Abstract. The receptor tyrosine kinase p185<sup>c-neu</sup> can be constitutively activated by the transmembrane domain mutation Val<sup>664</sup>→Glu, found in the oncogenic mutant p185<sup>c-neu</sup>. This mutation is predicted to allow intermolecular hydrogen bonding and receptor dimerization. Understanding the activation of p185<sup>c-neu</sup> has assumed greater relevance with the recent observation that achondroplasia, the most common genetic form of human dwarfism, is caused by a similar transmembrane domain mutation that activates fibroblast growth factor receptor (FGFR) 3. We have isolated novel transforming derivatives of p185<sup>c-neu</sup> using a large pool of degenerate oligonucleotides encoding variants of the transmembrane domain. Several of the transforming isolates identified were unusual in that they lacked a Glu at residue 664, and others were unique in that they contained multiple Glu residues within the transmembrane domain. The Glu residues in the transforming isolates often exhibited a spacing of seven residues or occurred in positions likely to represent the helical interface. However, the distinction between the sequences of the transforming clones and the nontransforming clones did not suggest clear rules for predicting which specific sequences would result in receptor activation and transformation. To investigate these requirements further, entirely novel transmembrane sequences were constructed based on tandem repeats of simple heptad sequences. Activation was achieved by transmembrane sequences such as [VVVEVVA]∞ or [VVVEVVV]∞, whereas activation was not achieved by a transmembrane domain consisting only of Val residues. In the context of these transmembrane domains, Glu or Gln were equally activating, while Lys, Ser, and Asp were not. Using transmembrane domains with two Glu residues, the spacing between these was systematically varied from two to eight residues, with only the heptad spacing resulting in receptor activation. These results are discussed in the context of activating mutations in the transmembrane domain of FGFR3 that are responsible for the human developmental syndromes achondroplasia and acanthosis nigricans with Crouzon Syndrome.

The <i>c-neu</i> oncogene was initially isolated from a rat ethylnitrosourea-induced neuro/glioblastoma and encodes a receptor tyrosine kinase belonging to the epidermal growth factor receptor (EGFR)<sup>1</sup> family; the oncogenic mutant, referred to as p185<sup>c-neu</sup>, is closely related to its wild-type cellular homologue, referred to as p185<sup>c-neu</sup> (Shih et al., 1981; Schechter et al., 1984; Bargmann et al., 1986a, b; Dougall et al., 1994). Like other members of the EGFR family, p185<sup>c-neu</sup> consists of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain (Drebin et al., 1984; Ullrich and Schlessinger, 1990). As for other receptor tyrosine kinases, ligand binding to the wild-type p185<sup>c-neu</sup> receptor induces receptor dimerization, leading to tyrosine kinase activation and subsequent downstream signaling events (Peles et al., 1991; Dougall et al., 1994; Stein et al., 1994; for review see Heldin, 1995).

Activation of p185<sup>c-neu</sup> arises from structural changes, such as the Val<sup>664</sup>→Glu mutation in the transmembrane domain (Bargmann et al., 1986b) or deletions in the extracellular juxtamembrane region (Siegel et al., 1994). Activated p185<sup>c-neu</sup> resembles a ligand-stimulated receptor, as evidenced by its increased tyrosine phosphorylation of other proteins, as well as elevated levels of autophosphorylation (Bargmann and Weinberg, 1988a; Segatto et al., 1988; Stern et al., 1988; Weiner et al., 1989a; Peles et al., 1991; Ben-Levy et al., 1992; Cao et al., 1992; Qian et al., 1995). In addition, p185<sup>c-neu</sup> is primarily found in an aggregated form while wild-type p185<sup>c-neu</sup> is monomeric (Weiner et

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1. Abbreviations used in this paper: BPV, bovine papilloma virus; ECL, enhanced chemiluminescence; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; TCRα, T cell receptor α chain.
The membrane domain did not correlate with transformation.

This study also found that the lateral position 0 (P0), corresponding to Ala

The importance of the subdomain surrounding Glu was further examined by the mutations Val→Gly and Gly→Val, which both abolished transforming activity (Cao et al., 1992). This study also found that the lateral position and rotational orientation of Glu in the transmembrane domain did not correlate with transformation.

A subdomain containing a loosely defined sequence motif has been noted in the transmembrane domains of many other receptor tyrosine kinases (Sternberg and Gullick, 1990). This motif consists of a five-residue segment: position 0 (P0), corresponding to Ala in p185 neu, exhibits a small side chain such as Gly, Ala, Ser, or Thr; position P3 displays an aliphatic side chain like Ala, Val, Leu, or Ile, but is occupied by Glu in activated p185 neu, and position P4 exhibits either Gly or Ala. The presence of this motif in many different receptor tyrosine kinases suggests an important role in facilitating ligand-induced receptor dimerization.

For many years, p185 neu represented the sole example of a receptor tyrosine kinase that is activated by mutation within its transmembrane domain. Recently, however, it has become clear that some human developmental abnormalities are due to transmembrane domain mutations in fibroblast growth factor receptor (FGFR) 3. Achenondroplasia, the most common genetic form of human dwarfism, results from a single amino acid change in the transmembrane domain at silent NheI and SacI restriction sites (Webster et al., 1993). Transfection frequencies were determined by cotransfection protocol (Chen and Okayama, 1987), as described previously (Ma et al., 1995) for pools of different mutant pools of transmembrane domain at silent NheI and SacI restriction sites (Webster et al., 1993). Transfection frequencies were determined by cotransfection protocol (Chen and Okayama, 1987)

Materials and Methods

Construction of Degenerate Pools and Additional Mutants

Oligonucleotides were synthesized encoding the p185 neu transmembrane domain with degenerate codons targeted to the presumptive α-helical positions designated “a,” “d,” and “e,” shown in Fig. 1. In Pool 1, codons for Val, Ala, Gly, and Glu were targeted to each of these positions. In Pool 2, the codons for Val, Ala, Gly, and Glu, but not Val, were targeted to these positions. The degenerate oligonucleotides were synthesized on an oligonucleotide synthesizer (Applied Biosystems Inc., Foster City, CA) and then amplified by PCR. The degenerate pools were ligated into a pSV2-derived vector encoding p185 neu (pSV2neuNhel/SacI), replacing the wild-type transmembrane domain at silent NheI and SacI restriction sites (Webster and Donoghue, 1996). The Nhel site corresponds to bases 1973–1978, and the SacI site corresponds to bases 2114–2119, in the published nucleotide sequence encoding p185 neu (Bargmann et al., 1986a).

The synthetic oligonucleotide sequence used to generate the degenerate pools is shown below. Silent restriction sites for NheI (GCTAGC) and SacI (GAGCTC) embedded within the sequence are shown in bold. Extra residues were added at both ends to facilitate restriction digestion of the PCR-amplified double-stranded product. The first and last triplet codons show corresponding to Ala–Glut (GCT of NheI site) and Leu (CTC of SacI site), respectively, in the published amino acid sequence of p185 neu (Bargmann et al., 1986a):

5′-GCCAGAGA-GTGAGGCGG-CTGACA, TTCATCATT-AXA-ATG-G5′

were PCR amplified using standard conditions with the following primers: a sense strand primer, corresponding to the 5′-end of the degenerate oligonucleotide sequence shown above and spanning the Nhel site, 5′-GCCAGAGC-GTCAAGGACGTTGAGCCG-3′; and an antisense strand primer, corresponding to the complement of the 3′-end of the degenerate oligonucleotide sequence shown above and spanning the SacI site, 5′-GGTC-TCAAGCAGCTACAGTTTCC-3′.

Additional mutants described in the text were constructed by synthesizing pairs of single-stranded oligonucleotides to create double-stranded DNA fragments with Nhel and SacI cohesive overhangs, for ligation into pSV2neuNhel/SacI (Webster and Donoghue, 1996).

Focus Assays

NIH3T3 cells were transfected using a modified calcium phosphate transfection protocol (Chen and Okayama, 1987), as described previously (Ma et al., 1995). Transfection frequencies were determined by cotransfect-
ing with 0.1 μg of pSV2neo, and half of the cells were subsequently split into media containing G418 (Maher et al., 1993). For each DNA sample, the number of foci was normalized with respect to the number of Neo-resistant colonies and then expressed as a percentage of the transformation efficiency obtained with pSV2neoNT, encoding p185neo with the mutation Val664→Glu. All mutants were assayed at least three times in independent experiments for transformation efficiency.

Isolation of Transmembrane Domains from Degenerate Pools

At 9 d after transfection of degenerate Pool 1 into NIH3T3 cells, individual foci of transformed cells were expanded and RNA was isolated. cDNA was prepared using the following primer: 5′-ATACGCTTCA-TCTAGAATTCTTGG3′, complementary to bases 2323–2347 in the published nucleotide sequence encoding p185neo (Bargmann et al., 1986a). This cDNA was used for PCR with primers described above, and the products digested with NheI and SacI. For the mutants designated DEG.1 through DEG.5, each recovered transmembrane domain was ligated into pSV2NeoNheI/SacI and sequenced, and its ability to activate p185neo to a transforming phenotype was reconfirmed by transfection into NIH3T3 cells. This protocol ensured that transformation was due to a unique sequence, containing a single transmembrane domain, rather than a mixture of sequences. The nontransforming isolates, DEG.6 through DEG.10, were recovered in the same way but were negative for NIH3T3 transformation when assayed as individual clones.

Indirect Immunofluorescence

Transiently transfected Cos-1 cells (Chen and Okayama, 1987) were fixed in 3% paraformaldehyde/PBS for 10 min and then permeabilized in 0.5% Triton X-100/PBS for 5 min (Lee and Donoghue, 1992). Cells were incubated with mouse monoclonal antibody 7.16.4 to an extracellular epitope of p185neo (c-neu [AB-4] clone 7.16.4 from Oncogene Science Inc., Manhasset, NY) followed by FITC-conjugated goat anti-mouse antiserum (Boehringer Mannheim Corp., Indianapolis, IN). To detect cell surface expression of p185neo and derivatives, cells were fixed with paraformaldehyde and incubated without permeabilization, as described previously (Hannink and Donoghue, 1986; Lee and Donoghue, 1992).

For double-label experiments to detect both intracellular and cell surface expression, cells were fixed as described above and then treated with mouse mAb 7.16.4 (1:50 dilution) against an extracellular epitope of p185neo, followed by FITC-conjugated goat anti-mouse antiserum (Boehringer Mannheim Corp.) at 1:1,500 dilution. The same cells were then permeabilized with 1% Triton X-100/PBS and treated with a rabbit polyclonal p185neo C-18 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:3,000 dilution, which was detected with rhodamine-conjugated goat anti-rabbit antiserum (Boehringer Mannheim Corp.) at 1:4,000 dilution.

Immunoprecipitation, In Vitro Kinase, and Dimerization Assays

Cos-1 cells were split at a density of ~2 × 10^5 cells per 60-mm plate and transfected with 10 μg of DNA the next day (Chen and Okayama, 1987). After 2 d, the transfected cells were labeled for ~7 h with 100 μCi of [3H]Ser and [35S]Met each per ml in DME lacking Cys and Met. The cells were rinsed with TS buffer and lysed in 0.5 ml NP-40 lysis buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 5 mM EDTA, 10 mg/ml aprotinin, 10% glycerol), and clarified lysates were prepared (Maher et al., 1993). Immunoprecipitation was carried out using mAb c-neu, reduced samples prepared as above were run on a 4–12% gradient gel, transferred to nitrocellulose, blotted with mouse antiphosphotyrosine mAb 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY) and detected by ECL. The membrane was then reprobed with polyclonal p185neo C-18 antiserum to examine receptor expression, which was again detected by ECL.

Results

Pools of Mutants with Degenerate Transmembrane Domains

The model proposed by Sternberg and Gullick (1989) postulated the transmembrane domain of p185neo as an α-helical sequence in which Glu664 promotes activation by creating a hydrogen bond between two receptor molecules. To further characterize these interactions, we constructed pools of degenerate oligonucleotides that permitted variability in the transmembrane domain at positions P0, P3, and P4 in the Sternberg and Gullick notation, which are predicted to be of greatest structural importance based on a comparison of transmembrane domains from many growth factor receptors (Sternberg and Gullick, 1990). The repeating heptad motif present in interacting α-helical domains can also be designated (abcdefg)_n, in which the “a” and “d” positions are occupied by the residues at the interface where the two α-helical domains interact.

In degenerate Pool 1 (Fig. 1B), we randomly targeted codons for Val, Ala, Gly, or Glu to heptad positions “a,” “d,” and “e.” This choice allowed us to examine the potential effects of multiple Glu residues targeted to a variety of positions. The pool of degenerate oligonucleotides was amplified by PCR and then ligated into a pSV2-derived vector encoding p185neo (pSV2NeoNheISacI), replacing the wild-type transmembrane domain. The DNA was transfected into NIH3T3 cells, and the resulting foci were expanded. Subsequently, to recover individual transmembrane domains from foci of transformed cells, RNA was prepared from expanded cells and subjected to reverse transcriptase–PCR as described in Materials and Methods, and the resulting sequences were then subcloned as unique plasmids. Individual transmembrane domains recovered in this fashion were next ligated back into pSV2NeoNheISacI and reassayed for transformation of NIH3T3 cells. This ensured that transformation would be due to a unique sequence, rather than a mixture of transmembrane sequences.

The sequences of the transforming mutants isolated from this pool, designated DEG.1 through DEG.5 (Fig. 2C), were completely distinct in comparison with other mutants previously described. Interestingly, none of the activated mutants except DEG.1 exhibited a Glu at residue 664. Moreover, all of these mutants, except for DEG.2, exhibited multiple Glu residues spaced at intervals of seven residues, or multiples of seven residues. These results were intriguing considering that Glu resi-
were synthesized, amplified, and cloned into pSV2
Oligonucleotides coding for degenerate transmembrane domains (Sternberg and Gullick, 1990), referred to as P0-P4, corresponds found in many receptor tyrosine kinase transmembrane domains due of p185neu is boldfaced. The five-residue sequence motif borders of the transmembrane domain. The activating Glu resi-

The transmembrane domains of the transforming mutants DEG.1–DEG.5 suggested that there are many allowed sequences and those of the nontransforming mutants DEG.6–DEG.10 did not suggest clear rules for predicting which specific sequences will result in receptor activation and transformation. We therefore designed simple consensus repeats, containing a centrally located Glu residue, with most of the other residues substituted by Val, such as [VVVEVGL]n, [VVVEVAG]n, or [AVV-EGVL]n, (designated CONS.A, CONS.B, CONS.C, and CONS.D, respectively). Mutants were then constructed with the transmembrane domain composed entirely of these consensus sequences, repeated over the entire transmembrane domain of ~25 residues. Surprisingly, these constructs were transforming, with the CONS.A and CONS.D constructs exhibiting the greatest activity (Fig. 3 B). These results suggest that these simple repeating sequences contain all the information necessary for receptor activation. Evidently, there is little sequence specificity
Figure 2. Immunofluorescence of nontransforming isolates DEG.6–DEG.10. Double-label indirect immunofluorescence was used to detect either cell surface expression of p185c-neu-related proteins (right) or, after permeabilization of cells, intracellular expression (left). Conditions for the double-label immunofluorescence are described in Materials and Methods. (A) (A and B) mutant DEG.6; (C and D) mutant DEG.7; (E and F) mutant DEG.8; (G and H) mutant DEG.9; (I and J) mutant DEG.10. (B) (A and B) Mock transfected cells; (C and D) p185c-neu.
necessary for constitutive activation within the p185<sup>neu</sup> transmembrane domain itself.

It was also important to demonstrate that transformation was due to specific interactions involving Glu residues. Towards this end, we constructed a derivative of the CONS.C sequence in which the central Glu of each repeat was changed to Val. This mutant transmembrane domain, consisting entirely of Val residues, was designated CONS.C<sup>V</sup>, and was completely devoid of transforming activity (Fig. 3 B).

**Design of Derivative Transmembrane Domains Based on Consensus Heptad Repeats**

We also tested whether other residues could substitute for Glu in the context of the CONS.A heptad repeat (Fig. 3 C). We replaced Glu with Lys, Gln, Ser, and Asp, creating the mutants CONS.A<sup>E</sup><sup>ε</sup><sup>K</sup>, CONS.A<sup>E</sup><sup>ε</sup><sup>Q</sup>, CONS.A<sup>E</sup><sup>ε</sup><sup>S</sup>, and CONS.A<sup>E</sup><sup>ε</sup><sup>D</sup>. These mutants were designed to test whether other hydrogen-bonding residues could substitute in place of Glu. Only substitution with Gln allowed significant transforming activity, whereas Lys, Ser, and Asp were unable to activate the receptor. The observation that Gln can substitute for Glu in the mutant CONS.A<sup>E</sup><sup>ε</sup><sup>Q</sup> is consistent with the original characterization of p185<sup>neu</sup> showing that Val<sup>664</sup> in p185<sup>neu</sup> was equally effective at activation (Bargmann and Weinberg, 1988b).

As shown in Fig. 4, A–L, indirect immunofluorescence demonstrated that most of the mutants exhibited cell surface expression. The ability of these altered receptors to reach the surface indicates that the mutant transmembrane domains did not interfere with the proper localization of the protein. The only exception was CONS.A<sup>E</sup><sup>ε</sup><sup>D</sup>, which exhibited little or no cell surface staining, even though the protein is clearly being translated, as shown by the intracellular staining of permeabilized cells (Fig. 4, O). However, its reduced surface expression may be due to aggregation or interaction with another protein that is retained in the ER and/or Golgi, as discussed previously for some of the nontransforming mutants, DEG.7, DEG.9, and DEG.10.

**Spacing and Number of Glu Residues**

The design of the degenerate pools of transmembrane domains, as well as the design of the subsequent consensus repeat clones described above, assumed without any real evidence that there would exist a fundamental heptad structural motif resulting from the α-helical transmembrane domain. To directly examine this premise, we constructed a series of clones in which the Glu residues were substituted by other residues capable of hydrogen bonding, such as Lys, Gln, Ser, or Asp. For example, CONS.A<sup>E</sup><sup>ε</sup><sup>S</sup> is identical to CONS.A except that the Glu residues are substituted by Gln. Transformation by each isolate was quantitated as a percentage of p185<sup>neu</sup>. Results represent the average values from three independent experiments, normalized by cotransfection with pSV2neo, and presented as −, +, or ++ as described in Fig. 1. Surface expression was determined by indirect immunofluorescence, as described in text.

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**Table 1.**

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*Figure 3.* Consensus sequences and derivatives. (A) The p185<sup>c-neu</sup> and p185<sup>neu</sup> sequences are shown. The locations of the presumptive heptad repeats, along with the letters indicating the heptad positions, are specified. Vertical lines designate the probable borders of the transmembrane domain. Glu<sup>664</sup> is in boldface. (B) Consensus sequence mutants were constructed as described in the text. The placement of the Glu residues in each mutant is identical. Based on the variations found in the transforming isolates from degenerate Pool 1, the heptad position g in CONS.B and CONS.C was mutated from Ala in CONS.A to Gly and Val, respectively. The repeating heptad in CONS.D is heptad 2 from p185<sup>neu</sup> with Thr<sup>662</sup> changed to Val. The mutant CONS.C<sup>E</sup><sup>ε</sup><sup>V</sup> has a transmembrane domain composed entirely of Val residues and was constructed as a negative control. Glu residues are boldfaced. (C) Derivatives of CONS.A mutant. Derivatives were designed in which the Glu residues were substituted by other residues capable of hydrogen bonding, such as Lys, Gln, Ser, or Asp. For example, CONS.A<sup>E</sup><sup>ε</sup><sup>S</sup> is identical to CONS.A except that the Glu residues are substituted by Gln. Transformation by each isolate was quantitated as a percentage of p185<sup>neu</sup>. Results represent the average values from three independent experiments, normalized by cotransfection with pSV2neo, and presented as −, +, or ++ as described in Fig. 1. Surface expression was determined by indirect immunofluorescence, as described in text.
Figure 4. Indirect immunofluorescence of consensus mutants and derivatives. Indirect immunofluorescence using the monoclonal antibody 7.16.4 directed against the extracellular region of p185 neu and a fluorescein-conjugated goat anti-mouse secondary antibody revealed both cell surface and intracellular protein expression. A–L show nonpermeabilized cells to examine cell surface expression. (A) p185 neu; (B) p185 neu; (C) CONS.A; (D) CONS.B; (E) CONS.C; (F) CONS.D; (G) CONS.A E2; (H) CONS.A E3; (I) CONS.A E4; (J) CONS.A E5; (K) CONS. A E6; (L) CONS.A E7. M–O show cells after permeabilization to examine intracellular expression. (M) p185 neu; (N) p185 neu; (O) CONS.A E8.
Most of the transforming DEG mutants described earlier contained multiple Glu residues (Fig. 1 C), as did the transforming mutants CONS.A–CONS.D, which all contained three Glu residues (Fig. 3 B). The occurrence of multiple Glu residues is in contrast to p185 neu, which contains only a single Glu residue in the transmembrane domain at position 664. We therefore examined whether multiple Glu residues are in fact required for activity.

This issue was addressed through two different sets of mutants, one constructed in the background of CONS.A, [VVVEVVA], and another set in the background of CONS.C, [VVVEVVV]. These two sets of mutants yielded somewhat different results. As shown in Fig. 6 A, “single-Glu” derivatives of the CONS.A sequences were designed, each possessing only a single Glu residue located at different positions, and designated CONS.AE2, CONS.AE3, and CONS.AE4. None of these mutants was transforming, indicating that within the context of the CONS.A heptad repeat, a single-Glu residue is not sufficient to provide activation. This result is in contrast to the single Glu664 residue that leads to activation of p185 neu, suggesting that there may be sequence information within the transmembrane domain, in addition to the Val664 → Glu mutation, that stabilizes dimerization (Cao et al., 1992). We considered the possibility that these mutant proteins might fail to reach the cell surface and that this defect might explain their inactivity in transformation assays. However, all of the single-Glu proteins were expressed at the cell surface, as shown by indirect immunofluorescence in Fig. 4, G and H, for CONS.AE2 and CONS.AE4.

Several mutants were also designed in the CONS.C background that had either one, two, three, or four Glu residues, designated CONS.C1xE–CONS.C4xE. The clone containing three Glu residues, CONS.C3xE, is the original CONS.C clone. As shown in Fig. 6 B, the mutants with either two or four Glu residues, designated CONS.C2xE and CONS.C4xE, exhibited the greatest activity, almost twice as much as the mutant with three Glu residues. However, in this series of mutants, even the presence of a single Glu residue, as in CONS.C1xE, led to significant transformation above background.

It is curious that a single Glu residue is activating in the mutant CONS.C1xE (Fig. 6 B), but not in the very similar

![Figure 5](https://jcb.rupress.org/figure/5)

**Figure 5.** Importance of the spacing of Glu residues. (A) A series of clones, based on the mutant CONS.C3xE, were constructed varying the spacing between two Glu residues, from a minimum spacing of two residues (diad mutant) to a maximum spacing of eight residues (octad mutant). (B) Transformation by each isolate shown in (A) was quantitated as a percentage of p185neo. Numerical results of transformation assays are presented graphically and represent the average values from two independent experiments, normalized by cotransfection with pSV2neo.

![Figure 6](https://jcb.rupress.org/figure/6)

**Figure 6.** Importance of the number of Glu residues. (A) Derivatives of CONS.A. Three derivatives were constructed, each having a single Glu residue. Depending upon the position of the Glu residue, these mutants were designated CONS.AE2, CONS.AE3, and CONS.AE4. (B) Derivatives of CONS.C. Derivatives were constructed in the background of CONS.C having from one to four Glu residues. These clones are designated CONS.C1xE, CONS.C2xE, CONS.C3xE, and CONS.C4xE. Note that CONS.C3xE is the parental CONS.C mutant presented in Fig. 3. Transformation by each isolate was quantitated as a percentage of p185neo. Results represent the average values from three independent experiments, normalized by cotransfection with pSV2neo, and presented as −, +, or ++ as described in Fig. 1. For transformation results shown in B, numerical values are also shown in parentheses as the percentage of p185neo.
The active mutant has a background CONS.C motif of [VVVEVVV], whereas the inactive mutant has a background CONS.A motif of [VVVEVVA]; these sequences differ only by the Val→Ala substitution in the last position of the repeat motif. This suggests that the ability of a single Glu residue to activate a particular transmembrane domain, as occurs in the case of p185<sup>neo</sup>, may be critically dependent upon other specific details of the transmembrane domain that are not immediately obvious. These results may help to explain the negative results of earlier studies in which repositioning of the Glu<sup>664</sup> residue to other locations in the transmembrane domain did not result in transformation (Cao et al., 1992).

**Figure 7.** Immunoprecipitation/kinase assay of consensus mutants. Lysates from transfected cells, labeled metabolically with [35S]Cys and [35S]Met, were subjected to immunoprecipitation using monoclonal antibody 7.16.4, as described in Materials and Methods. (A) Kinase assay. Immunoprecipitated lysates were subjected to in vitro kinase reactions using γ-[32P]ATP. 32P-labeled proteins were detected by SDS-PAGE and autoradiography. Exposure time was 21 h. (B) Expression. To demonstrate equivalent levels of protein expression for different mutants, identical aliquots of immunoprecipitated lysates as used in A were analyzed by SDS-PAGE followed by fluorography to detect 35S-labeled proteins. Exposure time was 3 d. The samples shown in lanes 2, 4, and 6 represent nontransforming clones, while the samples shown in lanes 3, 5, and 7 represent transforming derivatives. Lane 1, mock; lane 2, p185<sup>c-neu</sup>; lane 3, p185<sup>neo</sup>; lane 4, CONS.CE<sup>→V</sup>; lane 5, CONS.C; lane 6, CONS.AE<sup>→S</sup>; lane 7, CONS.AE<sup>→Q</sup>.

**Figure 8.** Detection of phosphotyrosine in immunoprecipitated receptors. Lysates were prepared from transfected cells and were subjected to immunoprecipitation using monoclonal antibody 7.16.4, as described in Materials and Methods. Immunoprecipitated lysates were analyzed on a 4–12% gradient gel under reducing conditions, transferred to nitrocellulose, probed with monoclonal phosphotyrosine antiserum 4G10 (A) or with polyclonal p185<sup>c-neu</sup> C-18 antiserum (B), and specific proteins were detected by ECL. The samples shown in lanes 2, 4, and 6 represent nontransforming clones, while the samples shown in lanes 3, 5, and 7 represent transforming derivatives. A shows detection of phosphotyrosine, and B shows detection of receptor expression. Samples are: lane 1, mock; lane 2, p185<sup>c-neu</sup>; lane 3, p185<sup>neo</sup>; lane 4, CONS.CE<sup>→V</sup>; lane 5, CONS.C; lane 6, CONS.AE<sup>→S</sup>; lane 7, CONS.AE<sup>→Q</sup>.

**Kinase Activity of Mutants**

Transformation by the neu oncogene is dependent upon p185<sup>neo</sup> having a functional kinase domain, and p185<sup>neo</sup> exhibits increased turnover, autophosphorylation, tyrosine phosphorylation of intracellular substrates, and activation of PLC-γ (Bargmann and Weinberg, 1988a; Stern et al., 1988; Weiner et al., 1989a,b; Peles et al., 1991; Cao et al., 1992; Brown et al., 1994). Moreover, transformation and SH2-dependent signaling by p185<sup>neo</sup> requires intermolecular receptor association that is mediated by the transmembrane domain, as demonstrated by functional complementation between truncated kinase-active p185<sup>c-neu</sup> and full-length kinase-inactive p185<sup>neo</sup> (Qian et al., 1994, 1995). Another indication of receptor dimerization and aggregation is provided by measurement of ligand-binding using chimeras consisting of the ligand-binding domain of EGFR substituted into either p185<sup>c-neu</sup> or p185<sup>neo</sup>; the Val→Glu mutation in the transmembrane domain of such EGFR/Neu chimeras results in the conversion of low-affinity ligand-binding sites into high-affinity binding sites, consistent with an oligomerized state of the oncogenic receptor (Ben-Levy et al., 1992).

To confirm kinase activation of the mutants described here, selected mutants were examined for receptor activation using an immunoprecipitation/kinase assay of transfected Cos-1 cells. As shown in Fig. 7A, p185<sup>neo</sup> exhibited approximately threefold greater autophosphorylation than
p185c-neu (lanes 3 and 2, respectively). The mutants CONS.CE→V and CONS.C were examined, as they represent an interesting pair of closely related mutants. The first of these is inactive in transformation assays, whereas the latter mutant is active. In the immunoprecipitation/kinase assay, the biologically active mutant CONS.C exhibited a similar increase in autophosphorylation compared with the inactive receptor CONS.CE→V (lanes 5 and 4, respectively). We also examined another pair of closely related mutants, CONS.AE→S and CONS.AE→Q, where once again the first mutant is inactive in transformation assays, but the latter mutant is active. Once again, in the immunoprecipitation/kinase assay, the biologically active mutant CONS.AE→Q exhibited an increase in autophosphorylation compared with the inactive mutant CONS.AE→S (lanes 7 and 6, respectively). In this experiment, similar levels of protein expression were achieved for p185c-neu, p185neu, and the various mutants examined, as demonstrated by 35S-metabolically labeled/immunoprecipitated proteins from the same lysates (Fig. 7B). Thus, consistent with prior experimental results from other laboratories (Bargmann and Weinberg, 1988a; Stern et al., 1988; Weiner et al., 1989a,b; Cao et al., 1992), biologically active derivatives constructed in this work exhibited increased levels of kinase activity, as determined by receptor autophosphorylation in immunoprecipitation/kinase assays.

Immunoprecipitated samples were also examined for the presence of phosphotyrosine by immunoblotting. Fig. 8A demonstrates that two transforming mutants, CONS.C (lane 5) and CONS.AE→Q (lane 7), exhibit significant incorporation of phosphotyrosine into immunoprecipitated receptors, as did the positive control, p185neu (lane 3). Little or no phosphotyrosine was associated with the nontransforming mutants, CONS.CE→V (lane 4) and CONS.AE→S (lane 6), or with the nontransforming control, p185c-neu (lane 2). As a control for receptor expression levels, B demonstrates approximately equivalent levels of receptor expression, determined by immunoblotting using polyclonal p185c-neu C-18 antiserum.

These same mutants were also examined for receptor dimerization using gradient gel electrophoresis under nonreducing conditions (Weiner et al., 1989b; Burke et al., 1997). Fig. 9B presents these results, while the same lysates were also analyzed under reducing conditions as a control, shown in A. Two transforming mutants, CONS.C (lane 5) and CONS.AE→Q (lane 7), exhibited significant bands of dimeric receptors, as did the positive control, p185neu (lane 3). Importantly, no dimerization was observed for the nontransforming mutants analyzed here, CONS.CE→V (lane 4) and CONS.AE→S (lane 6), or for the nontransforming control, p185c-neu (lane 2). The ability to detect receptor dimerization for the transforming mutants, but not for the biologically inactive mutants, indicates that the consensus sequences described here promote receptor dimerization, and that in this respect they are similar to the parental transforming clone, p185neu.

Discussion

Function of the Transmembrane Domain

The results presented here demonstrate that activation of p185c-neu can be achieved by a transmembrane domain with very little sequence specificity. Glu residues in the appropriate positions of a simplified transmembrane domain, such as [VVVEVVV]n, lead to receptor activation as demonstrated by biological transformation (Fig. 3), increased kinase activity (Fig. 7), increased incorporation of phosphotyrosine (Fig. 8), and receptor dimerization (Fig. 9). Receptor activation is conferred by as few as one or as many as four Glu residues within some of these repeats. Moreover, a periodic heptad spacing seems to be critical
for activation; this was demonstrated by systematically varying the spacing between two Glu residues from two to eight, with only the heptad spacing resulting in activation (Fig. 5 B). Thus, an apparently complex system can be reduced to a repeated sequence motif. Furthermore, in these simplified transmembrane domains, Gln is able to substitute efficiently for Glu, as shown by the mutant CONS.AE→Q. This is consistent with previous observations (Bargmann and Weinberg, 1988b) that Gln, but not Ser, Lys, or Asp, was able to substitute for Glu at position 664 in receptor activation (Fig. 3).

From the transforming isolates of degenerate Pool 1, it is clear that the location of Glu at position 664 is not critical for activation (Fig. 1), in contrast to prior results suggesting that the primary structure around Glu664 plays an important role. For instance, mutants with Glu663 or Glu665 were inactive (Bargmann and Weinberg, 1988b). More recently, Cao et al. (1992) analyzed the requirements for activation of the p185neu transmembrane domain; all of their transforming mutants retained Glu at position 664, and a mutant (designated lil670VEG) with the motif VEG seven residues COOH-terminal, was nontransforming.

Our isolation of the transforming mutants DEG.2, DEG.3, DEG.4, and DEG.5, all of which lack a Glu at position 664, would suggest that there exists considerable flexibility with regard to the allowed placement of the Glu residue(s) that may mediate activation. The isolation of these transforming mutants also demonstrates that the construction of a degenerate pool of oligonucleotides encoding transmembrane domains provides a powerful approach to identifying and isolating those rare sequences that may allow receptor activation.

Our data show that activation of p185c-neu can be mediated by Glu residues spaced seven amino acids apart in a repeating heptad. Analysis of the sequences of the isolates from Pool 1 (Fig. 1) is consistent with this proposal. Most of the transforming isolates contain Glu residues separated by multiples of seven amino acids. In the nontransforming isolate, DEG.6, which was expressed at the cell surface (in contrast to DEG.7 and DEG.10), there are Glu residues spaced seven amino acids apart, but there is also a Glu that does not follow that pattern. This may suggest that the presence of a Glu in a nonheptad motif may cause a disruption in the interaction between receptor molecules that prevents transforming ability, although it is difficult to infer absolute rules because of the other sequence variations exhibited by the DEG mutants.

The Val→Glu mutation that activates p185c-neu is also activating when introduced into the corresponding position of the Drosophila EGFR homologue (DER), resulting in increased kinase activity (Wides et al., 1990). Although it was initially reported that the corresponding Val627→Glu mutation does not activate the human EGFR transmembrane domain (Kashles et al., 1988), a more recent study suggests that this mutation is activating (Beguinot et al., 1995). Additionally, a Val→Glu mutation at position 659 in c-erbB-2, the human Neu homologue that is overexpressed in many breast cancers, results in elevated tyrosine kinase activity (Segatto et al., 1988). These examples provide further evidence for activation of EGFR family members by appropriate introduction of a strongly polar residue in their transmembrane domains.

The hydrophobic environment of the membrane creates an energetic need to shield polar side chains such as that of Glu (even if uncharged), which can be accomplished by appropriate hydrogen bonding. Previously, it was proposed that there may be a requirement for Ala661 in p185neu to allow hydrogen bonding between the Glu664 side chain of one receptor with the carbonyl oxygen of Ala661 in the other. There is clearly no strict requirement for Ala at position 661, however, as shown by the fact that Val is tolerated at position 661 in many of the biologically active mutants described here. The presence of the branched side chain at Val661, which might interfere with side chain–backbone hydrogen bonding, argues against this model but does not exclude it. More likely, however, would be a model of side chain–side chain hydrogen bonding between the Glu of one subunit with the corresponding Glu of the other subunit. The choice between these two models will require further biophysical characterization. While the results presented in this work are certainly consistent with a coiled-coil arrangement for the interacting transmembrane domains of p185neu, other structural models cannot be excluded at the present time, such as one proposed for the homodimerizing transmembrane domain of glycoporphin A (Lemmon et al., 1992; Treutlein et al., 1992).

Relevance of p185neu Activation to Other Systems

As summarized in Fig. 10, prior studies have demonstrated that substitutions at position 664 of p185neu exhibit the following pattern of biological activity: Glu, Gln > Asp, Tyr ≥ Val, Lys, Gly, His (Bargmann and Weinberg, 1988b). These results are generally consistent with a role of polar or hydrophilic residues in promoting activation, although the failure of Lys and His to allow for activation remains
Transmembrane Domain Mutations in Human Developmental Syndromes

Transmembrane-mediated receptor activation clearly plays a fundamental role in several human developmental syndromes. Acrodysostosis, the most common genetic form of human dwarfism, results from a single amino acid change in the transmembrane domain of FGFR3, Gly380→Arg (Rousseau et al., 1994; Shiang et al., 1994). This mutation results in constitutive activation of FGFR3, leading to abnormal development at the growth plate of long bones (Webster and Donoghue, 1996). As depicted in Fig. 10, we have previously demonstrated that mutations at residue 380 of FGFR3 exhibit the following pattern of activation: Arg, Glu, Asp > Gln, His > Lys (Webster and Donoghue, 1996). Recently, Meyers et al. (1995) characterized an autosomal dominant mutation in FGFR3 that is manifested as two distinct developmental syndromes presented together: first, acanthosis nigricans, a hyperplastic epithelial proliferation syndrome resulting in thickened hyperpigmented skin; and second, Crouzon Syndrome, characterized by craniosynostosis or premature fusion of the cranial sutures, resulting in cranial malformation and ocular proptosis. The mutation responsible for these developmental anomalies maps to the transmembrane domain of FGFR3 and, surprisingly, involves a single substitution mutation creating a Glu residue (Ala91→Glu). Using FGFR3/Neu chimeras, we have recently demonstrated that the Ala91→Glu mutation also leads to constitutive receptor activation (Webster, M.K., and D.J. Donoghue, unpublished data).

It is clear that transmembrane domains play a more substantial role than serving as structural elements for membrane anchoring. The examples discussed above underscore the importance of understanding the molecular details of activating mutations in the transmembrane domains of receptors. The principles elucidated here are likely to be generally relevant to understanding activation and signal transduction by other receptor tyrosine kinases in addition to p185 neu.

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