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Correlation of the structure of the transmembrane domain of the *neu* oncogene-encoded p185 protein with its function

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ABSTRACT **The human homologue of the *neu* oncogene is frequently found in human tumors. Certain amino acid substitutions at position 664 in the transmembrane domain of the *neu* oncogene-encoded p185 protein product are known to cause malignant transformation of cells. Using conformational energy analysis based on ECEPP (empirical conformational energies for polypeptides program), we have previously determined the preferred three-dimensional structures for the transmembrane domain of the p185 protein with a transforming (glutamic acid) and a nontransforming (valine) substitution at the critical position 664 and found that the global minimum-energy conformation of this region in the nontransforming protein contains a sharp bend, whereas the global minimum-energy conformation for this region from the transforming protein is entirely α-helical. We now demonstrate that this result holds for other known nontransforming (glycine, histidine, tyrosine, and lysine) and transforming (glutamine) substitutions at position 664. Furthermore, a simple statistical thermodynamic analysis of the results indicates that ≈85% of each of the nontransforming sequences exist with the bend at positions 664 and 665, while ≈90% of each of the transforming sequences exist as an α-helix. About 9% of the nontransforming sequences exist as the α-helix. These results suggest that if the intracellular concentration of the normal protein is increased at least 10-fold, thereby increasing the α-helical form by this factor, cell transformation should result. This conclusion is directly supported by genetic experiments in which this level of overexpression of the normal protein was achieved with attendant cell transformation.**

The *neu* oncogene was originally identified in nitrosourea-induced neuroblastomas of the rat (1, 2). The human homologue of the *neu* gene, which is also termed c-*erbB2* or *HER2*, has since been identified in advanced human mammary carcinomas (3, 4) and in other human cancers of glandular origin (5–7). The *neu* gene encodes a protein of 185 kDa (p185), which shares extensive homology with the epidermal growth factor receptor (8) and similarly has three principal domains: an extracellular receptor domain, a single transmembrane domain, and an intracellular domain with tyrosine kinase activity (9). It has been hypothesized that activation of p185 results in the stimulation of cytoplasmic tyrosine kinase activity causing signal transduction in the cell (8, 10).

Transforming *neu* genes differ from their nontransforming counterparts by selective amino acid substitutions at position 664 in the transmembrane domain of the p185 protein (10). The *neu* genes encoding glutamine or glutamic acid at position 664 are found to produce cell transformation *in vitro* with a high degree of efficiency, whereas *neu* genes encoding valine, histidine, tyrosine, lysine, or glycine at position 664 do not produce cell transformation except at elevated levels of expression (10). Thus, the region around position 664 is important in regulating the activity of the transforming *neu* oncogene protein products and their nontransforming counterparts. For example, it is possible that activating mutations at position 664 lead to signal transduction into the cell in the absence of a growth factor–receptor interaction, thus accounting for their transforming effect. We have previously proposed that this effect may be due to discrete conformational changes in the transmembrane domain of p185 induced by the activating amino acid substitutions at position 664 (11).

The amino acid sequence of the mostly hydrophobic transmembrane domain of p185 (amino acid residues 650–683) (8, 12) is Glu-Gln-Arg-Ala-Ser-Pro-Val-Thr-Phe-Ile-Ile-Ala-Thr-Val-Xaa-Gly-Val-Leu-Leu-Phe-Leu-Ile-Leu-Val-Val-Val-Val-Gly-Ile-Leu-Ile-Lys-Arg-Arg, where the critical residue 664 is indicated by Xaa. Using conformational energy analysis, we have previously determined the preferred three-dimensional structures of the region of p185 around position 664 with valine (nontransforming) and glutamic acid (transforming) at this position (11). The results indicated that the global minimum energy conformation for this region of the nontransforming protein with valine at position 664 contained a sharp bend (CD* conformation at residues 664 and 665), which was distinctly different from the global minimum-energy conformation for this region of the transforming protein with glutamic acid at position 664, which was entirely α-helical (11). In this paper, we demonstrate, using a statistical thermodynamic analysis of all of the minima obtained for the transmembrane domain from Phe-658 to Phe-669 with seven different substitutions at position 664, that this initial observed trend holds rigorously and that overexpression of the normal proteins should result in cell transformation, in agreement with the results of recent experiments suggesting a close correlation between the structure and the function of this protein.

METHODS

Conformational analysis was performed on the hydrophobic dodecapeptides, Phe-Ile-Ile-Ala-Thr-Val-Gln-Gly-Val-Leu-Leu-Phe and Phe-Ile-Ile-Ala-Thr-Val-Xaa-Gly-Val-Leu-Leu-Phe where Xaa = His, Tyr, Lys, or Gly, for the nontransforming and Gln for the transforming *neu* gene-encoded proteins, respectively. The general methods used are based on the program ECEPP (empirical conformational energy for polypeptides program) (13). This approach has been successfully used to compute the allowed conformations of the naturally occurring amino acids (14), short oligopeptides (15), long constrained oligopeptides (16, 17), and long unconstrained polypeptides (18, 19), including other oncogene-encoded protein sequences such as from *ras* (20–24) and *myc*, *fos*, and *jun* (25). In the current studies, hydration effects were not taken into account since it was felt that they would

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not influence the conformation of this hydrophobic dodecapeptide, which is presumably shielded from solvent in the hydrophobic core of the lipid bilayer of the cell membrane. These techniques, when applied to hydrophobic peptides up to 26 amino acids, yield structures that agree well with available experimental data (18, 19, 26, 27).

In this study, the chain build-up procedure was used, in which each peptide was built up by the successive addition of single amino acid residues from the amino to the carboxyl terminus. Thus, all the single residue minima for the first two amino acids (phenylalanine and isoleucine) were combined and subjected to energy minimization. The conformers, whose energies lay within a cutoff energy (5.0 kcal/mol; 1 cal = 4.184 J) of that of the global minimum, were retained and then combined with all of the single residue minima for the next residue (isoleucine) in the chain. This process was repeated iteratively until all residues for the initial hexapeptide sequence (from Phe-658 to Val-663) were added. The initial hexapeptide, with several consecutive hydrophobic residues, had been previously identified (11) as a likely nucleation sequence with few allowed low-energy conformations. At this point, the five different heptapeptides were generated by adding glutamine, histidine, tyrosine, lysine, or glycine in all their single residue minima to the low-energy conformers of the initial hexapeptide. The heptapeptides were then subjected to energy minimization and the low-energy conformers (within 5 kcal/mol of the global minimum) were retained. In each case, the procedure was then repeated sequentially for the remaining five amino acid residues (glycine, valine, leucine, leucine, and phenylalanine) to complete the respective dodecapeptides. After the addition of these five residues beyond the critical residue 664, the process was terminated because the addition of further residues did not influence the distribution of low-energy conformations as expected from the results of previous analyses (28).

At all stages of the chain build-up procedure, all of the dihedral angles of the chain were allowed to vary in the energy minimization protocol and the amino and carboxyl termini of each peptide contained *N*-acetyl and $NHCH_3$ groups, respectively, to include any effects of neighboring residues not included in a particular peptide.

For each peptide, from the energy distribution of minima, it was possible to compute residue probabilities for individual conformational states or combinations of conformational states (where conformational states are described by backbone dihedral angles as defined in ref. 14) from the partition function for the minima for each sequence as described in detail (29).

RESULTS

In the generation of the allowed conformations for the p185 dodecapeptides, the distribution of low-energy conformations for the peptide from the transforming (Gln-664) and nontransforming (His-664, Tyr-664, Lys-664, and Gly-664) proteins followed two distinct patterns. In the case of the Gln-664 peptide, the global minimum-energy conformation for the heptapeptide was entirely α-helical. With the addition of subsequent amino acid residues, other low-energy conformations were seen, but the α-helical conformation around Gln-664 was always the predominant form, and, for the final dodecapeptide, the completely α-helical conformation was the global minimum energy structure as found previously for the Glu-664 peptide. This was not the pattern found for the other amino acid substitutions at position 664. For example, in the case of the His-664 and Tyr-664 peptides, the global minimum-energy conformation for the heptapeptides had the structure AAAAAAC (see ref. 14 for a definition of the single-letter conformation code for amino acid residues). In both cases, with the addition of Gly-665, the global minimum energy conformation was AAAAAACD*, with a hairpin turn structure (CD*) at positions 664 and 665. With the addition of subsequent amino acid residues up through the final dodecapeptide stage, the conformation with the bend at positions 664 and 665 (CD* conformation or the closely related CG conformation) remained the predominant form and the global minimum-energy conformation. In the case of the Lys-664 and Gly-664 peptides, the global minimum-energy conformation at the heptapeptide stage was entirely α-helical. However, with the addition of the subsequent amino acid residues, the conformation with the bend at positions 664 and 665 became the predominant form, and, in both cases, the global minimum-energy conformation for the dodecapeptide contained the CD* bend at these positions. This was the same pattern as found previously for the nontransforming Val-664 peptide.

Thus, the global minimum energy conformation for the transmembrane hydrophobic dodecapeptide from the transforming (Gln-664 or Glu-664) oncogene-encoded p185 proteins is significantly different from the global minimum-energy conformations for the same region from the nontransforming (His-664, Tyr-664, Lys-664, Gly-664, and Val-664) proteins. These differences are clearly shown in Fig. 1, in which the global minimum-energy conformations for the

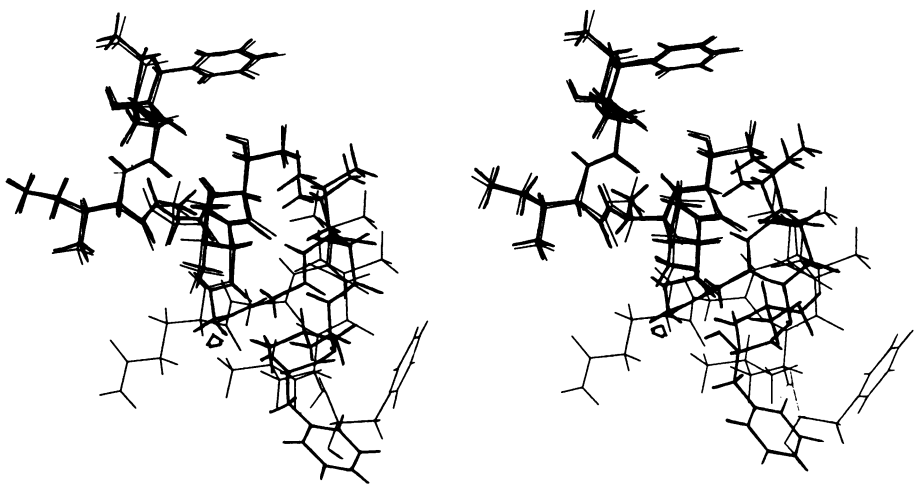

FIG. 1. Stereoviews of the superposition of the global minimum-energy conformation of the transmembrane dodecapeptide from the transforming p185 protein with glutamic acid at position 664 (lightface) on the global minimum-energy conformation of the transmembrane dodecapeptide from the nontransforming p185 protein with glycine at position 664 (boldface). Amino termini are at the top. Arrows indicate the C^{α} of residue 664, after which the two structures diverge.

Table 1. Probabilities for bend and helical conformations in the p185 sequences from positions 658–669

Amino acid residue at position 664	CD* or CG†	AA‡	Transforming activity
1. Gln (0.79)	0.05	0.90	+
2. Glu (0.76)	0.06	0.89	+
3. Gly (0.75)	0.87	0.09	−
4. His (0.77)	0.88	0.08	−
5. Lys (0.75)	0.85	0.10	−
6. Val (0.72)	0.84	0.10	−
7. Tyr (0.73)	0.86	0.09	−

The probability of the global minimum conformation for each peptide is listed in parentheses. The global minimum-conformation for the transforming peptides 1 and 2 was found to be AAAAAAAA-AAAA, while that for the nontransforming peptides 3–6 was AAAA-AACD*AAAC and for the nontransforming peptide 7 it was AAAA-AACGAAAC. The single-letter conformation code for amino acid residues is defined by ranges of dihedral angles as described in ref. 14. The ability to transform cells at normal levels of expression is indicated by +. Overexpression of the normal proteins has also been shown to cause cell transformation when increased at least 5- to 10-fold. This is consistent with these probabilities, which predict that an ≈10-fold increase of the normal protein would be necessary to achieve the total number of p185 molecules in the AA state that would be equivalent to that same number of molecules for the transforming protein at normal levels of expression.

†Probability that the CD* or CG bend conformation exists at positions 664 and 665.

‡Probability that the AA helical conformation exists at positions 664 and 665.

Gln-664 (transforming) and Gly-664 (nontransforming) dodecapeptides are displayed in stereoview superimposed upon one another.

It should be noted that all of the dodecapeptides from nontransforming proteins did exhibit higher-energy conformations that were identical (all α-helix) to the global minimum-energy conformation for the transforming Gln-664 and Glu-664 peptides. Also, the transforming peptides were found to have higher-energy minima that contained the CD* bend at residues 664 and 665. We therefore elected to examine the thermodynamic distribution of the minima for each sequence. We computed the most probable conformation for each sequence and the most probable conformation for the two critical residues 664 and 665 as described (29). The results of this analysis are summarized in Table 1. It may at once be noted that the global minima for the two classes of peptides (transforming and nontransforming) are distinctly different, as expected with the nontransforming sequences adopting the CD* (or closely related CG) bend conformation at positions 664 and 665, while the transforming sequences adopt the AA conformation at these positions. In fact, based on the distribution of all conformations for the Gln-664 peptide, the probability of the AA conformation at positions 664 and 665 is 90%, whereas the probability of the CD* at these positions is only 5%; similar results are obtained for the transforming Glu-664 peptide as shown in Table 1. On the other hand, for the Gly-664 peptide, the probability of the AA conformation at positions 664 and 665 is only 9%, whereas the probability of the CD* conformation at these positions is 87%; similar results are obtained for the other nontransforming peptides as shown in Table 1. There is, therefore, a distinct difference in conformational preference between transmembrane peptides from transforming and nontransforming p185 proteins in the region around positions 664 and 665.

DISCUSSION

The fact that the transmembrane domains of five nontransforming *neu*-encoded proteins adopt the same global minimum-energy conformation with high probability, which is distinctly different from the global minimum-energy conformation adopted with high probability by the transmembrane domains of two transforming *neu*-encoded proteins (which are identical to each other), suggests that there exists a normal conformation (bend at positions 664 and 665) and a transforming conformation (all α-helical) for this region of the *neu* p185 protein. In addition, since it is predicted that it is the α-helical conformation at positions 664 and 665 that is critical to transforming activity, it would be expected that amino acid substitutions in p185 at these positions with high helix-propagation potential (e.g., leucine) would result in a transforming protein. These predictions could thus be tested in site-specific mutagenesis experiments in which the codon for leucine is generated in the *neu* gene corresponding to positions 664 and 665 in p185 and by observing for cell transformation.

Furthermore, it is noteworthy that all five of the peptides from nontransforming *neu*-encoded proteins have higher-energy conformations, which are identical to the global minimum-energy conformation of the two peptides from the transforming *neu*-encoded proteins. This suggests that even in normal cells a small amount of the activated form of the protein will always be present, but this amount is apparently insufficient to cause cell transformation. However, it has been demonstrated that overexpression of the normal human *neu* protooncogene (encoding valine at position 664) can cause transformation of NIH 3T3 cells in culture (30, 31). Furthermore, as noted above, it appears that a considerable proportion of certain human tumors, including cancers of glandular origin, particularly mammary carcinomas, contain an amplified *neu* gene (4–7). This suggests that amplification alone of the *neu* gene and resulting overexpression of its "normal" protein product may play a critical role in the development of some types of human cancer, as has been demonstrated for other oncogenes. The conformational energy analyses provide a possible explanation of why overexpression of these proteins might have a transforming effect on cells. Amplification of *neu* genes (which when expressed at normal levels do not cause cell transformation, such as those encoding valine, histidine, tyrosine, lysine, or glycine at position 664) with overexpression of their protein products would increase the total amount of p185 in cells existing in the normal conformation (bend at positions 664 and 665), but it would also result in an increase in the total amount of p185 in cells existing in the higher-energy transforming conformation (all α-helical). If some minimal level of p185 in the α-helical conformation is required for cell transformation, this effect might be mimicked by the increased amounts of protein with valine, histidine, tyrosine, lysine, or glycine at position 664 that would exist in the minority α-helical conformation when these proteins are overexpressed. Based on the probability findings that ≈9% of the nontransforming p185 proteins will adopt the transforming AA conformation at positions 664 and 665, one can predict that overexpression of normal *neu* genes by ≈10-fold should result in a transforming effect. In fact, it has been shown in NIH 3T3 cells in culture that overexpression of the normal *neu* gene by 5- to 10-fold does have a transforming effect (30). On the other hand, amplification and overexpression of the normal gene at lower levels (1- to 4-fold) in NIH 3T3 cells is not transforming (31). Thus, it appears that there is a certain critical mass of normal p185 necessary to produce malignant transformation of cells and that this critical mass may correlate with the percentage of normal p185 molecules expected to adopt the transforming helical conformation. These conclusions are also consistent with the results of stereochemical modeling of the p185 transmembrane domain with either valine or glutamic acid at position 664 (32). These results suggest that the α-helical conformation of the transmembrane domain of p185 would

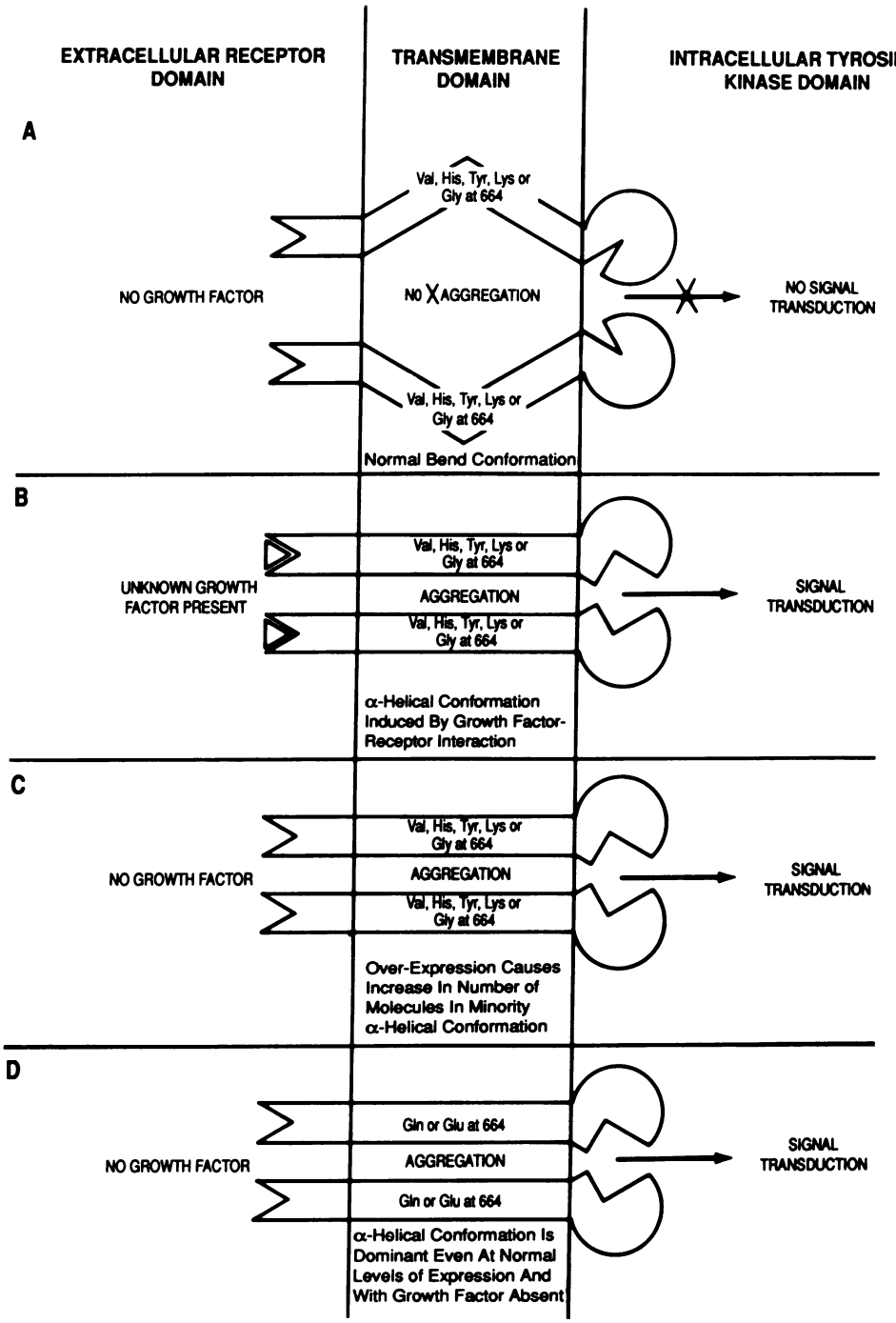

FIG. 2. Schematic representation of the proposed mechanism of activation of p185 based on conformational changes in the transmembrane domain. (*A*) In the absence of a posited but as yet unidentified growth factor, nontransforming p185 molecules favor a bend conformation in the transmembrane domain that prevents aggregation of p185 molecules and signal transduction into the cell. (*B*) When the posited growth factor is present and interacts with the extracellular receptor domain, it is proposed that a conformational change to an α-helix is induced in the transmembrane domain allowing aggregation and signal transduction. (*C*) With overexpression of nontransforming *neu* genes, sufficient numbers of p185 molecules exist in the minority α-helical conformation to allow significant aggregation and signal transduction even in the absence of a growth factor–receptor interaction. (*D*) Transforming p185 molecules always favor the α-helical conformation, thus allowing aggregation and signal transduction at normal levels of expression and without a growth factor–receptor interaction.

promote the aggregation of p185 molecules in the membrane based on the formation of a dimer of p185 transmembrane α-helices; such an arrangement might permit favorable extracellular–extracellular or intracellular–intracellular domain associations (33). Such a model of p185 dimerization is consistent with the proposed mechanism of action for p185 in which aggregation of the molecules activates the tyrosine kinase domain, causing signal transduction into the cell (10, 33), and is further supported by the recent finding that transforming amino acid substitutions at position 664 (such as glutamic acid) do in fact lead to aggregation of *neu*-encoded proteins (34) and that ligand-induced dimerization of p185 is necessary for signal transduction (35).

The above conclusions are summarized in Fig. 2. In this figure, the two situations are represented in which transmembrane domains that form the bend at positions 664 and 665 do not dimerize and do not transduce signals, and, conversely, those transmembrane domains that exist in the α-helical

form, including positions 664 and 665, dimerize and hence cause cell transformation through continuous cell signal transduction. Thus, the critical step in the contribution of the *neu* oncogene to the multistage process of cell transformation may be the adoption of the α-helical conformation by the transmembrane domain of its p185 protein.

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