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A subdomain in the transmembrane domain is necessary for p185^{neu*} activation

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The *neu* proto-oncogene encodes a protein highly homologous to the epidermal growth factor receptor. The *neu* protein (p185) has a molecular weight of 185 000 Daltons and, like the EGF receptor, possesses tyrosine kinase activity. *neu* is activated in chemically induced rat neuro/glioblastomas by substitution of valine 664 with glutamic acid within the transmembrane domain. The activated *neu protein (p185*) has an elevated tyrosine kinase activity and a higher propensity to dimerize, but the mechanism of this activation is still unknown. We have used site-directed mutagenesis to explore the role of specific amino acids within the transmembrane domain in this activation. We found that the lateral position and rotational orientation of the glutamic acid in the transmembrane domain does not correlate with transformation. However, the primary structure in the vicinity of Glu664 plays a significant role in this activation. Our results suggest that the Glu664 activation involves highly specific interactions in the transmembrane domain of p185.**

Key words: *erb2/neu*/receptor/transmembrane domain/tyrosine kinase

Introduction

The *neu* proto-oncogene encodes a 185 kDa surface glycoprotein designated p185 that possesses tyrosine kinase activity (Bargmann *et al.*, 1986b; Coussens *et al.*, 1985; King *et al.*, 1985; Schechter *et al.*, 1985; Stern *et al.*, 1986; Yamamoto *et al.*, 1986). *neu* is closely related to, but distinct from, c-*erbB*, the gene encoding the epidermal growth factor (EGF) receptor (EGFR) (Coussens *et al.*, 1985; Bargmann *et al.*, 1986b). *neu* is often activated in chemically induced neuroectodermal tumors in BDIX rats (Padhy *et al.*, 1982; Bargmann *et al.*, 1986b). The activating mutation creates an allele denoted *neu** with a single nucleotide substitution. Remarkably, the resulting amino acid replacement (Val664 to Glu664) lies within the predicted transmembrane domain (Bargmann *et al.*, 1986a). The transforming *neu**-encoded protein, p185*, has elevated tyrosine kinase activity, turns over rapidly, and forms dimers with high frequency (Bargmann and Weinberg, 1988a; Stern *et al.*, 1988; Weiner *et al.*, 1989a,b). In these respects it resembles the ligand-activated EGFR and other activated receptor tyrosine kinases. These results lend credence to the simple model that the *neu** activating mutation locks the receptor into an active configuration normally achieved upon hormone binding.

neu can be oncogenically activated in other ways besides point mutation. Amplification of the human *neu* proto-oncogene (also called c-*erbB2* and *HER2*) occurs with high frequency in certain carcinomas, especially breast and ovarian adenocarcinomas, and may correlate with poor prognosis (King *et al.*, 1985; Slamon *et al.*, 1989). The finding that overexpression of human *neu* in the apparent absence of ligand results in transformation of NIH3T3 cells (DiFiore *et al.*, 1987; Hudziak *et al.*, 1987) distinguishes p185 from other receptors and is consistent with a role for overexpressed p185 in human cancers. Furthermore, like the EGFR, deletions of the N-terminal and C-terminal domains partially release the oncogenic potential of *neu* (Bargmann and Weinberg, 1988b).

Two models have been proposed to explain the means by which the signal generated by hormone binding to receptor ecto-domains traverses the plasma membrane and stimulates the intracellular tyrosine kinase (reviewed by Ullrich and Schlessinger, 1990). One model is that ligand binding to the extracellular domain causes a conformational change which is transmitted through the transmembrane domain and causes a corresponding change in the intracellular domain. The second model is that ligand binding induces receptor oligomerization which in turn stimulates the intrinsic receptor kinase activity. For the EGF receptor, this latter model is supported by several observations: (i) cross-linking agents such as antibodies and lectins can stimulate the EGF receptor (Yarden and Schlessinger, 1987a); (ii) EGF stimulates oligomerization of EGF receptors both *in vitro* and in living cells (Cochet *et al.*, 1988); (iii) the isolated oligomeric form of EGF receptor has a higher kinase activity than the monomeric form (Yarden and Schlessinger, 1987b; Boni-Schnetzler and Pilch, 1987); (iv) EGF stimulates transphosphorylation between different forms of the EGF receptor (Honegger *et al.*, 1990), and (v) a kinase-deficient mutant of the EGF receptor dominantly inhibits normal EGF receptor activity through formation of hybrid oligomers (Kashles *et al.*, 1991). Studies of the insulin (Boni-Schnetzler *et al.*, 1986), platelet derived growth factor (PDGF) (Heldin *et al.*, 1989) and colony stimulating factor I (CSF-1) (Li and Stanley, 1991) receptors suggest that clustering is a general mechanism for transmembrane signal transduction. However, despite a wealth of correlative evidence it has been difficult to prove definitively that clustering is both necessary and sufficient for receptor activation. Furthermore, the domain(s) that nucleate clustering have not been identified, nor is it certain how hormone binding regulates clustering. Resolution of these questions is important because the minimal clustering domain represents an important therapeutic target for receptor-mediated diseases.

Since p185 is highly homologous to the EGF receptor, it has been hypothesized that the Glu664 activation works to stimulate oligomerization by stabilizing protein-protein

interactions in the transmembrane domain (Bargmann *et al.*, 1986a). Consistent with this model, p185* has a higher propensity to form dimers than normal p185 (Weiner *et al.*, 1989b).

The role of the transmembrane domain in receptor tyrosine kinase activation is uncertain. It is generally perceived as a passive component that merely connects the extra- and intracellular portions of the receptor. However, the finding that *neu** encodes what is likely to be an intramembrane amino acid substitution has focussed interest on this portion of the protein.

Substitution of almost any amino acid at position 12 in the Harvey *ras* protein leads to oncogenic activation, consistent with the idea that these *ras* activations cause loss of function (Seeburg *et al.*, 1984). In contrast, activation of p185 at position 664 is highly amino acid specific (Bargmann and Weinberg, 1988b). Mutation of Val664 to four other amino acids, glycine, histidine, lysine and tyrosine, does not activate p185. Glu664 and Gln664 fully activate p185 while Asp664 induces a partial activation (in human *neu*, aspartic acid is as active as glutamic acid in the corresponding position; Segatto *et al.*, 1988). The strong amino acid specificity for activation at position 664 suggests that Glu664 is a gain-of-function mutation. However, simply placing a glutamic acid in the transmembrane domain does not activate p185: when the residues adjacent to Val664, Val663 or Gly665, are replaced with glutamic acid individually, the mutated *neu* protein is not activated (Bargmann and Weinberg, 1988b). The failure of Glu663 and Glu665 mutations to activate p185 might have two different explanations. The first is that the position of the glutamic acid relative to other elements must be conserved. Either the lateral position or the rotational orientation of the glutamic acid might be important. An alternative model is that the amino acid context of Glu664 is critical for transformation and that Glu663 or Glu665 mutants are inactive because the specific structure required for activation is disrupted.

We tested these models by constructing mutant *neu* genes to identify constraints on the activation of p185*. Our results suggest that an oligopeptide within the transmembrane domain of p185* is involved in a highly specific way in oncogenic activation. This has general implications for the role of the transmembrane domain in signal transduction by all members of the receptor tyrosine kinase superfamily.

Results

Is the conservation of spacing to the endo-domain sufficient?

If conservation of the distance of glutamic acid to the cytoplasmic domain (17 amino acids from Glu664 to the basic anchor sequence) is sufficient to activate p185, glutamic acid at position 663 (rather than 664) would activate the protein if combined with a compensating deletion to restore this spacing. To test this, we changed Val663 to Glu and deleted Val666 (Table I, mutant 1). By the same logic, changing Gly665 to Glu would activate in the context of an insertion. Thus we changed Gly665 to Glu and inserted a glycine at position 669 (mutant 2). Site-directed mutagenesis of *neu* was performed using synthetic oligonucleotides and the mutated genes were expressed in NIH3T3 cells using a retrovirus vector (Bargmann *et al.*, 1988b). Virus stocks encoding mutant p185s were assayed for focus inducing activity and used to generate stable cell lines for biochemical analysis. Neither of the mutant genes had transforming

Table I. Positional mutations in p185 transmembrane domain

	659	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	focus formation
extracellular																							intracellular
p185	I	I	A	T	V	V	G	V	L	L	F	L	I	L	V	V	V	V	G	I	L	I	
p185* (v664E)	i	i	a	t	v	**E**	g	v	l	l	f	l	i	l	v	v	v	v	g	i	l	i	
1. v663E; v666del			i	i	a	t	**E**	v	g	()l	l	f	l	i	l	v	v	v	g	i	l	i	—
2. g665E; 669insG	i	i	a	t	v	v	**E**	v	l	l	**G**	f	l	i	l	v	v	v	g	i	l	i	—
3. i671E		i	i	a	t	v	v	g	v	l	l	f	l	**E**	l	v	v	v	g	i	l	i	—
4. lil670VEG		i	i	a	t	v	v	g	v	l	l	f	**V**	**E**	**G**	v	v	v	g	i	l	i	—
5. v664E; lil670VEG		i	i	a	t	v	**E**	g	v	l	l	f	**V**	**E**	**G**	v	v	v	g	i	l	i	+
6. v664E; v673del			i	i	a	t	v	**E**	g	v	l	l	f	l	i	l()v	v	v	g	i	l	i	+
7. v664E; i659del		(	)	i	a	t	v	**E**	g	v	l	l	f	l	i	l	v	v	v	g	i	l	i	+

The wild-type rat p185 transmembrane domain sequence (Bargmann *et al.*, 1986b) is shown at the top using the one-letter amino acid code. Amino acid number within the p185 polypeptide is indicated. Mutant p185s are shown below, beginning with p185*. Amino acid substitutions and insertions are shown in upper case, and wild-type amino acids in lower case. Parentheses show the sites of amino acid deletions. Results of focus assays based on data in Table II and similar unpublished data are shown on the right.

activity as measured in focus assays (Table II). This showed that conservation of the lateral position of Glu relative to the endo-domain is not sufficient for *neu* activation.

Is conservation of rotational orientation of Glu sufficient?

In one model for p185* activation, protonated Glu664 carboxyl groups form interchain hydrogen-bonded dimers within the transmembrane domain (Sternberg and Gullick, 1989). If so, then keeping Glu side chains on the appropriate face of the transmembrane domain might be sufficient for activation. Ile671, approximately two turns down from Glu664 (within 20° assuming that the transmembrane domain is an α-helix), was changed to Glu (mutant 3). Since amino acids adjacent to Glu664 might be involved in activation, the tripeptide Leu670-Ile-Leu, was changed to Val-Glu-Gly in mutant 4. Neither of the two mutations activated p185 (Table II). Since the latter mutations might cause gross conformational changes of p185*, we made the same tripeptide substitution in p185* (mutant 5). Interestingly, this tripeptide substitution in p185* did not interfere with the transforming activity of p185* (Table II). These results showed that rotational orientation of Glu is not sufficient to explain p185 activation. Furthermore they demonstrated that the Glu664 activating mutation is compatible with major non-conservative substitutions (including a second acidic residue) in the neighborhood of Ile671.

Is conservation of position necessary for transformation?

Having determined that conservation of the position of Glu is not sufficient for transformation, we next determined whether it is necessary. *neu** deletion mutants were used to determine if there are strict constraints on relative location of the activating glutamic acid: in mutant 6, Val673 (downstream of Glu664) was deleted, changing the position and rotational orientation of Glu664 relative to the intracellular domain. In mutant 7, Ile659 (upstream of Glu664) was deleted, altering the position and rotational orientation of Glu664 relative to the extracellular domain. Both mutants were fully active in inducing focus formation in NIH3T3 cells (Table II). These results indicated that conservation of neither the position nor rotational orientation relative to the extracellular domain or the intracellular domain is necessary for *neu** activation.

A subdomain of the p185 transmembrane subdomain is involved in Glu664 activation

These experiments refuted the positional models and led to the alternative that a specific protein structure in the transmembrane domain of p185* is involved in Glu664 activation. We therefore examined a series of second-site mutations in *cis* with Glu664 to identify other amino acids critical for this interaction (Table III). Most of the substitutions did not interfere with *neu** activation: moving from the N-terminus towards the C-terminus, deletion of Ile659 (mutant 7), substitution of Ile659-Ile660 with valines (mutant 11), a non-conservative mutation of Ile660 to Gly (mutant 10), and Thr662 to Ile (mutant 8) all spared transforming activity. Similarly, mutations of Val666 to Gly (mutant 12), Leu667 to Gly (mutant 14), leucines 667–668 to valines (mutant 13), Leu670-Ile-Leu to Val-Glu-Gly (mutant 5) and deletion of Val673 (mutant 6) did not interfere with Glu664 activation of p185* either.

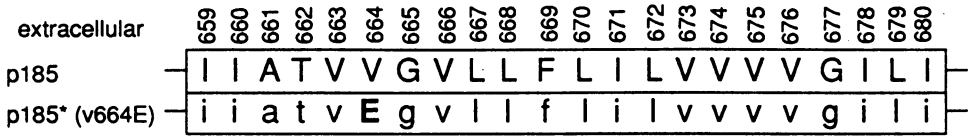
In contrast, three amino acids were sensitive to substitutions, Ala661, Val663 and Gly665. Changing Val663 to Gly (mutant 17), Gly665 to Val (mutant 15) or exchanging the two amino acids to conserve composition (mutant 16)

Table II. Focus induction by p185 mutants

Mutant	Number foci	Number G418^r colonies	Foci/G418^r colonies
Experiment 1			
neu	0	980	0
1	0	1040	0
2	0	1300	0
3	0	880	0
*neu**	130	210	0.6
Experiment 2			
neu	0	1130	0
1	0	1250	0
2	0	1060	0
3	0	940	0
4	0	1010	0
5	280	480	0.6
6	300	420	0.7
7	190	210	0.9
9	14	1810	0.01
15	0	1170	0
16	0	1820	0
*neu**	170	220	0.8
Experiment 3			
neu	0	1230	0
8	300	640	0.5
10	320	800	0.4
11	130	440	0.3
12	210	520	0.4
13	150	370	0.4
14	130	250	0.5
17	0	1180	0
*neu**	330	710	0.5
Experiment 4			
neu	0	1130	0
4	0	1000	0
5	130	320	0.4
6	68	180	0.4
7	280	540	0.5
8	30	52	0.6
9	4	320	0.01
10	150	480	0.3
11	195	470	0.4
12	140	200	0.7
13	150	360	0.4
14	130	280	0.5
15	0	980	0
16	0	1450	0
17	0	1020	0
Experiment 5			
neu	3	3640	0.001
18	4	2520	0.002
*neu**	520	2240	0.5

Plasmid encoding p185 mutants were transfected into ψ-2 cells and virus stocks harvested, and used to infect 100 mm cultures of NIH3T3 cells (Bargmann and Weinberg, 1988b). Infected cells were seeded onto four plates each at 1:4 and 1:40 dilutions. Duplicate plates were assayed for focus formation or selected for G418 resistance. The numbers of foci and G418-resistant colonies per original infected plate were extrapolated from at least two plates each in an individual experiment.

Table III. Second-site mutations in p185*



		focus formation
5. v664E; lil670VEG	i i a t v E g v l l f V E G v v v v g i l i	+
6. v664E; v673del	i i a t v E g v l l f l i l()v v v g i l i	+
7. v664E; i659del	()·i a t v E g v l l f l i l v v v v g i l i	+
8. t662L; v664E	i i a l v E g v l l f l i l v v v v g i l i	+
9. a661l; v664E	i i L t v E g v l l f l i l v v v v g i l i	+/−
10. i660G; v664E	i G a t v E g v l l f l i l v v v v g i l i	+
11. ii659VV; v664E	V V a t v E g v l l f l i l v v v v g i l i	+
12. v664E; v666G	i i a t v E g G l l f l i l v v v v g i l i	+
13. v664E; ll668VV	i i a t v E g v V V f l i l v v v v g i l i	+
14. v664E; l667G	i i a t v E g v G l f l i l v v v v g i l i	+
15. v664E; g665V	i i a t v E V v l l f l i l v v v v g i l i	−
16. vvg663GEV	i i a t G E V v l l f l i l v v v v g i l i	−
17. v663G; v664E	i i a t G E g v l l f l i l v v v v g i l i	−
18. *neu*/CD4	i i a t v E g I I A G L L L F I G L G I F F	−

All mutants have glutamic acid at position 664. Results of focus assays based upon Table II and similar data are shown at right. Mutant 9 ('+/−') had a substantially reduced focus-inducing activity. Results of alterations in various amino acids are summarized at the top. Some of these amino acids were only altered in combination with other changes.

completely abolished transforming activity. Another mutation, from Ala661 to Leu (mutant 9), drastically inhibited the transforming activity of p185* to ~3−5% of normal focus activity and yielded smaller foci.

Are amino acids 661−665 sufficient for transformation?

The above results indicated that a segment of p185* encompassing Ala661−Gly665 is involved in p185* activation. To determine if this domain is sufficient for p185* activation we replaced amino acids 667−680 downstream with an unrelated transmembrane sequence from CD4 (mutant 18). This hybrid p185*−CD4−p185* protein did not induce transformation of NIH3T3 cells (Table II), indicating that there are other still undefined determinants necessary for p185* activation.

The differences in the transforming activity of mutated p185s are due to their intrinsic protein structure

We performed several controls to be certain that differences in focus activity derived from primary protein structure. Since highly overexpressed p185 can transform NIH3T3 cells (DiFiore *et al.*, 1987; Hudziak *et al.*, 1987), we first determined whether cell transformation correlated with protein abundance. Cells were labeled to steady-state with [^{35}S]cysteine and p185 was immunoprecipitated (Figure 1, left panels). Although p185 expression was roughly comparable in polyclonal cultures expressing the various mutants, cells expressing non-transforming mutants showed consistently higher expression of p185 than cells expressing transforming *neu* mutants (Figure 1, left panels). This is consistent with the observation that p185* (Glu664) turns over more rapidly than non-transforming p185 (Stern *et al.*, 1988) and suggests that all of the transforming mutants will retain this property. For mutants 1−7 and 15−17, we also characterized several independent monoclonal cell lines (data not shown). The expression of p185 varied ~10- to 30-fold among different cell clones but cells expressing each mutant had uniformly transformed or non-transformed morphologies that correlated completely with results of the focus assays. This result is consistent with earlier data showing that transformation by p185* occurs even at very low abundance and is evident over a wide range of p185* expression (Stern *et al.*, 1986). Non-mutated p185 is not transforming at these levels (Hung *et al.*, 1986).

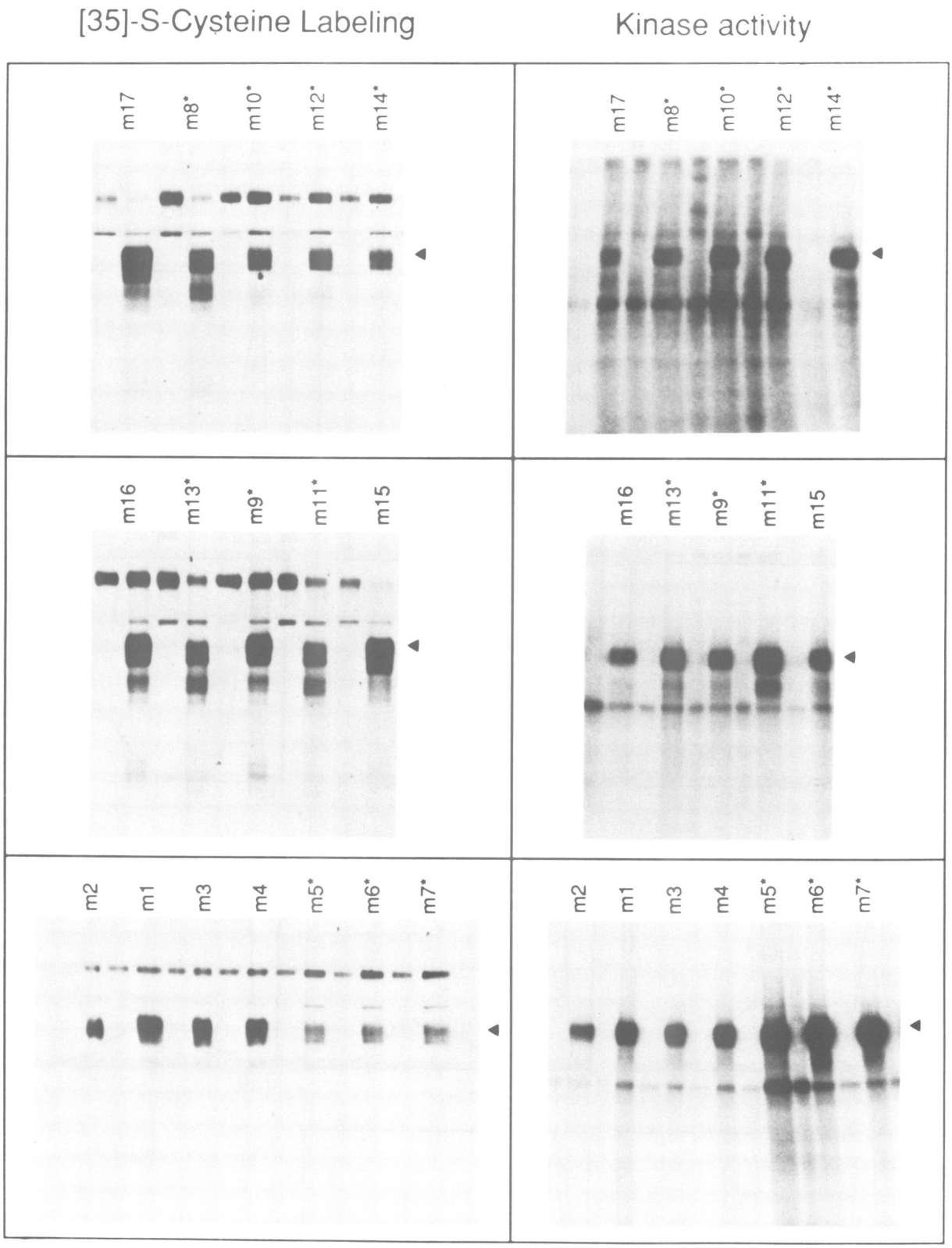


Fig. 1. Expression and kinase activity of p185 mutants. Polyclonal cultures of NIH3T3 cells infected with mutant viruses were seeded in duplicate plates and assayed in parallel for p185 expression by [^{35}S]cysteine labeling (left panels) and p185-associated kinase activity (right panels). Left panels: subconfluent cells were labeled with 200 μCi [^{35}S]cysteine (Amersham) overnight. The cells were lysed with RIPA buffer and immunoprecipitated with either normal serum (left each pair) or anti-p185 monoclonal antibody 7.16.4 (right each pair). The fluorographed gel was exposed to preflashed film at −70°C for 3 days. Right panels: immune complex kinase assays were performed as described (Stern *et al.*, 1986). Lysates were split and immune complexes were prepared with normal serum (left each pair) or 7.16.4 anti-p185 (right each pair). 15 μCi of [γ^{32}P]ATP (Amersham) was used at a final concentration of 10 μM ATP in each assay and the reactions were incubated for 10 min at 30°C. Gels were exposed to preflashed film at −70°C for 5 days with an intensifying screen. Mutants, described in Tables I and III, are indicated at top. Asterisks denote mutants with at least some transforming activity (Table II; mutant 9 has weak activity). Arrowheads indicate the positions of p185 proteins. All samples were analyzed on 7.5% acrylamide−0.175% bisacrylamide SDS−polyacrylamide gels.

Since surface expression of p185* is probably required for activity (Bargmann and Weinberg, 1988b), another trivial explanation for the non-transforming mutants is that the mutated p185s are not expressed at the cell surface. Surface expression of p185 in cells was determined by flow microfluorimetry of non-permeabilized cells stained with anti-p185. All cells expressing p185 mutants showed substantial labeling of p185 on the cell surface that was at least comparable with (usually greater than) that of *neu**-transformed B104-1-1 cells used as a positive control (Figure 2; similar data, not shown, we obtained for mutant 18 using cell surface immunofluorescence). These results showed that the non-transforming p185 mutants are transported to the cell surface.

Mutations in the transmembrane domain might cause gross changes of p185 conformation that disrupt tyrosine kinase activity. Immune complex kinase assays were performed to verify that the p185 mutants were still functional. All mutants showed autophosphorylation activity comparable with wild-type p185 (Figure 1, right panels. Similar results were

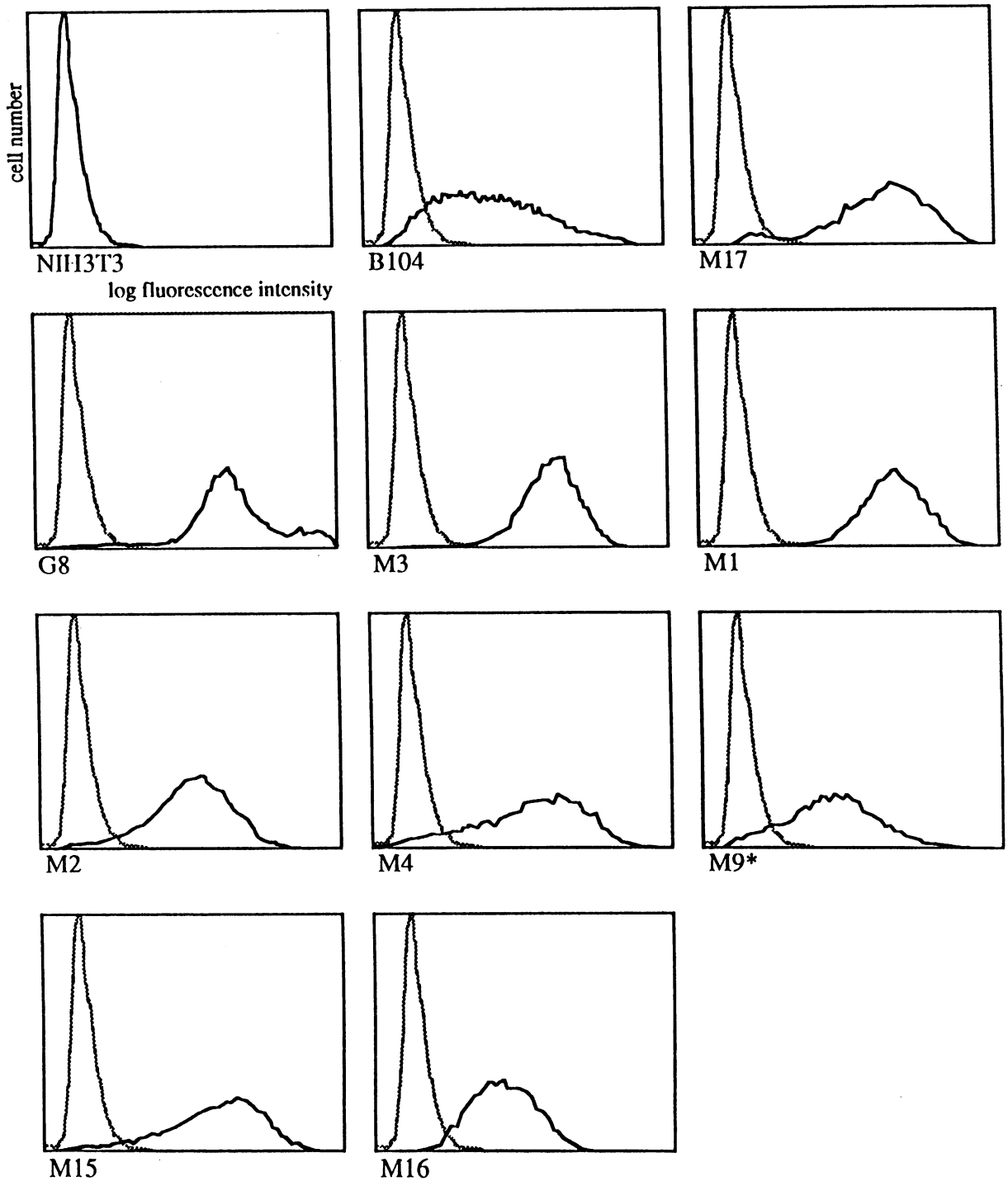


Fig. 2. Surface expression of p185 mutants. Non-permeabilized cultures were stained with anti-p185 antibody 7.16.4 followed by fluoresceinated anti-mouse antibody and analyzed by flow microfluorimetry. Each histogram is superimposed on that of the negative control NIH3T3 cell lines analyzed in parallel. 'B104' denotes the positive control *neu**-transformed cell line B104.1.1.

obtained for mutant 18). Although the steady-state level of expression of transforming mutants was consistently lower than non-transforming mutants (Figure 1, left panels), they were generally associated with higher autophosphorylation activity in this assay (Figure 1, right panels). This extended the previous observations that transforming p185* has a higher basal kinase activity (Stern *et al.*, 1988; Bargmann and Weinberg, 1988a).

As a final measure of activity of the mutant p185s we determined whether focus induction correlates with tyrosine phosphorylation of cellular substrates. Anti-phosphotyrosine immunoblots of total cellular protein extracts were compared for the various mutants (Figure 3). We have previously found that p185* induces tyrosine phosphorylation of several substrates (Stern *et al.*, 1988). One of the bands, the 62 kDa GAP-associated protein, is the fastest migrating protein marked with an arrowhead in Figure 3. Tyrosine phosphorylation of this protein correlates well with p185 activity, supporting the idea that it is a p185 substrate (Cao *et al.*, 1991). This and other tyrosine phosphorylated proteins were consistently observed in cells expressing fully transforming *neu* mutants. In cells carrying non-transforming *neu* mutants and the weakly transforming mutant 9, no significant tyrosine phosphorylation of these proteins was seen. Thus focus induction correlated well with transformed morphology, reduced steady-state expression of p185, increased immune complex kinase activity and increased tyrosine phosphorylation of cellular proteins.

Discussion

Our finding that a small subset of amino acids within the p185* transmembrane domain is highly sensitive to substitutions is a departure from previous conclusions concerning the related EGFR. Our data suggest that there is a functional element that involves near-contiguous amino acids within the transmembrane domain of p185*. This element encompasses amino acids Ala661, Val663, Val664 and Gly665.

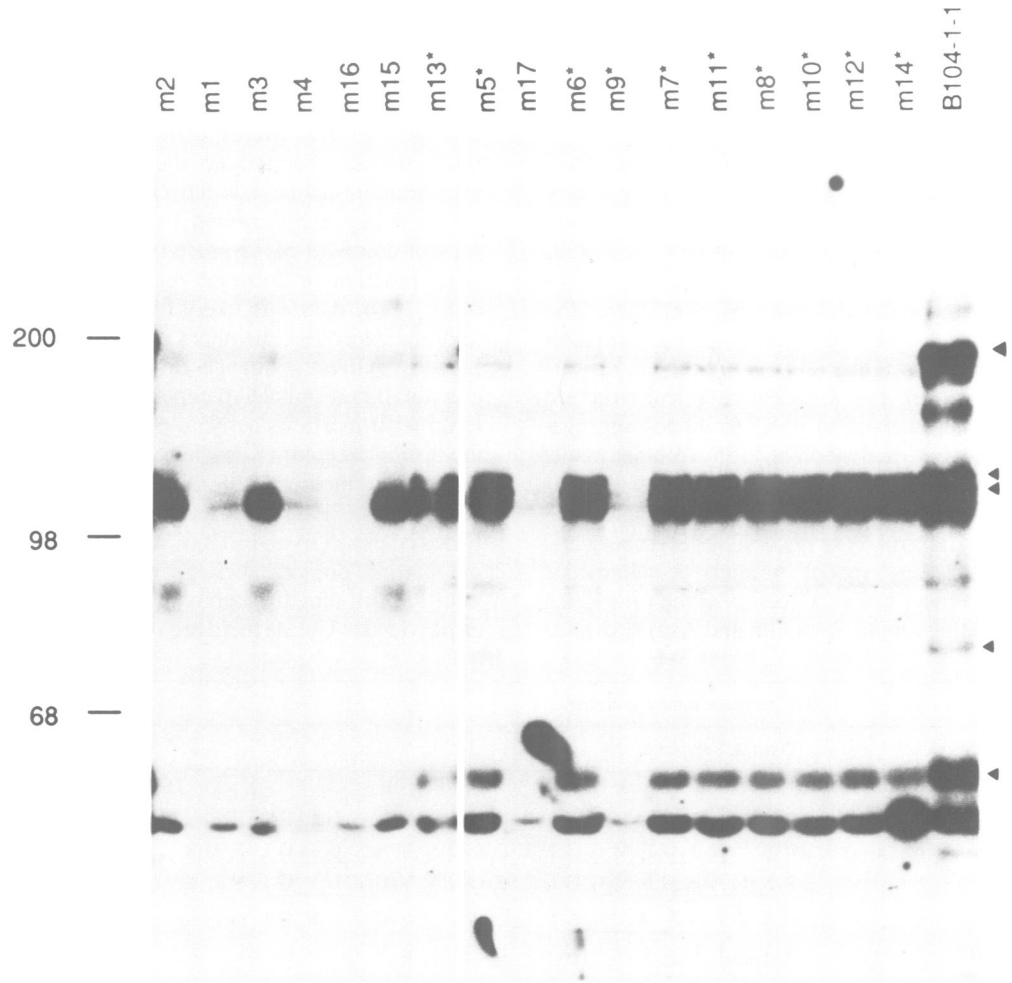


Fig. 3. Anti-phosphotyrosine immunoblots of protein extracts isolated from cells containing p185 mutants. Total protein extracts of cells were prepared as described (Kamps and Sefton, 1988). The protein loaded on each track was normalized by Coomassi brilliant blue staining. Even transfer of protein to the nitrocellulose filter was verified by staining with 0.01% Ponceau red. An extract from the positive control cell line B104.1.1 was used as a marker only. It was prepared independently and was not normalized for protein content. Immunoblotting with anti-phosphotyrosine antibody was performed as described (Kamps and Sefton, 1988). The filter was exposed to preflashed film with an intensifying screen for 3 days. The topmost arrowhead indicates p185, and the bottom arrowhead the 62 kDa GAP-associated protein (Cao *et al.*, 1991). Remaining arrows mark other immunoreactive proteins specific to transformed cells. Cell lines analyzed are indicated at the top. Asterisks mark mutants with at least some transforming activity.

Although mutations at other positions did not affect activation by Glu at position 664, replacement of much of the transmembrane domain with an unrelated CD4 sequence disrupted p185* activation. This result suggests that additional determinants are also necessary for activation.

*Activation of p185**

The amino acid specificity of Ala661–Gly665 suggests that this region is involved in a protein–protein interaction. The simplest model is that this element facilitates oligomerization of p185*. Other data are compatible with a model in which the p185* transmembrane domain itself nucleates oligomerization: the Glu664 mutation greatly enhances focus induction by a truncated p185 lacking most of the ecto-domain (Bargmann *et al.*, 1988b). This truncated protein is competent to form complexes with full-length p185* (H.Cao and D.F.Stern, unpublished data). Thus most of the ecto-domain is dispensible for p185* activation and dimerization. Furthermore, a mutated form of p185* bearing an extra unpaired cysteine just outside the transmembrane domain forms homodimers with high efficiency in intact cells (H.Cao *et al.*, submitted for publication). Assuming that the cross-link is formed at the site of the novel cysteine residue, this suggests that in a p185* dimer the transmembrane domains are in close proximity.

Structural models for the formation of the postulated inter-p185* dimers have been proposed based upon the primary structure of the transmembrane domain. One model is based on conformational energy analysis (Brandt-Rauf *et al.*, 1989, 1990). The global minimum-energy conformation of non-transforming substitutions at position 664 (Val, Gly, His, Lys, Tyr) contains a sharp bend, whereas the conformation of transforming proteins (Glu, Gln) is entirely α-helical. According to this model, the complete α-helical structure of the transmembrane domain of p185 facilitates aggregation of p185s and leads to activation of p185. Another model is that the Glu664 carboxyl group forms hydrogen bonds with its homolog on a second p185* molecule, stabilizing lateral interactions (Bargmann *et al.*, 1988b; Sternberg and Gullick, 1989). A final model predicts a symmetric interaction between two p185 α-helices in which protonated Glu664 (and activating amino acids Gln and Asp) hydrogen bonds with the Ala661 carbonyl oxygen on the other helix (Sternberg and Gullick, 1989, 1990). None of these models has yet been subjected to rigorous testing.

Although we cannot directly evaluate the structural models, our data do provide a test for a generalized consensus sequence that was deduced from the latter model (Sternberg and Gullick, 1989, 1990). This consensus sequence requires a small side chain at position 0 ('P0'; p185 Ala661), an aliphatic side chain at P3 (p185 position 664), and a small side chain (Gly or Ala) at P4. Such 'Sternberg–Gullick' consensus sequences are underlined in Table IV in a compilation of EGF receptor family transmembrane domain sequences.

Our data are consistent with the Sternberg–Gullick consensus in demonstrating the importance of Ala661 and Gly665 as predicted. However, the consensus sequence places no constraints on P2, while we found that substituting Gly for Val at this position (mutant 17) interferes with transformation. Another small discrepancy is that no Sternberg–Gullick consensus sequence is evident in the *Drosophila* homolog DER, but a valine to glutamic acid mutation activates DER (Wides *et al.*, 1990; the valine is shown in bold type in Table IV).

Signaling by non-mutated receptors

Since the *neu** activating mutation occurs in the transmembrane domain, it is not entirely surprising that other amino acids in this region of p185* are important. The present data will be of greatest interest if the same amino acids function in hormone-regulated signaling by normal p185. If so, then it is likely that the results will extend at minimum to other members of the EGF receptor family. Indeed, indirect evidence implicates the transmembrane domain of non-mutated receptors in signaling. First, amino terminal truncations activate *neu, erb B* (EGFR), *ros* and the insulin receptor (Khazaie *et al.*, 1988; Bargmann *et al.*, 1988b; Birchmeier *et al.*,1986; Matsushime *et al.*, 1987; Wang *et al.*, 1987). Furthermore, C-terminally truncated forms of the EGF and PDGF receptors lacking more of the cytoplasmic domains form growth factor-dependent dimers with the full-length receptors (Kashles *et al.*, 1991; Ueno *et al.*, 1991). Unless there are two independent mechanisms

Table IV. Transmembrane domains of EGF receptor family members

Gene	Species	Predicted sequence	Reference
		1 2 3 4 5	
ERBB	hu	P S I A T G M V G A L L L L V V A L G I G L F M R	Ullrich *et al.*, 1984
erbB	mu	P S I A T G I V G G L L F I V V V A L G I G L F M R	Avivi *et al.*, 1991
erbB	ch	P S I A A G V V G G L L C L V V V G L G I G L Y L R	Lax *et al.*, 1988
neu	hu	L T S I V S A V V G I L L V V V L G V V F G I L I K	Coussens *et al.*, 1985
neu	ra	V T F I I A T V V G V L L F L I L V V V V G I L I K	Bargmann *et al.*, 1986b
ERBB3	hu	L T M A L T V I A G L V V I F M M L G G T F L Y W R	Kraus *et al.*, 1989
X-mrk	Xi	S S L A V G L V S G L L I T V I V A L L I V V L L R	Wittbrodt *et al.*, 1989
C-let-23	Ce	T R M V I I G S V L F G F A V M F L F I L L V Y W R	Aroian *et al.*, 1990
DER	Dr	N M I F I I T G A V L V P T I C I L C V V T Y I C R	Livneh *et al.*, 1985

Twenty-five amino acids are shown aligned solely by the location of the first basic amino acid presumed to identify the junction with the cytoplasm. There is no attempt to identify the more ambiguous junction with the ecto-domain. All glycines are shown in bold. Sternberg–Gullick consensus sequences (G, A, S, T or P at PO; A, V, L, I, at P3; G or A at P4; Sternberg and Gullick, 1990) are underlined. '1, 2, 3, 4, 5' indicate the positions of amino acids 661–665 in rat *neu*. Val664 in rat and its equivalent in human *neu* are shown in bold type. *DER* kinase activity is activated by replacement of the valine in bold type with glutamic acid (Wides *et al.*, 1990). 'hu' denotes human genes, ra, rat; mu, murine; ch, chicken, fe, feline; gp, guinea pig; Dr, *Drosophila melanogaster*, Ce, *Caenorhabditis elegans*; and Xi, *Xiphorus maculatus*.

for receptor activation, these experiments limit the possible location of critical oligomerization domains. If there is a single domain that nucleates clustering, it must be located within or near the transmembrane domain.

The conclusion that the transmembrane domain of the EGF receptor is a passive element is based primarily on mutational studies (Kashles *et al.*, 1988; Carpenter *et al.*, 1991). Our results are consistent with these reports in showing that signaling is not prevented by insertions and deletions that alter the spacing of amino acids in the transmembrane domain, and that the transmembrane domain is generally insensitive to point mutations. Such data have led to the conclusion that there is no functional structure within receptor transmembrane domains. The inconsistency between our results and these studies on the EGF receptor is the striking sensitivity of certain amino acids in p185* to substitution. Because there has been no similar effort yet to scan the EGF receptor transmembrane domain with mutations, there are several possible explanations for this difference. (i) The 'element' we have identified may operate only in mutated p185* and not p185. (ii) The element may be present in *neu* proteins, but not other receptor kinases. (iii) There may be a functionally homologous region in the EGF receptor that has not yet been altered among the mutations that have been tested. However, mutations have been made in two amino acids of the EGFR that are apparently homologous to critical amino acids in p185*. Assuming that the amino acids TGMVG in the EGFR are homologous to ATVVG in rat *neu* (Table IV), the equivalents of p185 Val664 and Gly665 (EGFR Val627 and Gly628) have been mutated to several other amino acids. Mutations at the 664 analogue including substitution with Glu either failed to affect (Carpenter *et al.*, 1991) or slightly activated the EGFR (in the presence of ligand; Kashles *et al.*, 1988). Mutation of EGFR Gly628 to Lys or to Glu did not grossly interfere with regulation by EGF (Carpenter *et al.*, 1991) even though mutation of the analogous Gly in p185* to Val (mutant 15) interfered with transformation. (This Gly is also predicted to be important by the Sternberg–Gullick rules.)

If the domain that we have identified is important in signaling it should be evolutionarily conserved. Comparison of receptor transmembrane domains is difficult because they are highly enriched for a small number of hydrophobic amino acids. The position of glycine is a useful indicator of sequence conservation among transmembrane domains because few glycines are present in each transmembrane domain. Members of the EGF receptor family are all characterized by the presence of Gly residues ~17 amino acids upstream from the anchor sequence (Table IV). (A similar conservation of glycine position may occur in FGF receptor and *eph* families; data not shown.)

Receptor tyrosine kinases are not the only molecules in which single transmembrane domains evidently have specific functions. The transmembrane domain of integral membrane proteins has been implicated in Golgi complex retention, endoplasmic reticulum (ER) retention, degradation and protein assembly (Machamer and Rose, 1987; Bonifacino *et al.*, 1990). A short transmembrane sequence is necessary and sufficient for retention and degradation of the T cell receptor α-subunit (TCRα) in the ER and directs the interaction of TCRα with CD3-δ. It is noteworthy that two charged amino acids in the transmembrane domain are part of the determinant for ER retention and degradation (Bonifacino *et al.*, 1990). Similarly, the sites of interaction of Fcγ RIIIA-α and the ζ chain have been mapped to the transmembrane domain of the ζ chain. TCRζ and Fcε RIγ normally exist as dimers and resemble p185* in having an acidic residue flanked by a small side chain amino acid, with both flanked by aliphatic amino acids (Kurosaki *et al.*, 1991).

In summary, our results suggest that the transmembrane domain plays an active role in signal transduction by integral membrane proteins. In the *neu* protein, a segment encompassing amino acids 661–665 contains critical amino acids. We predict that the glycine-containing motif in the EGFR family is involved in signal transduction, possibly by directly facilitating dimerization of these receptors.

Materials and methods

Cells

DHFR/G8 cells are NIH3T3 cells transfected with a genomic clone of *neu* and express high levels of p185 (Hung *et al.*, 1986). B104-1-1 cells are NIH3T3 cells transformed by transfection with *neu** (Padhy *et al.*, 1982). Cells were grown in Dulbecco–Vogt modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS).

Mutagenesis

Site-directed mutagenesis and expression of *neu* was performed with appropriate synthetic oligomers as described (Kunkel *et al.*, 1985; Bargmann *et al.*, 1988b). The synthetic oligomers used were: mutant 1, 5′-CATTGCAACTGAAGTGGGCCTGCTGTTCC-3′ mutant 2, 5′-CAACTGTAGTGGAGGTCCTGCTGGGATTCCTGATCTTAG-3′; mutant 3, 5′-CTGCTGTTCCTCGAGTTAGTGGTGG-3′; mutants 4 and 5, 5′-GTCCTGCTGTTCGTCGAAGGAGTGGTGGTC-3′; mutant 6, 5′-CTTAGTGGTGGTCGGGATCCTAATCAAAC-3′; mutant 7, 5′-GACATTCATCGCGACTGTAGAGG-3′; mutant 8, 5′-CATTCATCATTGCGATCGTAGAGGGCG-3′; mutant 9, 5′-GTGACATTCATAATATTATCTGTAGAGG-3′; mutant 10, 5′-GACATTCATCGGCGCCACTGTAGAGG-3′; mutant 11, 5′-GGTGACATTCGTCGTCGCGACTGTAGAGG-3′; mutant 12, 5′-GTAGAGGGCGGCCTGCTGTTC-3′; mutant 13, 5′-CTGTAGAGGGAGTCGTGGTGTTCCTGATC-3′; mutant 14, 5′-GTAGAGGGCGTCGGCCTGTTCCTGATC-3′; mutant 15, 5′-GCAACTGTAGAAGTCGTTCTGCTGTTCC-3′; mutant 16, 5′-CATCATTGCAACCGGTGAGGTCGTCCTGCTG-3′; mutant 17, 5′-CATTGCAACTGGAGAGGGAGTCCTGCTGTTC-3′ and mutant 18, GCAACTGTAGAGGGCATCATCGCCGGCCTCCTGCTTTTCATTGGCCTAGGCATCTTCTTCAAACGAAGGAGAC. A *NdeI*(1895)–*BglI*(2377) fragment derived from a mutagenized Bluescript subclone was recloned in the pDOL-*neu* expression vector, which expresses *neu* under control of a MoMuLV LTR, and carries a selectable SV-neo cassette (Bargmann and Weinberg, 1988b). Mutants were verified by sequencing the novel junctions in the final constructs, the regions covered by the mutagenic oligonucleotides, and nearly all of the DNA from the *NdeI* site through the transmembrane domain coding sequences.

pDOL-*neu* DNAs encoding mutant p185s were introduced into ψ-2 cells using the calcium phosphate method with glycerol shock. Transfected cells were selected with G418 (Geneticin-Gibco). Virus was harvested and used to infect NIH3T3 cells (Bargmann and Weinberg, 1988b). Cells were passaged 2 days later to assay for focus formation and parallel cultures were selected for G418 resistance. Data shown in Table II are representative of a larger series of experiments in which the activity of each mutant was determined from at least four NIH3T3 cultures (usually six or eight) infected independently.

Antibodies

Monoclonal anti-p185 antibody 7.16.4, was a generous gift from M.I.Greene, or was purchased from Oncogene Science (anti-*neu* antibody 4). Polyclonal rabbit anti-phosphotyrosine (raised against poly[Gly-Ala-phosphotyrosine] linked to keyhole limpet hemacyanin) was produced and affinity purified in this laboratory as described (Kamps and Sefton, 1988).

Immunoprecipitation, immunoblotting and immune complex kinase assay

Most of these procedures were performed as previously described (Stern *et al.*, 1986; Stern and Kamps, 1988). For [^{35}S]cysteine labeling

experiments, immunoprecipitations were performed using RIPA (10 mM sodium phosphate, pH 7.0; 1% Triton X-100; 0.1% SDS; 1% sodium deoxycholate; 150 mM sodium chloride; 1% aprotinin) containing 100 μM sodium orthovanadate. Immune complex kinase assays were performed using the milder TG buffer (1% Triton X-100; 10% glycerol in PBS) (Stern *et al.*, 1986).

Flow microfluorimetry

Near-confluent cultures in 100 mm dishes were detached with PBS–25 mM EDTA, transferred to Eppendorf tubes, and fixed and stained using antibody 7.16.4 and fluorescein-labeled secondary antibody (Dobashi and Stern, 1991). Incubations were performed at room temperature with rotation. Washes were performed with centrifugation at 2000 r.p.m. for 3 min in a volume of 500 μl. Surface expression was analyzed by Robert D.Edwards using an EPICS Profile machine (Coulter Cytometry). At least 10 000 cells were analyzed on each run.

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