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Interaction of the Neu/p185 and EGF Receptor Tyrosine Kinases: Implications for Cellular Transformation and Tumor Therapy

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Abstract Growth factor receptors such as the epidermal growth factor receptor (EGFR) and the $p185^{c\text{-}neu}$ protein serve vital roles in the transduction of differentiation, developmental, or mitogenic signaling within normal cells. Two methods of analysis suggest that the inappropriately high expression of either protein tyrosine kinase promotes malignant transformation. First, data from in vitro experiments indicate that overexpression of either EGFR or $p185^{c\text{-}neu}$ (or the human homolog c-erbB-2) transforms cell-lines. Second, analysis of primary tumors and tumor cell-lines derived from many epithelial tissues (breast, stomach, ovary, and pancreas) show growth factor receptor gene amplification and elevated protein levels. The physical and functional interaction of $p185^{c\text{-}neu}$ and EGFR leads to the formation of a highly active, heterodimeric tyrosine kinase complex which synergistically activates cellular transformation. Anti-receptor antibodies have shown potential utility for the down modulation of these cell-surface proteins and suppression of the malignant phenotype. Design of organic antibody "mimetics" based on the structure of antireceptor antibodies may provide useful therapies and biological reagents to affect growth factor receptor function. © 1993 Wiley-Liss, Inc.

Key words: neu/p185 protein, c-erbB-2, epidermal growth factor receptor, transformation

One biological consequence of aberrant regulation or expression of normal growth control mechanisms is the malignant transformation of cells. This uncontrolled growth of cells can occur as the result of activation of oncogenes or loss of tumor suppressor gene function. Oncogenic proteins exert a positive, dominant effect on cellular growth while tumor suppressor proteins have a recessive, negative effect. Oncogenes were originally described as capable of conferring the transformed phenotype from retroviruses to nontransformed cells. Transforming oncogenes were derived from cellular proto-oncogenes which have been conserved in evolution, and are thought to play roles in normal cell growth and development [Bishop, 1991].

Neoplasia can result from various genetic events causing activation of the malignant property of proto-oncogenes. These genetic alterations include proto-oncogene rearrangement, duplication, point mutations, insertional mutagenesis, and aberrant expression. This review will focus on the inappropriate expression of one class of cellular proto-oncogenes, the class I growth factor receptors. This group includes such proteins as the epidermal growth factor receptor (EGFR) and the $p185^{c\text{-}neu}$ tyrosine kinases [Yarden and Ullrich, 1988]. Much of the work that has defined the involvement of both neu/p185 and EGFR in cellular transformation in in vitro models and in malignancies will be described, with emphasis on a unique mechanism of cellular transformation resulting from the synergistic interaction of $p185^{c\text{-}neu}$ and EGFR. In addition, the cell-surface localization of these two proteins has allowed antibody- and mimetic-based strategies of anti-tumor therapy to be developed, and these strategies will also be discussed.

EPIDERMAL GROWTH FACTOR RECEPTOR

The epidermal growth factor receptor (EGFR) is a 170 kDa cell-surface glycoprotein consisting of an extracellular ligand binding domain, a

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single transmembrane region, and an intracellular domain. The cytoplasmic domain can be divided into two regions, the tyrosine kinase domain and the carboxy-terminal 200 amino acids which contain at least 4 tyrosine residues which are sites of autophosphorylation by the kinase (Fig. 1). The EGF receptor is able to bind both EGF and transforming growth factor alpha (TGF-α) with a concomitant modulation of kinase activity. A model for receptor kinase activation has been suggested in which ligand binding promotes dimerization of two EGFRs, activating the tyrosine kinases resulting in autophosphorylation of the tyrosine residues by an intermolecular reaction [Ullrich and Schlessinger, 1990]. Autophosphorylation increases the affinity for substrates, allowing cellular substrates to bind the activated receptor and become tyrosine phosphorylated. Receptor signaling through these cellular substrates ultimately promotes cell division and proliferation. This model has been tested by examining the kinetics of dimerization and activation of EGFR [Canals, 1992]. Both dimerization and tyrosine kinase activation were second order with respect to EGFR concentration, implicating dimerization as an activating event for the kinase domain and suggesting that dimers are important structures in the propagation of mitogenic signals from the cell surface to the nucleus.

The tissue levels of EGF and TGF-α appear to be regulated as are expression levels of EGFR. EGF is a potent mitogen which is found in many human tissues and is elevated during mammary gland development and breast maturation. The expression of TGF-α appears to be deregulated in malignant tissues, showing elevated levels in human breast adenocarcinomas. Studies have demonstrated that introduction of high levels of EGFR into NIH 3T3 cells induces the transformed phenotype. Partial transformation occurs in vitro, only in the presence of physiological levels of EGF [DiFiore et al., 1987a]. These results suggest the possibility that inappropriate EGFR expression combined with ligand might promote malignant transformation. The presence of EGFR in breast cancers has been shown to be associated with a poorer patient prognosis. Studies by several groups [reviewed in Gullick, 1990] indicate a correlation between EGFR expression and the presence of positive axillary lymph nodes in patients with breast cancer. Further support for the role of EGFR in malignancies is the observation of gene amplification and protein overexpression of EGFR in glioblastomas [Fleming et al., 1992].

THE NEU/P185 PROTEIN

The neu oncogene encodes a 185 kDa glycoprotein composed of three distinct domains: a 640 amino acid extracellular domain including two cysteine-rich subdomains, a 24 amino acid amphipathic transmembrane helix, and a 580 amino acid cytoplasmic domain which includes the tyrosine kinase domain and autophosphorylation sites (Fig. 1). The $p185^{c\text{-}neu}$ protein is highly homologous to the EGFR, both in amino acid sequence and overall structural organization. The carboxy terminal-120 amino acids contains the greatest sequence divergence between the two tyrosine kinases, and is thought to confer specific regulation of the cognate kinase domains [DiFiore et al., 1990a], although the role of the carboxy-termini in signaling and/or substrate specificity remains controversial [DiFiore et al., 1990b; Mikami et al., 1992]. Considering the high percentage of proline, glycine, and serine residues within this region (20, 10, and 10%, respectively), it is possible that the carboxy terminus of the $p185^{c\text{-}neu}$ receptor adopts an unusual structure potentially important for the specificity of $p185^{c\text{-}neu}$ signal transduction.

The neu oncogene (encoding the transforming $p185^{c\text{-}neu}$ protein) was originally identified as a result of DNA transfection/focus formation assays using DNA isolated from a rat neuroblastoma. This oncogene becomes activated in the rat by a point mutation within the transmembrane region changing a valine to a glutamic acid at position 664. Our working hypothesis is that this amino acid substitution promotes the dimerization/aggregation of $p185^{neu}$ which results in elevated tyrosine kinase activity of $p185^{neu}$ and ultimately in cellular transformation. Comparisons of kinase activity of the proto-oncogenic and oncogenic p185 demonstrate that the latter is more highly phosphorylated on tyrosine residues in intact cells and more active as a kinase in immune complex kinase assays. This increase in enzymatic activity is apparent within the plasma membrane and results in increased phosphorylation of cellular substrates. To directly test the linkage between the tyrosine kinase activity and cellular transformation, our laboratory altered the ATP binding site within the kinase domain of oncogenic neu by changing lysine 757 to a methionine via site directed mutagenesis. Transfected cell lines expressing this

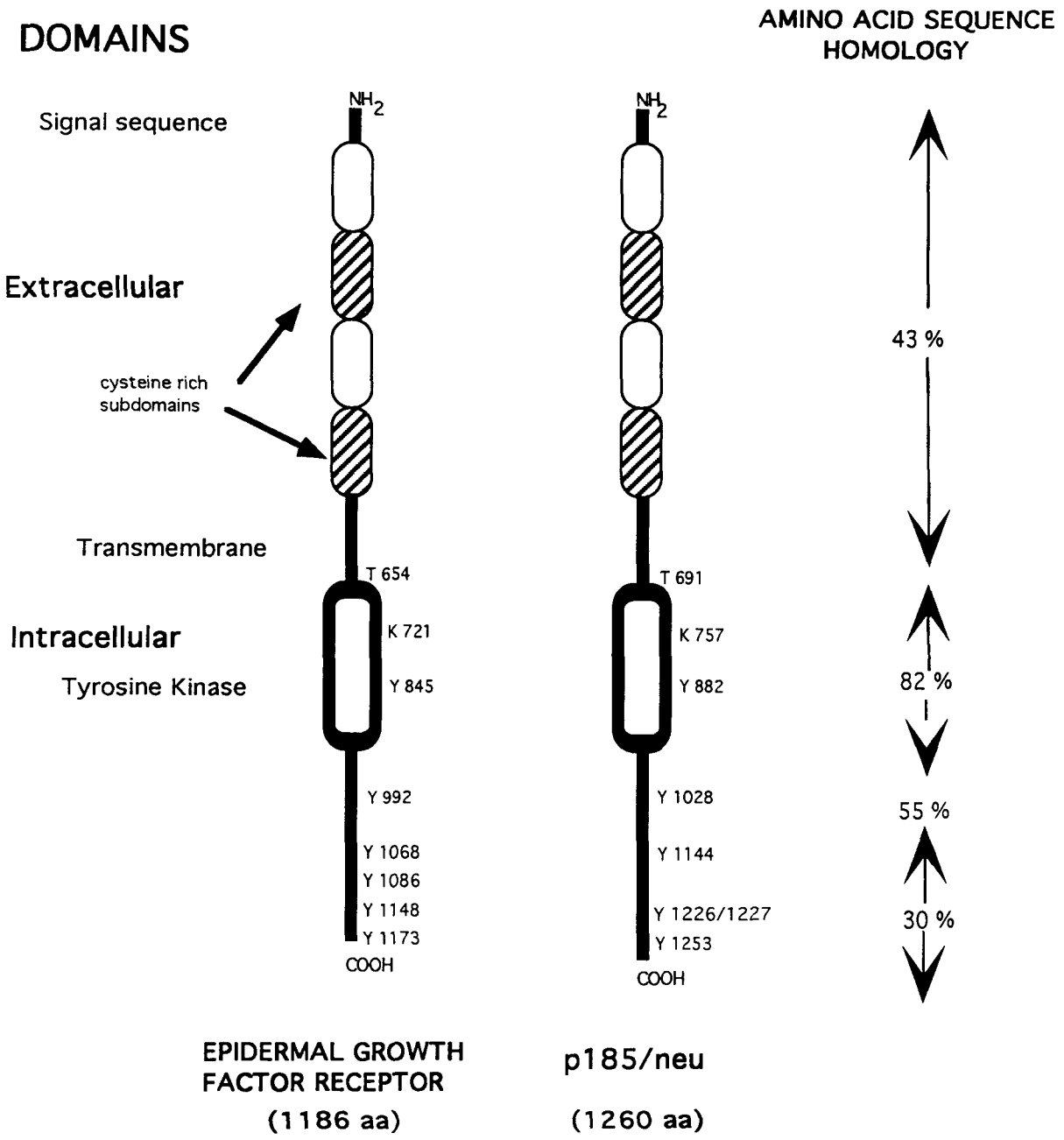


Fig. 1. Schematic illustration and comparison of amino acid homology between the human epidermal growth factor receptor (EGFR) and the rat neu/p185 protein. The numbering of amino acids is according to [Bargmann et al., 1986].

mutant neu protein were not transformed, demonstrating that a functional tyrosine kinase is necessary for the transforming potential of $p185^{neu}$ [Weiner et al., 1989a].

Similar to the EGFR, phosphorylation of $p185^{neu}$ regulates its tyrosine kinase activity. Substitution of phenylalanine for tyrosine residues within the carboxy-terminus of $p185^{neu}$ decreases the transforming potential [Akiyama et al., 1991, Segatto et al., 1990, Mikami et al., 1992], establishing a vital functional role for phosphorylation of $p185^{neu}$ tyrosines.

The marked increase in the oncogenic $p185^{neu}$ tyrosine kinase activity appears to be related to an increase in a multimeric, aggregated form of the protein. Using bifunctional cross-linking reagents, we observed [Weiner et al., 1989b] that the oncogenic form of neu/p185 aggregates into

high molecular weight dimers, while the proto-oncogenic protein exists predominately in the monomeric form. The high molecular weight aggregates were also observed in the absence of cross-linkers under nonreducing gel electrophoresis (but not under reducing conditions), suggesting a role for disulfide bonding in this aggregation. The amino acid substitution within the transmembrane region of $p185^{neu}$ shifts the equilibrium from the monomeric to the aggregated protein, in effect mimicking the ligand-induced aggregation and activation seen with EGFR. A recent study using a chimeric protein (composed of the extracellular domain of EGFR and the transmembrane and cytoplasmic domain of neu/p185) has indicated that the oncogenic mutation mediates dimerization of the chimeric protein and establishes a high affinity ligand binding site [Ben-Levy et al., 1992]. These studies of the type I receptor tyrosine kinases, neu/p185, and EGFR suggest that the physical association of these proteins affects their enzymatic activation and, potentially, the malignant transformation of cells.

The tissue-specific and developmental expression of neu has been examined in rats and humans. Studies have examined the expression of the neu proto-oncogene in a variety of rat tissues at different stages of pre- and post-natal development [Kokai et al., 1987]. Immunohistochemical and RNA blot analyses detected $p185^{c\text{-}neu}$ expression at the mid-gestational stage in tissues including the nervous system and secretory epithelium. In adult animals, secretory epithelial tissues (intestine, mammary gland, lung, kidney, and pancreas) and basal cells of the skin expressed $p185^{c\text{-}neu}$. Differential expression of the neu gene in fetal neuronal (but not adult) cells suggests involvement of $p185^{c\text{-}neu}$ in the development of this tissue type. Moreover, the oncogenic mutation described above occurs after treatment of pregnant BDIX rats with ethylnitrosourea at day 15 of gestation. This transplacental carcinogenesis leads to the same mutation of neu in multiple independent neurogenic tumors. It is likely that neu gene expression at this critical point in neuronal development increases its susceptibility to mutation leading to neuroblastoma formation. Human tissue distributions show no expression in adult nervous tissue, but do indicate expression in secretory epithelium such as the mammary gland, pancreas, intestine, salivary gland, and ovary [Press et al., 1990; Prigent et al., 1992].

RELATIONSHIP BETWEEN HUMAN NEU (C-ERBB-2) AND CANCER

The human homologue of neu, c-erbB-2, is amplified and overexpressed in a significant number of adenocarcinomas. This observation was first described in a human gastric tumor [Akiyama et al., 1986]. Slamon and colleagues [1989] examined the levels of c-erbB-2 RNA, DNA, and protein in breast and ovarian adenocarcinomas and correlated c-erbB-2 amplification with a poor clinical outcome. Amplification of the c-erbB-2 gene and subsequent overexpression of $p185^{c\text{-}erbB\text{-}2}$ was identified in 25–30% of primary breast and ovarian tumors. Tumors with higher gene copy numbers of c-erbB-2 correlated with a poorer patient prognosis. Some, but not all, studies have confirmed these results and have been the subject of several reviews [Maguire and Greene, 1990; Gullick, 1990]. C-erbB-2 overexpression also appears to be associated with some lung [Kern et al., 1990], colon [Cohen et al., 1989], and a high percentage of pancreatic adenocarcinomas [Williams et al., 1991b].

Although the oncogenic point mutation observed in rat neu has not been observed with c-erbB-2 in human tumors, amplification/overexpression of this protein appears to be related to malignancy. The role of c-erbB-2 amplification in human cancer can be explained by a model in which there is an equilibrium between the monomeric and dimeric/aggregated forms of the protein. As the quantity of $p185^{c\text{-}erbB\text{-}2}$ protein increases, the equilibrium is shifted to the dimeric/aggregated state resulting in activation of the tyrosine kinase and inappropriate cellular signaling. Evidence for this model stems from in vitro tumorigenesis assays using cell-lines overexpressing the normal proto-oncogenic forms of c-erbB-2 or rat neu (Table I). Using focus formation, anchorage-independent growth, and tumor formation in athymic mice as the criteria for transformation, studies have shown that c-erbB-2 will mediate transformation when expressed at high levels in NIH 3T3 cells [DiFiore et al., 1987b; Hudziak et al., 1987]. Overexpression of the proto-oncogenic form of rat neu, using SV40 promoter-driven expression vectors, failed to cause transformation of NIH 3T3 cells [Hung et al., 1986] or NR6 cells which are devoid of endogenous EGFR [Kokai et al., 1989]. Apparently, a critical level of overexpression is necessary to achieve a significant growth advantage and transformation of NIH 3T3 cells, since

TABLE I. Transformation Parameters of Cell-Lines Expressing Type I Growth Factor Receptors*

Cell-line (promoter/genes expressed)	Cell-line characteristics [Parental cell]	Receptor no.	Transformation results Focus formation	Soft agar growth	Tumor growth	Reference
G8 (SV40/rat c-neu cDNA)	[NIH 3T3]	3.4×10^{5}[a]	No	No	No	[Hung et al., 1986]
B104-1-1 (rat neu DNA)	[NIH 3T3]	1.4×10^{5}[a]	Yes	Yes	Yes	[Kokai et al., 1989]
NV (SV40/rat neu cDNA)	[NR6]	1.7×10^{5}[a]	No	No	No	[Kokai et al., 1989]
SV40 neuN (SV40/rat c-neu cDNA)	[NIH 3T3]	N.D.	No	No	No	[Di Marco et al., 1990]
LTR-2 neuN (LTR/rat c-neu cDNA)	[NIH 3T3]	20 × SV40 neuN[b]	Yes	Yes	Yes	[Di Marco et al., 1990]
SV40-erbB-2 (SV40/c-erbB-2 cDNA)	[NIH 3T3]	N.D.	No	No	No	[DiFiore et al., 1987; Di Marco et al., 1990]
LTR-2-erbB-2 (LTR/human c-erbB-2 cDNA)	[NIH 3T3]	20 × SV40 erbB-2[b]	Yes	Yes	Yes	[DiFiore et al., 1987; Di Marco et al., 1990]
NR6/HER-2 (LTR/human c-erbB-2 cDNA)	[NR6]	N.D.	N.D.	Yes	Yes	[Chazin et al., 1992]
NE19 (SV40/human EGFR cDNA)	[NR6]	2.5×10^{5}[c]	Yes	No	No	[Kokai et al., 1989]
LTR-EGFR (LTR/human EGFR cDNA)	[NIH 3T3]	N.D.	Yes[d]	Yes[d]	No	[DiFiore et al., 1987]
M1 (SV40/human EGFR cDNA and SV40/rat c-neu cDNA)	[NR6]	1.4×10^{5}[c] 1.3×10^{5}[a]	Yes	Yes	Yes	[Kokai et al., 1989]

*N.D. = not determined.
[a]Cell surface levels of rat neu/p185 protein were determined by ^{125}I-anti-p185$^{c\text{-}neu}$ monoclonal antibody (7.16.4) binding assays.
[b]Protein levels determined by densitometric scanning of Western blots.
[c]EGFR levels determined by ^{125}I-EGF binding.
[d]Positive results were observed only in the presence of EGF.

transformation was achieved when LTR-based expression vectors were used [DiMarco et al., 1990]. It was estimated that the more powerful LTR promoters allowed 20-fold higher expression levels of p185$^{c\text{-}neu}$ relative to SV40-based systems. A recent study by Chazin et al. (1992) confirms that overexpression of c-erbB-2 by LTR vectors is sufficient to transform cells in the absence of EGFR. However, it should be pointed out that the level of expression is extraordinary. In sum, these data demonstrate that overexpression of c-erbB-2 or the normal form of rat neu (p185$^{c\text{-}neu}$) is functionally linked to the transformation of cells in vitro and establishes a potential role for c-erbB-2 overexpression in human malignancies.

Using the normal and oncogenic form of rat neu/p185 purified from transfected insect cells, our laboratory has demonstrated that the oncogenic form has a higher propensity to form aggregates as measured by sucrose density gradient centrifugation. In a direct test of the above model, increasing the concentration of the normal p185$^{c\text{-}neu}$ protein shifted the equilibrium toward the aggregated state. Moreover, analysis of enzyme kinetics indicated that the aggregated proteins had a significant increase in V_{max} relative to the monomeric protein (LeVea et al., manuscript submitted). Taken together, these studies indicate that the inappropriate expression of the normal form of p185$^{c\text{-}erbB\text{-}2}$ can mediate aberrant cellular signaling and oncogenic transformation both in vitro and in vivo.

INTERACTION OF NEU/P185 AND THE EGF RECEPTOR

The physical and functional interaction of neu/p185 and EGFR has been suggested by a number of different observations, and more recently has been demonstrated by direct experiments. During early attempts to identify a ligand for p185$^{c\text{-}neu}$, Stern and colleagues [1986] revealed that although EGF does not bind to the p185$^{c\text{-}neu}$ receptor, EGF treatment of Rat-1 cells (expressing both EGFR and p185$^{c\text{-}neu}$) increased the tyrosine phosphorylation of p185$^{c\text{-}neu}$. This observation suggested that the p185$^{c\text{-}neu}$ receptor could serve as a substrate for EGFR, and was confirmed by a number of groups [Akiyama et al., 1988; Kokai et al., 1988; Stern and Kamps,

1988]. The EGF-mediated tyrosine phosphorylation of p185$^{c\text{-}neu}$ was rapid and dose-dependent, and was not observed either in cell-lines which lacked EGFR or in cells in which EGFR was down-modulated, indicating a direct role for EGFR. The functional consequence of this interaction after EGF treatment appears to be the concomitant activation of the in vitro kinase activity of neu/p185 [Kokai et al., 1988], establishing a unique regulatory pathway between two receptor tyrosine kinases. These data suggested that neu/p185 is indirectly activated by EGFR or can directly serve as a substrate for the EGFR tyrosine kinase resulting in neu/p185 kinase activation. The phosphorylation of neu/p185 after EGF treatment in both normal and transformed cell lines indicates that this phenomenon could represent a normal regulatory mechanism for neu/p185.

To examine this functional cross-talk between the two transmembrane tyrosine kinases further, our laboratory created mouse fibroblast cell-lines transfected with either rat p185$^{c\text{-}neu}$, human EGFR, or both proteins simultaneously (Table I) [Kokai et al., 1989]. The moderate overexpression of these proteins in a background lacking endogenous neu/p185 and EGFR allowed us to examine the direct interaction of these two proteins. Cell-lines expressing either of the tyrosine kinases individually (using SV40 expression vectors) exhibited normal morphology and were not transformed (as has been described previously) as assayed by anchorage-independent growth in soft agar and tumor formation in athymic mice. The M1 cell-line, which expressed both EGFR and p185$^{c\text{-}neu}$ at moderate levels exhibited a transformed morphology and formed tumors in vivo. EGFR and p185$^{c\text{-}neu}$ expression were examined in stable transfectants using ^{125}I-EGF and anti-neu mAb 7.16.4 binding, respectively. As shown in Table I, the combined expression levels of the two tyrosine kinases in the co-expressing line M1 did not significantly exceed the expression levels of either protein in individually expressing lines. This result has been observed for multiple clones expressing similar levels of the two proteins. Thus, neither EGFR nor p185$^{c\text{-}neu}$ expressed at moderate levels were able to transform NR6 cells, but when co-expressed at these levels, transformation occurred. This observation suggested that a novel functional and/or structural interaction of the two kinases occurs in these cells.

To define this synergistic interaction we and others have determined how these two proteins associate and interact. Since EGF was able to mediate the tyrosine phosphorylation of p185$^{c\text{-}neu}$ (see above) it was hypothesized that the two proteins were physically linked. To directly examine this, cell lines expressing both p185$^{c\text{-}neu}$ and EGFR were treated with homobifunctional cross-linking reagents, and subsequent immunoprecipitation with anti neu/p185 or EGFR antibodies identified a high molecular weight heterodimeric complex of p185$^{c\text{-}neu}$ and EGFR (Fig. 2A). The heterodimeric complex of human c-erbB-2 and EGFR has also been detected in SKBR-III breast cancer cells [Goldman et al., 1990]. More rigorous analysis of the intermolecular association of p185$^{c\text{-}neu}$ and EGFR was performed using non-ionic lysis and membrane fractionation in the absence of cross-linkers and provided further evidence for this interaction (Fig. 2B). This figure illustrates the high molecular weight heterodimer after nonreducing gel electrophoresis, and the co-immunoprecipitation of the cognate receptors under reducing conditions. The heterodimeric complex formation is stimulated by EGF addition, is reversible, and involves noncovalent interactions.

The physical interaction of p185$^{c\text{-}neu}$ and EGFR uniquely promotes increased mitogenic signal transduction resulting in cellular transformation. Evidence for this hypothesis comes from the analysis of in vivo tyrosine autophosphorylation and exogenous substrate phosphorylation which indicate that this heterodimeric EGFR/p185$^{c\text{-}neu}$ complex is a highly active tyrosine kinase [Qian et al., 1992]. Using immune complex kinase assays, the EGF-induced heterodimeric complex from co-expressing cell-lines showed greatly elevated kinase activity for auto- and exogenous (Histone H3) substrate phosphorylation relative to control cell lines expressing each individual receptor tyrosine kinase. In order to determine the level of in vivo tyrosine phosphorylation of the heterodimer relative to each homodimeric receptor, selective isolation of the EGFR/p185$^{c\text{-}neu}$ heterodimer was achieved using sequential immunoprecipitation with two antireceptor antibodies followed by antiphosphotyrosine Western blotting. This experiment indicated that the EGFR/p185$^{c\text{-}neu}$ complex contains approximately 50% of the total tyrosine phosphorylation levels present in the pool of heterodimeric and homodimeric species (compare specific heterodimer phosphotyrosine signal in lanes

2 and 5 to the pooled signal in lanes 3 and 4, Fig. 2C).

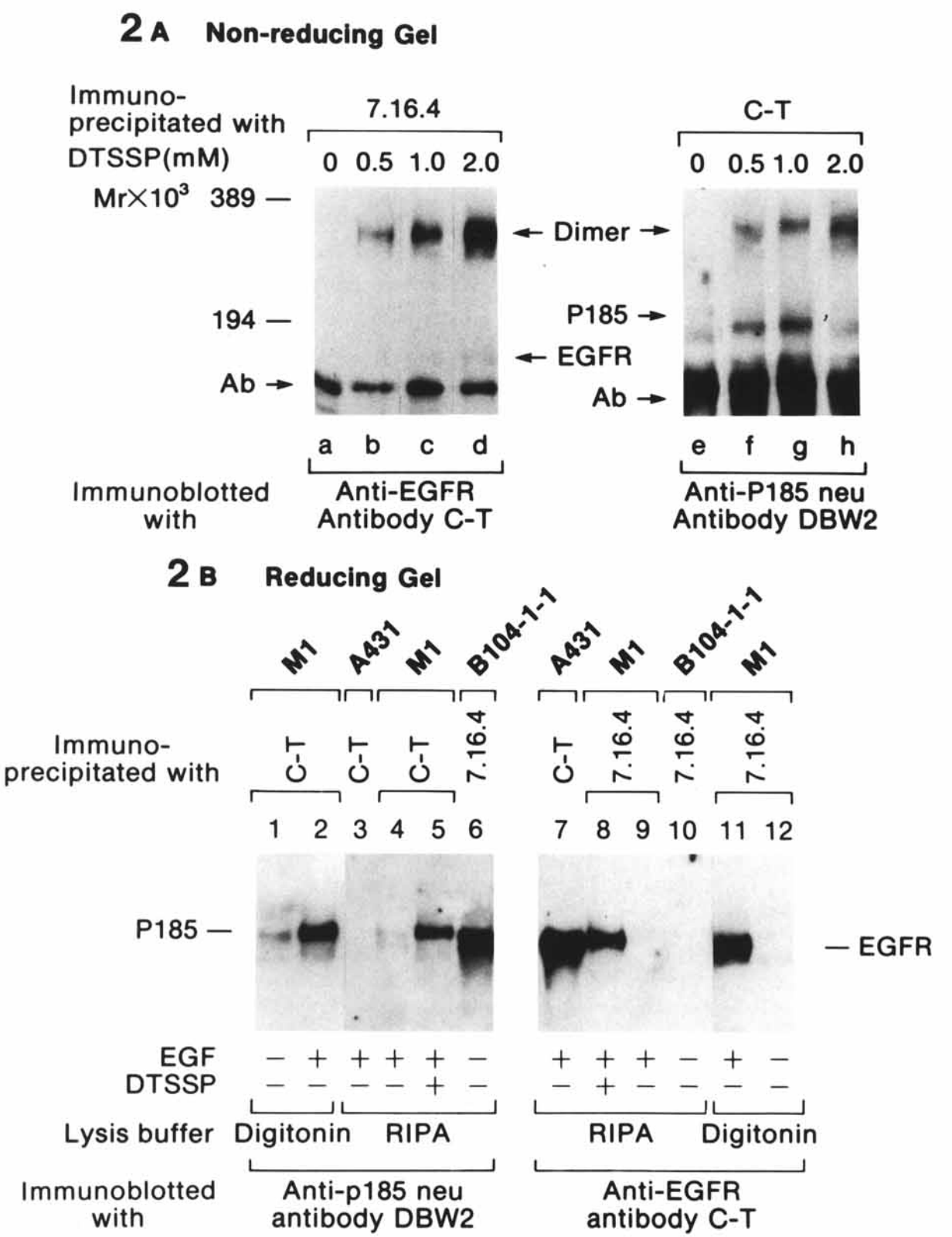
Further support for the functional interrelationship between the type I growth factor receptors comes from the observations that mutant forms of one receptor protein can regulate the normal function of the other receptor. A recent study [Spivak-Kroizman et al., 1992] indicated that a kinase-deficient EGFR mutant was able to heterodimerize with c-erbB-2, and tyrosine phosphorylation of both EGFR and c-erbB-2 was stimulated by EGF treatment. EGF also stimulated the formation of a heterodimer composed of wild-type c-erbB-2 and a EGFR mutant lacking the kinase domain and carboxy-terminus. However, tyrosine kinase activity was inhibited in this latter experiment, suggesting that the heterodimeric complex requires both kinase domains for activity. Other studies utilizing co-expression of wild-type EGFR and mutant $p185^{neu}$ proteins (kinase inactive or kinase deletion mutants) showed proper heterodimer formation, but loss of tyrosine kinase activity. The mutant $p185^{neu}$ proteins suppressed both EGF-stimulated DNA synthesis and cellular transformation in a dominant negative manner (Qian, Dougall, and Greene, unpublished data). These studies illustrate the utility of such an in vitro co-expression approach to examine the mechanism of growth factor receptor interaction and to determine the protein structures and interactions required.

The aggregation of the two different proteins promotes the formation of an active kinase complex with unique characteristics which can dramatically affect signal transduction and is reminiscent of the activated, aggregated $p185^{neu}$ oncogenic protein or homodimeric EGFR complexes [Ullrich and Schlessinger, 1990]. These studies suggest a mechanism by which EGFR and neu/p185 (or c-erbB-2) are transregulated, thereby promoting cellular transformation (see Fig. 3 for model). Upon dimerization, the tyrosine kinase of the heterodimeric EGFR/$p185^{c\text{-}neu}$ complex becomes activated, resulting in autophorylation of this protein complex. The synergistic contribution of the two proteins toward transformation implies that the EGFR/$p185^{c\text{-}neu}$ heterodimer stimulates distinct responses from either of the homodimers. The mechanism of this synergistic signal transduction is not clear at present, but our working hypothesis centers on the following possibilities. The affinity states of EGFR and neu/p185 (c-erbB-2) substrates toward their cognate receptors may be enhanced or the heterodimer stimulates substrates to a higher degree by virtue of the elevated kinase activity. Alternatively, the heterodimeric complex may recognize unique substrates not recognized by homodimeric proteins, which may affect unique responses in EGFR/$p185^{c\text{-}neu}$ transformed cells. The interaction of neu/p185 and EGFR may also modulate ligand binding and/or receptor down modulation and degradation differentially, as has been suggested by some studies [Wada et al., 1990a; Goldman et al., 1990]. The consequence of this latter interaction would presumably affect the maintenance of the activated kinase complex at the plasma membrane, and profoundly alter the normal signaling of these proteins. Continued study of the fundamental basis of this structural and functional interaction will provide crucial information regarding this novel transformation pathway. These data suggest that similar transmodulation mechanisms may also occur between other receptor kinases which utilize dimerization as an essential step in enzymatic activation [reviewed in Ullrich and Schlessinger, 1990]. The correlation of individual growth factor receptor overexpression and neoplasia, together with the synergistic contributions of neu/p185 and EGFR toward cellular transformation in vitro, suggests that the unusual situation in which multiple growth factor receptors are inappropriately expressed in vivo could be clinically important.

ANTIBODY DOWN MODULATION OF TRANSFORMED PHENOTYPE

Our laboratory has utilized monoclonal antibodies directed against the extracellular domain of the rat $p185^{neu}$ protein as a model system to examine the feasibility of antibody-mediated tumor therapy. Promising results from this system using mouse monoclonal antibodies (mAbs) to reverse the transformed phenotype induced by the oncogenic $p185^{neu}$ protein will be discussed, followed by an introduction to novel anti-tumor therapies utilizing functional domains derived from these antibodies.

Mouse mAbs raised against B104-1-1 cells (expressing rat $p185^{neu}$) recognize p185/neu on the cell-surface by flow cytometry and in cell lysates by immunoprecipitation [Drebin et al., 1984]. Treatment of the B104-1-1 cells with anti-neu mAb 7.16.4 dramatically inhibited the anchorage independent growth of these cells, a


2 A Non-reducing Gel
Immuno-precipitated with
7.16.4
C-T
DTSSP(mM)
0 0.5 1.0 2.0
0 0.5 1.0 2.0
Mr×10³ 389 —
194 —
Ab →
← Dimer →
P185 →
← EGFR
Ab →
a b c d
e f g h
Immunoblotted with
Anti-EGFR Antibody C-T
Anti-P185 neu Antibody DBW2
2 B Reducing Gel
M1
A431
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B104-1-1
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C-T
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1 2 3 4 5 6 7 8 9 10 11 12
P185 —
— EGFR
EGF
DTSSP
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Digitonin
RIPA
RIPA
Digitonin
Immunoblotted with
Anti-p185 neu antibody DBW2
Anti-EGFR antibody C-T

Figure 2A–B

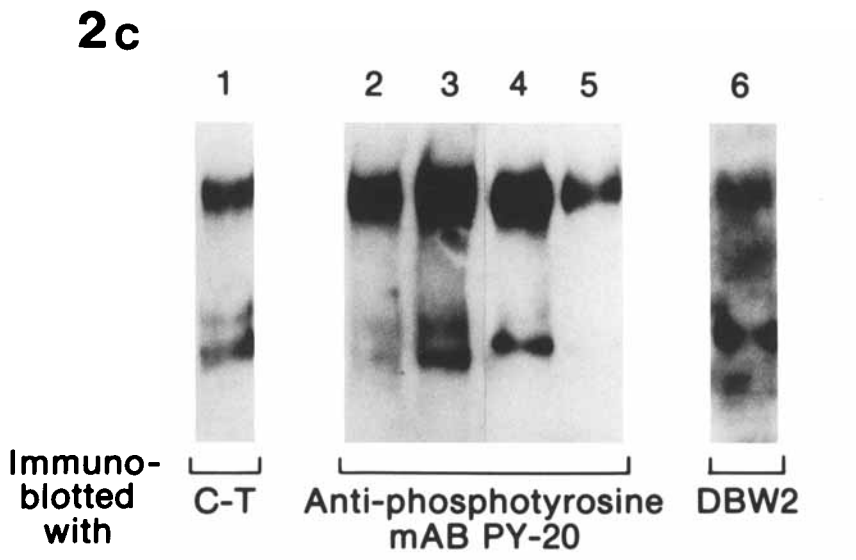

Figure 2C

unique characteristic of transformed cells (Fig. 4) [Drebin et al., 1985]. This effect was selective for the p185^{neu} protein since treatment with mAb 7.16.4 did not affect the phenotype of ras-transformed cells. Further experiments indicated that the reversion of transformation was correlated with the down modulation of p185^{neu} from the cell surface and increased degradation of this protein. We have extended these data to examine the effects of anti-neu mAbs on the growth of neu-mediated tumors in vivo. Treatment with anti-neu mAbs was able to significantly inhibit the tumorigenic growth of neu-transformed NIH 3T3 cells implanted into athymic mice. While none of the antibodies was able to completely eradicate tumors, multiple antibodies reactive with distinct epitopes of p185^{neu} resulted in synergistic anti-tumor effects and complete tumor growth inhibition in 50% of the treatment animals [Drebin et al., 1988a]. Long-term survival and tumor resistance was directly correlated with surface down modulation of p185^{neu}. Down modulation of p185^{neu} and the transformed phenotype was dependent on the presence of the mAb and was reversible upon removal of the antibody. This (mAb 7.16.4), and other, anti-neu mAbs (representing IgM, IgG2a, and IgG2b isotypes) exert a direct cytostatic effect on neu-transformed cells, indicating that antibody-mediated cytotoxicity or complement-mediated lysis are likely not to be involved in this reversion of transformation [Drebin et al., 1988b; Chazin et al., 1992]. The down modulation effects of these mAbs appears to be specific toward the oncogenic form of p185^{neu}; internalization of normal p185$^{c\text{-}neu}$ is not affected by anti-neu mAbs in vitro and toxicity has not been observed in vivo. Shepard et al. [1991] examined the abilities of several anti-c-erbB-2 mAbs against tumor cell-lines expressing various levels of c-erbB-2 protein and observed

Fig. 2. Analysis of EGFR/p185$^{c\text{-}neu}$ heterodimer in M1 cells. **A:** Chemical cross-linking analysis. Cells were treated with various concentrations of the bivalent covalent cross-linker DTSSP, and cell lysates were immunoprecipitated with antibodies reactive against neu/p185 (7.16.4) or EGFR (C-T) as indicated. Immunoprecipitates were resolved by 4–7.5% gradient nonreducing SDS PAGE and immunoblotted with specific antisera against EGFR (C-T) or neu/p185 (DBW2). The precipitation of the approximately 360 kDa complex by immunoprecipitation with one antireceptor antibody followed by immunodetection using the other, reciprocal antireceptor antibody identifies this complex as a heterodimer composed of both p185$^{c\text{-}neu}$ and EGFR. **B:** Analysis of the EGFR/p185$^{c\text{-}neu}$ heterodimer in the absence of chemical cross-linkers. Cells were treated as in A and lysed with buffers containing 1% SDS (**lanes 3–10**) or a non-ionic detergent, digitonin (**lanes 1, 2, 11, 12**). Immune complexes were resolved on 6% SDS PAGE under reducing conditions (which cleave the thio-labile cross-linker used in lanes 5 and 8). **C:** In vivo tyrosine phosphorylation of the EGFR/p185$^{c\text{-}neu}$ heterodimer. Lysates from cross-linker treated M1 cells were split and immunoprecipitated with either 7.16.4 or C-T. One aliquot of each (representing both homodimers and heterodimers) was boiled in sample buffer and analyzed by SDS PAGE (**lanes 3, 4**). Remaining aliquots were boiled in SDS buffer to inactivate antibodies and then reimmunoprecipitated with anti-EGFR antibody (C-T) (**lanes 1, 2**) or anti-Neu antibody DBW-2 (**lanes 5, 6**). Lanes 1, 2, 5, and 6 then represent only the heterodimeric species. After SDS PAGE, the blots were probed with the indicated antibodies. (Reproduced from Qian et al., 1992, with permission.)

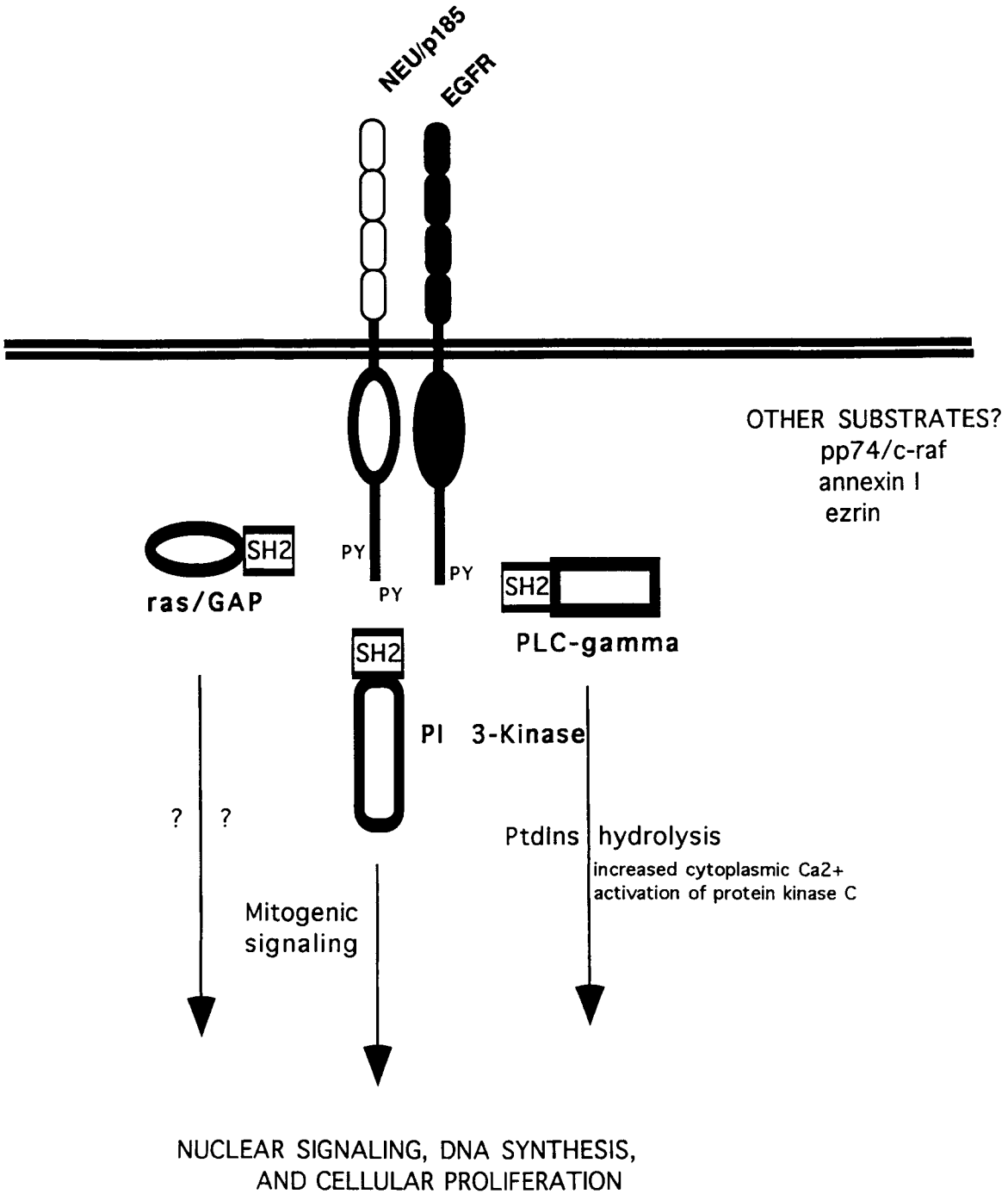

Fig. 3. Model for the heterodimerization and transregulation of EGFR and neu/p185. The physical interaction of the EGFR and neu/p185 proteins leads to kinase activation and high levels of tyrosine autophosphorylation (PY). This autophosphorylation recruits cellular substrates, by virtue of the substrate src-homology 2 (SH2) domains, to the heterodimeric complex. The substrates are then subsequently tyrosine phosphorylated leading to eventual mitogenic signaling within the nucleus. The identity of cellular substrates specific for the heterodimeric EGFR/p185$^{c\text{-}neu}$ heterodimer are currently unknown, and potential substrate interactions have been indicated.

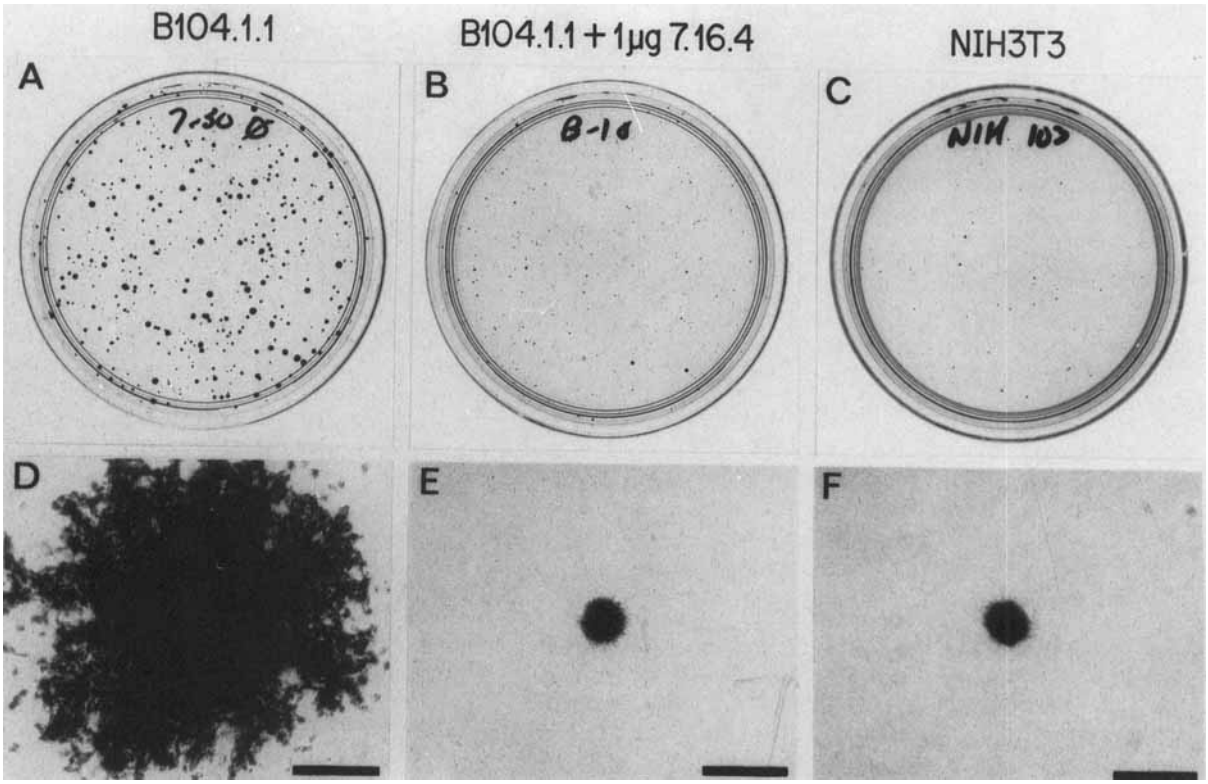

Fig. 4. Inhibition of soft agar formation by cells transformed by $p185^{neu}$ in the presence of antibody 7.16.4. B104-1-1 or NIH 3T3 cells were plated (1 × 103) in 0.18% agar and fed at weekly intervals with the indicated amount of anti-neu monoclonal antibody 7.16.4. Plates were photographed after 2 weeks of growth. **A–C:** Photographs of entire culture plates. **D–F:** Representative colonies photographed at ×40. (Reproduced from Drebin et al., 1985, with permission of the publisher.)

that antibody-mediated growth inhibition was dependent upon c-erbB-2 overexpression. The specific effects of anti-neu (c-erbB-2) antibodies toward oncogenic $p185^{neu}$ and overexpressed c-erbB-2 may then be due to a mechanism directed toward only the highly aggregated form of the protein.

The efficacy of anti-neu mAbs is apparently dependent upon the bivalent nature of the antibody and the ability to internalize receptor. We hypothesize that the antibody is cross-linking two $p185^{neu}$ proteins at the plasma membrane and altering the normal conformation of the dimeric receptor, affecting or stabilizing their oligomerization, rapid internalization, and eventual degradation. Support for this notion stems from experiments in which monovalent Fabs were ineffective at reverting transformation, but caused down modulation and inhibition of B104-1-1 cell growth in soft agar if a secondary IgG antibody was added. The selective criteria for anti-tumor antibodies include the ability to rapidly down modulate $p185^{neu}$ or $p185^{c\text{-}erbB\text{-}2}$ surface expression, affect degradation, and the inability to induce significant autophosphorylation/enzymatic activation. It is not entirely clear whether the tyrosine kinase becomes activated as a result of this cross-linking by anti-neu mAbs. Other studies have documented that anti-c-erbB-2 mAbs can stimulate tyrosine phosphorylation but are antagonistic toward cell growth and c-erbB-2-mediated tumorigenesis [Shepard et al., 1991]. The proposed mechanism of anti-tumor action by anti-c-erbB-2 antibodies in this study was to stimulate receptor internalization. Harweth et al. [1992] have recently reported anti-c-erbB-2 mAbs with partial agonist activity, but these mAbs still significantly inhibited the growth of tumor cells overexpressing $p185^{c\text{-}erbB\text{-}2}$. The inhibitory activity of these antibodies was correlated with efficient degradation of $p185^{c\text{-}erbB\text{-}2}$. The design and/or selection of higher affinity mAbs may prove to be more effective in mediating down modulation and subsequent degradation without the undesired activation of receptor signaling.

FUTURE PROSPECTS

The unique synergistic interaction between neu and EGFR results in transformation, and correlates with the c-erbB-2 and EGFR co-expression in highly malignant forms of breast and pancreatic cancer. Clinical studies of breast cancer patients indicate that overexpression of both c-erbB-2 and EGFR have more adverse effects than expression of either tyrosine kinase individually [Harris et al., 1989; Guerin et al., 1989; Gasparini et al., 1992]. Some human pancreatic tumor cell-lines have both elevated c-erbB-2 and EGFR expression [Korc et al., 1986; Cohen et al., 1989]. The expression of multiple growth factor receptors within one cell type is an example of inappropriate expression and likely contributes to the transformed phenotype of these human tumors. We are especially interested in antitumor therapies targeted specifically to these two cell-surface antigens. We have previously evaluated the hypothesis that growth of cells transformed by the synergistic interaction of neu/p185 and EGFR would be inhibited by immunologic down modulation of one or the other receptor. Anti-EGFR and anti-neu/p185 mAbs inhibited growth of tumor cells expressing both growth factor receptors [Wada et al., 1990b]. Moreover, this study illustrated that simultaneous administration of two anti-receptor antibodies had synergistic antitumor effects and inhibition of focus formation. These results implicate the involvement of both tyrosine kinases in the malignant transformation of these cells; however, the effect of mAb administration on the interaction of the two proteins has not been examined. Novel antibody constructs such as heterobifunctional anti-$p185^{neu}$/anti-EGFR antibodies can be developed using recombinant DNA techniques or conventional chemical cross-linking and have the potential to target the EGFR/$p185^{neu}$ heterodimeric complex. The contribution of the EGFR/$p185^{neu}$ kinase interaction toward malignant transformation of these cells could be elucidated by specific down regulation of the heterodimer using a heterobifunctional antibody construct. The results suggest that antireceptor effectors may have potentially useful applications in neoplasias in which growth factor receptor density is aberrantly high.

Although these studies have confirmed the feasibility of immunotherapeutic approaches toward $p185^{neu}$- and $p185^{neu}$/EGFR-expressing tumors, successful clinical application of this conventional immunotherapy is uncertain. The failure of antibody therapy in vivo is due, in part, to the proteinaceous nature of mAbs. Antibodies themselves are immunogenic, sensitive to proteolytic activity, and unable to cross blood-tissue barriers. Compounds which retain the binding and biological activities of antibodies, but lack the inherent drawbacks of mAbs would prove very useful as therapeutic reagents.

Our laboratory is examining the efficacy of constrained peptide or organic-mimetic analogs based on antibody structure to mediate the same biologic effects seen with antireceptor mAbs [Williams et al., 1991a]. Once the sequence and structure of anti-receptor antibodies have been determined, peptide analogs derived from complementary determining regions (CDRs) of the immunoglobulin can be generated which have the potential to mediate biological effects. The synthesis of overlapping peptides deduced from the CDR structure allows the determination of important residues for activity. Substitution of individual amino acids, coupled with computer-aided modeling, specifically define critical residues for both binding and biological efficacy. Utilizing this developmental strategy, we synthesized organic mimetics of CDR beta-turns or loops known to be important in ligand-receptor interactions. We have used this technology to construct active antibody analogs which bind to a cellular receptor. This mimetic is water soluble, resistant to proteolysis, and is nonimmunogenic. Binding affinities have been demonstrated in the low micromolar range and biological activity was confirmed using inhibition of DNA synthesis of activated lymphocytes as an assay [Saragovi et al., 1991].

The design and synthesis of mimetics derived from anti-c-erbB-2 and/or anti-EGFR mAbs could prove useful in the development of organic anti-tumor therapeutic modalities. CDR structural mimetics will be designed from the most biologically active anti-c-erbB-2 and anti-EGFR mAbs. Organic CDRs will be screened for the ability to limit antibody binding to the cell surface receptor, efficient down modulation of the cognate growth factor receptor, and reversion of cellular transformation. Theoretically, dimeric mimetics would increase the affinity toward one receptor species, and in the case of the EGFR/$p185^{c\text{-}neu}$ (or EGFR/$p185^{c\text{-}erbB\text{-}2}$) heterodimer, heterodimeric loop mimetics could also be generated which would have high affinity and specificity toward this complex.

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