophosphorylation site can alleviate the transforming potential of a C-terminally deleted Neu* (ΔCT), and since it also confers to the receptor coupling to the Ras–MAP kinase–Jun pathway, it is worthwhile considering the possibility that this signaling pathway is the major one that enables Neu* to transform cells. This possibility is strongly supported by the ability of anti-Ras antibodies to inhibit transformation and DNA synthesis in fibroblasts that express the P1 mutant. This model is consistent with the observation that inactivation of the function of Raf-1 abolished the induction of mitogenic signals in fibroblasts (Kolch *et al.*, 1991). However, the partial functional redundancy of the terminal autophosphorylation site suggests that additional, perhaps less efficient, biochemical pathways may culminate in cellular transformation by Neu*. These could involve PI3K and additional SH2-containing proteins that bind to Y1253 or to other individual autophosphorylation sites. The role of these sites, and the presumably regulatory intervening sequences of Neu, in generating the transformed phenotype remains to be elucidated.

Materials and methods

Materials

EGF was supplied by Toyobo (New York). Radioactive materials were purchased from Amersham (Buckinghamshire, UK). Unless otherwise indicated, all other chemicals were purchased from Sigma (St Louis, MO). Buffered solutions were prepared as described (Peles *et al.*, 1991). The NCT antiserum was raised against a synthetic peptide comprising the 15 C-terminal amino acids of the human Neu protein. Mouse monoclonal antibodies to rat Neu were either purchased from Oncogene Science (Ab-4) or have been described previously (Yarden, 1990). A monoclonal antibody to 5-bromo-2′-deoxyuridine (BrdU) was from Boehringer Manneheim. For detection of endogenous MAP kinase we used polyclonal antiserum 122 (Leevers and Marshall, 1992), and the 9E10 monoclonal antibody to Myc (Evan *et al.*, 1985) was used in Western blot analyses and immunoprecipitation of Erk2-Tag.

Expression vectors and transfection

Mutagenesis and deletions were carried out in the pBluescript plasmid (Stratagene, La Jolla, CA). These were verified by restriction analysis and nucleotide sequencing. The inserted DNA fragment was then cleaved out as a *Sal*I fragment and cloned into the compatible *Xho*I site of the pLSV expression vector. The ΔCT mutant of *neu* was generated by deletion of an internal *Nco*I fragment of ~900 bp of the rat *neu* cDNA (Bargmann *et al.*, 1986). This placed a termination codon downstream of the *Nco*I site at residue 3027. The same restriction site was used for generating the P1, P2,3 and P4 mutants. The P1 mutant was generated in pBluescript-*neu** plasmid from which a 3′ end *Nco*I–*Kpn*I fragment was deleted. A 140 bp *Pst*I–*Kpn*I fragment (starting at nucleotide 3756 of *neu* and containing a stop codon at nucleotide 3796) was then ligated to the *Nco*I site of the plasmid by using a double-stranded *Nco*I linker. The following sequences were used to synthesize double-stranded oligonucleotides that encoded the indicated autophosphorylation sites (Figure 1A). P2,3 upper oligo: 5′-C ATG TTT GAC AAC CTC TAT TAC TGG GAC CAG AAC TCA TAG TCG ACG TAC-3′; P2,3 lower oligo: 5′-GTC GAC TAT GAG TTC TGG TCC CAG TAA TAG AGG TTG TCA AA-3′; P4 upper oligo: 5′-C ATG CCC CAG CCC GAG TAT GTG AAC CAA TCA GAG GTT TAG TCG ACG TAC-3′; P4 lower oligo: 5′-GTC GAC TAA ACC TCT GAT TGG TTC ACA TAC TCG GGC TGG GG-3′.

Each oligonucleotide contained an *Nco*I restriction site at the 5′ end and *Sal*I and *Kpn*I sites at the 3′ end. The P5 mutant was generated by an internal deletion within the *neu* cDNA, that was created by digestion with *Bgl*II at nucleotide 3245. The exonuclease activity of Bal31 was then used to obtain a series of deletion mutants which were sequenced. The P5 clone was chosen because it contained a termination codon 43 codons 3′ to the last codon of the ΔCT mutant. The point mutants Y1253F and P1F were generated by oligonucleotide-directed mutagenesis using a single-strand pBluescript-*neu* plasmid and a kit from Amersham. Stable cell lines were generated by co-transfection with a *neu*-expression vector and a second plasmid (pSV2/neo) that drives the expression of the *neo* gene. The calcium phosphate precipitation method was used. Following transfection, a glycerol shock (15% glycerol in DMEM for 2 min) was carried out and the cells were subcultured in medium containing 0.8 mg/ml G418 (Gibco, Bethesda, MD). Resistant colonies were individually grown and assayed for binding of monoclonal antibodies to rat Neu. Transient expression of COS-1 cells was performed as described (Howe *et al.*, 1992).

Transformation assays

Focus formation assays and soft agar colony formation assays were performed as described (Ben-Levy *et al.*, 1992). Tumor growth in nude mice was determined as follows: 5×10^6 cells overexpressing the Neu receptor mutants were washed in phosphate buffered saline (PBS) and resuspended in 0.3 ml PBS. Cells were injected subcutaneously into CD1/Nu male mice (6 weeks old) and tumor volumes were measured every 7 days.

Immunofluorescence of Neu-expressing cell lines

Cells growing on glass coverslips were fixed in 4% formaldehyde for 20 min and permeabilized in 0.2% Triton X-100 for 15 min. A mouse monoclonal antibody to Neu (Ab-4) was added at 1:100 dilution and incubated with the cells for 1 h. A secondary donkey antibody to mouse immunoglobulins (DTAF-labeled antibody, Jackson ImmunoResearch Laboratories) was used for detection. The coverslips were then mounted in Moviol mountant containing 0.1% *p*-phenyldiamine. Images were recorded on a Bio-Rad MRC 600 confocal imaging system, that was used in conjunction with a Nikon Optiphot fluorescence microscope.

Microinjection and BrdU incorporation experiments

Cells were microinjected with the Ras-specific neutralizing monoclonal antibody Y13-259 or normal rat immunoglobulin G (ChromPure, Jackson ImmunoResearch Laboratories), each at 10 mg/ml. BrdU (10 μg/ml) was added to the culture medium after 20 h and the cells were maintained in culture for an additional 24 h. Cellular fixation and permeabilization were performed by incubation in 4% formaldehyde (20 min) followed by 15 min in 0.2% Triton X-100. Staining with a mouse monoclonal antibody to BrdU (Boehringer Manneheim, 1:20 dilution) was carried out for 90 min in the presence of DNase I (1 mg/ml). This was followed by a 90 min incubation with two fluorescently labeled donkey antibodies: a DTAF-labeled antibody to mouse immunoglobulins (Jackson ImmunoResearch Laboratories, 1:500 dilution), and a Texas Red-labeled antibody to rat immunoglobulins (Jackson ImmunoResearch Laboratories, 1:250 dilution). Mounting and imaging were as described for Neu-specific immunofluorescence.

Analysis of MAP kinase activation

Analysis of MAP kinase/Erk-2 activation in NIH-3T3 cell lines or in COS-1 cells was performed on lysates prepared from sparse exponentially growing cultures as previously described (Leevers and Marshall, 1992). MBP kinase activity was determined by assaying antiserum 122 immunoprecipitates of lysates from cell lines and antibody 9E10 immunoprecipitates of Erk2-tag transfected COS-1 cells using an *in vitro* kinase assay with [γ-^{32}P]ATP (Leevers and Marshall, 1992).

c-Jun transactivation assay

The calcium phosphate precipitation method was used to transfect into COS-7 cells 1 μg each of the tested *neu* plasmids, RSV-*jun* plasmid and a luciferase expression vector driven by the collagenase promoter. Carrier DNA (5 μg) was also included. Six hours after transfection, a 2 min glycerol shock was carried out. Twenty-four hours later the cells were deprived of serum for 16 h in serum-free medium and then harvested and washed twice in PBS. Reporter lysis buffer (Promega) was used to lyse the cells and the lysate was spun (12 000 *g*) for 10 min at 4°C. Luciferin and ATP were added to the cleared lysates and luciferase was determined in a luminometer by measurement of light emission as described (de Wet *et al.*, 1987).

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