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Three dimensional structure of the transmembrane region of the proto-oncogenic and oncogenic forms of the *neu* protein

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The *neu* proto-oncogene may be converted into a dominantly transforming oncogene by a single point mutation. Substitution of a valine residue at position 664 in the transmembrane region with glutamic acid activates the tyrosine kinase of the molecule and is associated with increased receptor dimerization. Previously we have proposed a model in which the glutamic acid side chain stabilizes receptor dimerization by hydrogen bonding. Other models have been proposed in which the mutation leads to a conformational change in the transmembrane region mimicking that assumed to occur following binding of a natural ligand. Synthetic peptides representing part of the transmembrane region were prepared. Some residues were replaced with serine in order to improve peptide solubility to allow purification and analysis. Both the peptides containing valine and glutamic acid dissolved in water and in an artificial lipid monolayer. The structures of the peptides were determined by NMR spectroscopy to be α-helical. No significant difference in conformation was observed between the two peptides. This result does not support the model proposing a conformational change. The receptor structures determined experimentally do allow alternative models involving receptor transmembrane region packing.

Key words: growth factor receptors/NMR spectroscopy/tyrosine kinase

Introduction

The rat *neu* gene was originally isolated as a dominantly transforming oncogene by transfection of DNA from the B104 rat neuroblastoma cell line (Shih *et al.*, 1981). This line was derived from a tumour induced by transplacental carcinogenesis employing the alkylating agent nitrosoethylurea (Schubert *et al.*, 1974). The primary structure of the predicted protein was found to resemble a transmembrane growth factor receptor with substantial sequence similarity to the EGF receptor (Bargmann *et al.*, 1986a). Comparison of the transforming gene with the proto-oncogene revealed that a single point mutation had changed a valine to a glutamic acid residue at position 664 in the sequence, predicted to be part of the transmembrane region of the protein (Bargmann *et al.*, 1986b). Subsequently, Bargmann and Weinberg (1988a) went on to show that activation by mutation was both dependent on the residue type and confined to position 664 in the sequence. Substitution with glutamic acid and glutamine were highly transforming and replacement with aspartic acid was weakly activating but substitution with lysine, histidine, glycine and tyrosine was without effect. Alteration of residues 663 or 665 to glutamic acid also had no effect. Biochemical analysis revealed that the Glu664 receptor had greatly enhanced tyrosine kinase activity (Bargmann and Weinberg, 1988b) which was essential for transformation (Weiner *et al.*, 1989a). Mutation of the human gene known as c-*erb*B-2 or HER2 at the same relative position was also activating (Segatto *et al.*, 1988) as was mutation of the *Drosophila* EGF receptor homologue (Wides *et al.*, 1990). Analogous substitutions in the transmembrane region of the human EGF receptor were, however, not transforming (Carpenter *et al.*, 1991).

The activating mutations of the *neu* gene appear to be confined to the chemical carcinogenesis model since no such mutations have been found in human breast, thyroid, brain, stomach or pancreatic cancers (Hall *et al.*, 1990; Lemoine *et al.*, 1990a, 1991; Tuzi *et al.*, 1991). Nevertheless, this system has revealed details of the mechanism of receptor activation which also may allow the development of selective antagonists. Ligand stimulation of the related EGF receptor is associated with receptor dimerization (Yarden and Schlessinger, 1987) and it was proposed that mutational activation of *neu* could be achieved by stabilizing receptors as dimers. The glutamic acid side chain in the interior of the cell membrane would be predominantly uncharged at neutral pH which would allow the formation of inter-receptor hydrogen bonds. A three dimensional model of such receptor interactions has been proposed (Sternberg and Gullick, 1989, 1990) in which the conformation of the transmembrane region was taken as an α-helix. The carboxyl group of Glu664 in one helix forms a hydrogen bond with the carbonyl oxygen of Ala661 in the other helix. A second, symmetric hydrogen bond is formed by the other Glu and Ala residues. Close packing of the helices is made possible by the small side chain of the glycine residue at position 665. Experimental support for this hypothesis was provided by the demonstration that mutant *neu* exists to a greater extent in the dimeric form than does the proto-oncogenic form (Weiner *et al.*, 1989b).

An alternative model of receptor activation relies on the mutation causing a conformational change in the receptor mimicking that predicted to occur as a consequence of ligand binding. A theoretical model for this has also recently been reported (Brandt-Rauf *et al.*, 1989, 1990). We thus undertook direct determination of the structure of the transmembrane region of both proto-oncogenic and mutant *neu* proteins by NMR to determine which model accounted for receptor activation.

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Results

Peptides 1 and 2 from the transmembrane region of oncogenic and proto-oncogenic *neu* were synthesized and found to be soluble in 100% trifluoroacetic acid but very poorly soluble in aqueous solvents (Table I). Thus the purity of these could not be determined by reverse phase HPLC. Two more peptides, 3 and 4, were then synthesized, in which particular hydrophobic residues were substituted with serine. This amino acid was selected as it is likely to be amphipathic in solvents of low polarity where the polar hydroxyl side chain may form a hydrogen bond with the main chain of an α-helix, thereby effectively reducing its hydrophilic character. Equally, in aqueous solvents the serine hydroxyl side chain would improve solubility. In addition, the phenylalanine residue at position 6 was altered to tyrosine. In the light of the model of specific receptor interaction in this region (Sternberg and Gullick, 1989) substitutions were made outside of this putative contact surface. Peptide 3 was still largely insoluble in water but peptide 4 dissolved well in 100 mM sodium phosphate buffer, pH 8.0. Since we wished to compare the structures of the oncogenic and proto-oncogenic molecules, we prepared two more peptides with an additional serine substitution, peptide 5 with valine in position 12 and peptide 6 with glutamate in position 12. These were both now soluble in water.

The changes introduced allowed the peptides to be analysed and purified by reversed phase HPLC and their sequences confirmed. It was possible, however, that they would no longer dissolved in non-polar environments and therefore not resemble the parent sequences or assume the same structures. We therefore tested their ability to transfer from an aqueous environment to a lipid monolayer employing the Langmuir trough apparatus. Peptide 5 or 6 was introduced into the aqueous phase and its effect on surface pressure of the monolayer was examined as an indication of its propensity to dissolve in a membrane environment. Figure 1 shows the effect of the peptides on the surface pressure of a monolayer of phosphatidylserine at various initial surface pressures up to 25 mN/m. Each peptide induced a marked change in surface pressure, although the more hydrophobic proto-oncogenic peptide 5 was slightly more surface active than the mutant peptide 6, as to be expected. The size of the change in surface pressure indicates that each peptide inserted efficiently into the monolayer, rather than merely interacting with the lipid head groups. The surface pressure of the plasma membrane of living cells is not known, but the membrane pressure of liposomes has been estimated to be 25 mN/m. The above results thus suggest that the synthetic peptides are able to dissolve efficiently in cell membranes at a surface pressure up to 25 mN/m.

NMR

The 2-D NMR experiments yielded the chemical shift assignments shown in Table II. Differences from random coil values (Wüthrich, 1986) of the α-CH chemical shifts are shown in Figure 2 for both the proto-oncogenic and oncogenic peptides at pH* (pH meter reading) 3.4 and 2.28 respectively.

$^3J_{NH\text{-}alphaCH}$ coupling constants were below 6 Hz for residues 5–15, with A9 having the lowest value (<4 Hz), in both peptides. There was a slow exchange of deuterium for amide hydrogen of residues Y6 to G13 for both peptides

Table I. Synthetic peptide sequences and their water solubility

Peptide																		Water solubility
1							F	I	I	A	T	V	*V*	G	V	L	L	F	L	I	no
2							F	I	I	A	T	V	*E*	G	V	L	L	F	L	I	no
3	A	S	P	V	T	Y	I	I	A	T	V	*V*	G	V	L	S	S	L			no
4	A	S	P	V	T	Y	I	I	A	T	V	*E*	G	V	L	S	S	L			yes
5	A	S	P	V	T	Y	S	I	A	T	V	*V*	G	V	S	L	F	S			yes
6	A	S	P	V	Y	T	S	I	A	T	V	*E*	G	V	S	L	F	S			yes
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18			

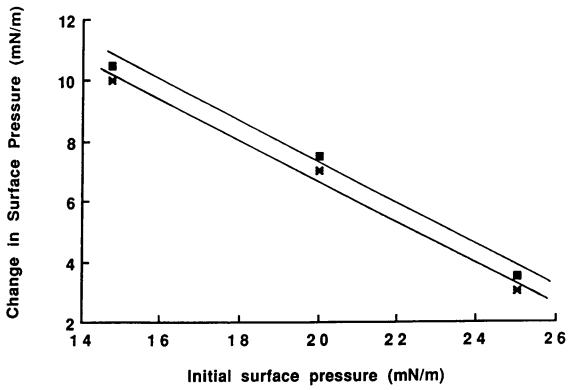

Fig. 1. Increase in surface pressure when *neu* peptides are injected beneath a phosphatidylserine monolayer. The peptides were at a final concentration in the aqueous phase of 5 μM in 10 mM Tris–HCl buffer, pH 7.4, containing 20 mM NaCl and 1 mM $CaCl_2$. ■ proto-oncogenic peptide 5, × oncogenic peptide 6.

[observed first order rate constant $k < 4 \times 10^{-3}$/min at 303 K and pH* 3 in d_2 trifluoroethanol (TFE) (2–4% water)] these protons remaining for more than 16 h, whereas the amide protons of the amino acid residues on either side of this central group exchanged in <9 h. A9 had the lowest observed rate constant in both cases. In an experiment with the oncogenic peptide at pH* 5.23 the amide protons exchanged much faster than at the lower pH* but the same pattern along the peptide chain was observed.

Structure

A total of 190 inter-residue distance restraints were used in the calculation of the structure of the oncogenic peptide and 154 for the proto-oncogenic peptide, additional restraints defining main chain hydrogen bonds were used in both cases. Initially, 40–50 oncogenic and proto-oncogenic structures were generated. Of these, only those with restraint energies <50 kcal/mol (equivalent to total restraint distance violations of <1 Å) were investigated further. This cut-off threshold was decided upon after examining the spread of restraint energies: 81% of the proto-oncogenic structures had restraint energies below this threshold as had 56% of the oncogenic structures. Further proto-oncogenic structures were discarded at random so that subsequent comparisons could be made between sets containing equivalent numbers of structures. The superimposition of these are shown in Figure 3. The side chains of the valine and glutamic acid residues have been included.

It should be noted that, as a control, similar structure calculations were carried out in the absence of hydrogen bond

Table II. Chemical shift assignments in p.p.m. for the proto-oncogenic peptide 5, pH 3.40

Residue	NH	αH	βH	γH	Others
Ala1		4.10	1.52		
Ser2	8.19	4.98	4.28, 3.99		
Pro3		4.48	2.51, 2.01	2.19, 2.11	δCH_2 3.92
Val4	7.48	3.82	2.00	1.07, 1.00	
Thr5	7.45	3.97	4.22	1.27	
Tyr6	7.86	4.31	3.14, 3.05		2,6H 7.07
					3,5H 6.78
Ser7	8.05	4.25	4.02, 3.82		
Ile8	8.03	3.78	1.91	1.81, 1.18	γCH_3 0.95
					δCH_3 0.87
Ala9	8.24 (8.30)	4.02 (4.10)	1.51		
Thr10	7.85 (7.90)	4.00	4.40	1.23	
Val11	8.04 (8.17)	3.71	2.26	1.12, 0.99	
Val/Glu12	8.63	3.61 (4.05)	2.14	1.07, 0.94	
			(2.28, 2.11)	(2.70,2.43)	
Gly13	8.15 (8.20)	3.97, 3.83			
		(3.97, 3.90)			
Val14	8.55 (8.41)	3.85	2.22	1.14, 1.01	
Ser15	8.13 (8.07)	4.25	4.17, 3.96		
			(4.10, 3.96)		
Leu16	8.10 (7.87)	4.22	1.60, 1.14	1.68	δCH_3 0.81
				(1.61, 1.19)	0.75
Phe17	7.97 (7.88)	4.76	3.39, 3.05		2,6H 7.35
					4H 7.23
					3,5H 7.29
Ser18	7.66	4.54 (4.61)	4.07		

The figures in brackets refer to observed shifts for the oncogenic peptide 6, pH 2.28, where values differed by >0.04 p.p.m.; solvent d_2-TFE/5% H_2O; 303 K.

restraints. These structures were also α-helical, although the helices were more loosely defined (results not shown).

*Effect of pH**

pH* titrations in d2-TFE (5% water) gave a pK_a for the titrating carboxy-terminal group of S18 of 4.8 ± 0.3 and 4.3 ± 0.3 for peptides 5 and 6 respectively. The pK_a of E12 in peptide 6 was 5.5 ± 0.2. These values are close to those expected in d_2-TFE (5% water), the pK_a of a carboxy-terminal group and a glutamate side chain being ~4.3 and 5.3 respectively.

Nuclear Overhauser effect (NOE) patterns and coupling constants were measured in experiments at pH* 6.36 (peptide 5) and pH* 7.07 (peptide 6). The observations were similar to those at lower pH* indicating α-helical structures. The carboxyl groups are negatively charged at the higher pH* and there was no evidence of structural change when the glutamate side chain carboxyl group became negatively charged.

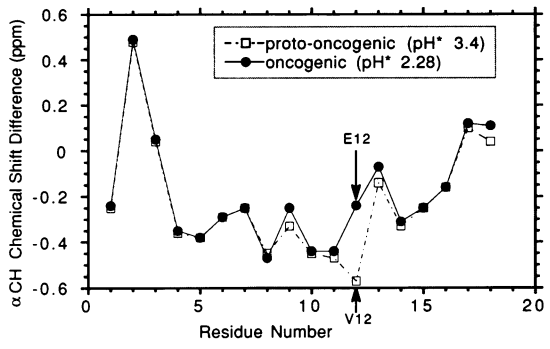

Fig. 2. Differences in α-CH chemical shifts from random coil values for proto-oncogenic and oncogenic peptides; 303 K; d_2-TFE (with 5% water).

Discussion

The transforming *neu* gene differs from the proto-oncogene by a single base change resulting in the replacement of a valine residue in the transmembrane region with glutamic acid (Bargmann *et al.*, 1986b). Site directed mutagenesis has demonstrated that both the position and the type of amino acid substitution are critical (Bargmann and Weinberg, 1988a). Several models were initially proposed to account for the mechanism by which the mutation could lead to receptor activation. These were that the mutation 'may affect the clustering of the receptor, stabilize an interaction between the receptor and a substrate or other effector in the membrane, or exert a physical stress that shifts the receptor slightly inward or outward in the membrane'. It was also suggested that the mutation might induce a conformational change in the receptor which resembled that induced by ligand binding (Bargmann *et al.*, 1986b).

Since no purified ligand is yet available which interacts with the *neu* protein, it is not yet known by what mechanism ligand binding normally activates the receptor. The only type 1 growth factor receptor for which ligands are available is the EGF receptor. Schlessinger and colleagues in a series of papers have clearly demonstrated that ligand binding promotes dimerization of the EGF receptor and stimulates tyrosine kinase activity (Ullrich and Schlessinger, 1990). Subsequently, it has been shown that PDGF promotes PDGF receptor dimerization and activation (Williams, 1989; Li and Schlessinger, 1991) and that CSF-1 promotes c-*fms*

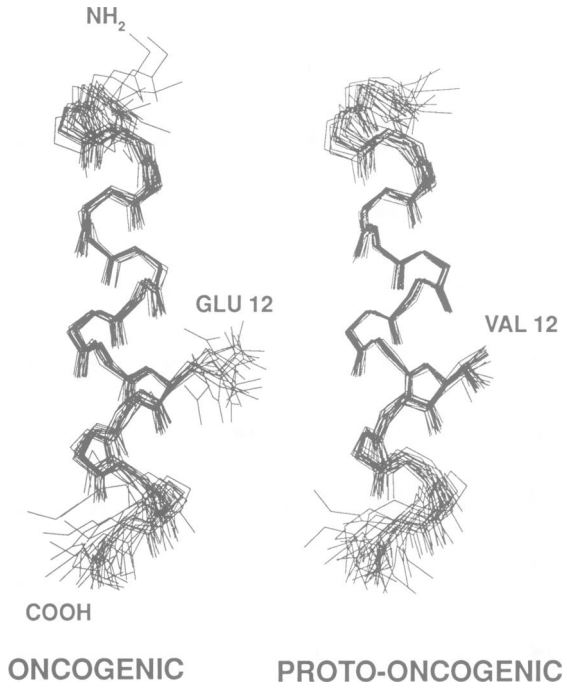

Fig. 3. Twenty-two oncogenic and 22 proto-oncogenic structures. Backbone atoms only are shown, except for the altered side chain (residue 12). In both cases the structures were calculated using NOE and hydrogen bond restraints. Structures are overlaid for backbone atoms (C, Cα, N and O) of residues 4–14.

dimerization and activation (Li and Stanley, 1991) suggesting that this may be a general mechanism of signalling for at least the type 1 and type 3 growth factor receptors. Experimental evidence has been provided that mutation of *neu* both activates the receptor's kinase activity and stabilizes receptors in a dimeric form (Weiner *et al.*, 1989b). This result tends to support a model in which receptor dimerization is the normal mechanism of activation of *neu* and that this is promoted by the mutation.

There are as yet no experiments reported which support the other models. At present there is no evidence to suggest that mutation stabilizes a receptor–substrate interaction. Indeed, much recent evidence demonstrates that known receptor substrates such as phospholipase Cγ, GAP and phosphatidyl inositol 3-kinase interact with specific intracellular sequences autocatalytically phosphorylated on tyrosine (Cantley *et al.*, 1991). Secondly, it is hard to envisage in the model proposing translocation of the membrane sequence that the position of the mutation would be critical. It has, however, been proposed using conformational energy predictions, that the amino acid substitution leads to a local conformational change in the transmembrane region (Brandt-Rauf *et al.*, 1989). In further work this model was developed to include seven different amino acid substitutions (Brandt-Rauf *et al.*, 1990). It was proposed that the non-transforming protein contained a sharp bend around position 664 which was distinctly different from the structure of the transforming protein which was entirely α-helical. We sought to address these alternative models experimentally by determining the three dimensional structure of peptides from within the transmembrane region encompassing the site of the activating mutation.

The NMR results show that the proto-oncogenic and oncogenic structures are both α-helical and are, within experimental limits, essentially identical. There are a number of well-defined side chains in both structures. For example, oncogenic χ_1 angles for 22 structures have $\sigma < 10°$ for residues 3, 5, 8–10 and 13. The observed 'fraying' of the helices toward the termini correlates with the number of inter-residue NOEs observed along the sequence (Figure 4). These observations suggests a real mobility/disorder of the terminal residues in solution. There is no evidence for any gross distortion of the helices dependent upon the type of amino acid side chain of residue 12. These results therefore do not support the model involving local conformational differences between the mutant and wild-type receptors.

Bargmann and Weinberg (1988a) originally suggested that 'the critical determinant of activation must be a property shared by glutamic acid and glutamine, which is deficient in aspartic acid and altogether absent in the other amino acid's. Sternberg and Gullick (1989, 1990) have proposed a theoretical model which explained this property. It was proposed that Glu, Gln and Asp could form hydrogen bonds in their protonated forms. In the hydrophobic membrane environment the pK_a for the acidic amino acids is raised such that at neutral pH a proportion of the residues will be unionized. The protonated side chain of each of a receptor pair may form hydrogen bonds with a main chain carbonyl oxygen residue in the other chain. Such bonding would stabilize the receptors as dimers. This model also accounts for the positional specificity of the mutation. In the NMR work reported here however, no direct evidence was found in support of dimerization of either peptide. Evidence for dimers might have been detected through anomalous NOEs and the observation of differences following ionization of the glutamate side chains since this would be expected to disrupt the putative intermolecular hydrogen bonds between them (Sternberg and Gullick, 1989). It should be noted, however, that a peptide in free solution has greater degrees of freedom than in a membrane environment, where the peptides would be constrained to parallel interactions. In solution, both parallel and anti-parallel dimerizations are possible. However, model building shows that the structures determined by NMR are quite capable of forming the proposed α-helical packing.

In summary we show that mutation of *neu* does not lead to conformational change in the transmembrane region. The data presented are consistent with alternative models involving receptor packing interactions but additional experiments are required to test these models directly. In order to do this we are currently preparing receptors with altered transmembrane regions and determining their properties.

Materials and methods

Peptide synthesis and purification

Peptides 1–6 (Table I) were synthesized by the F-moc-polyamide method on PepSyn KA resin. Temporary α-amino protection was provided by the 9-fluoroenylmethyloxycarbonyl (F-moc) group and *t*-butyl derived groups were used to protect the reactive side chains of glutamate, serine, threonine and tyrosine. 95% trifluoroacetic acid (TFA) was used to liberate the assembled peptide from the resin and remove the side chain protecting groups. The peptides were analysed by reversed phase HPLC using a Beckman C18 ODS column. For purification the peptides were dissolved in a mixture of 70:30 water:methanol and HPLC was carried out on a Beckman System Gold or a Waters system. The proto-oncogenic peptide 5 (Table I) was run on

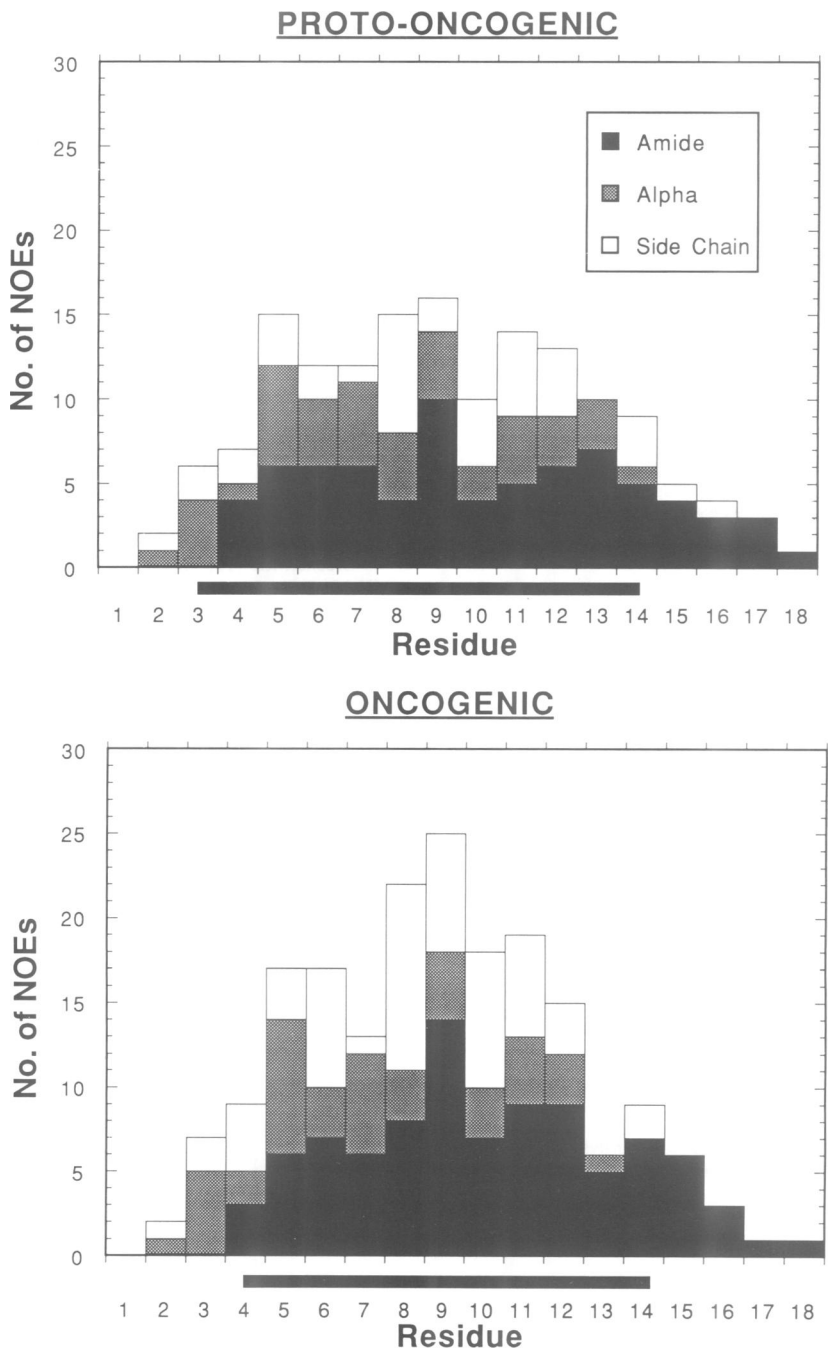

Fig. 4. Distribution of NOEs used in structure calculations. Each atom involved in an NOE is indicated; for example an αH-NH restraint is recorded for both the α and the amide proton. The horizontal black bar indicates residues with RMSDs <0.5 Å. RMSD here means the root mean square deviation of backbone atoms (C, Cα, N and O) from the energy minimized average structure.

a Dynamax C4 semi-preparative column (1 × 25 cm, particle size 12 μm) with a flow rate of 2 ml/min, loading ~1 mg each time. The oncogenic peptide 6 was run on a Dynamax C18 semi-preparative column (1 × 25 cm, particle size 12 μm) at 2 ml/min, loading ~4 mg each time. The eluent buffers were A: 0.1% TFA in water and B: 0.1% TFA in 80:20 acetonitrile:water. Eluent was monitored at a wavelength of 276 nm, where the absorbance of tyrosine dominates. Compositions of the purified peptides were confirmed by amino acid analysis and their sequences were confirmed by Edman degradation employing a gas-phase sequencer.

Langmuir trough

The ability of peptides 5 and 6 to transfer from an aqueous solvent to a lipid monolayer was determined employing a Langmuir trough apparatus (Verger and Pattus, 1982). Purified phospholipids were obtained from Avanti (Alabama, USA) and dissolved in chloroform at a concentration of 25 μg/ml. Lipid monolayers were formed by spreading 1 μl of the solution on a clean 10 mM Tris−HCl buffer, pH 7.4, containing 20 mM NaCl and 1 mM $CaCl_2$ subphase in a Teflon Langmuir trough with a surface area of 50 mm^2. This was followed by compression of the lipid monolayer to the desired surface pressure and injection of 10 μl of 10 mg/ml peptide under the monolayer to give a 20 ng/ml final concentration of peptide in the subphase. The surface pressure was measured at constant surface area with an automated surface barostat apparatus incorporating a Wilhelmy balance with a platinum plate and transducer (KSV2200, Finland). The Teflon trough had a total volume of 5 ml and was scrupulously cleaned before each experiment using ethanol and double-distilled water. All the measurements were taken at room temperature (22°C).

The phospholipid phosphatidylserine was spread to form monolayers with

surface pressures of either 15, 20 or 25 mN/m. Peptide was injected through the pre-formed monolayer into the subphase, with continuous stirring.

NMR

Approximately 1.8 mg of each HPLC purified transmembrane peptide were dissolved in 0.5 ml of d_2-TFE and used for collecting NMR spectra. The TFE had previously been distilled in water to exchange -OD for -OH, and the final solution contained ~5% water. This solvent has been used previously to mimic a membrane environment (Mulvey *et al.*, 1989) and does not induce a helical structure indiscriminately (Breeze *et al.*, 1991). Experiments were carried out on a Bruker AM 500 spectrometer at 303 K. The chemical shift reference was set at the centre of the residual CDH resonance of TFE at 3.88 p.p.m., with solvent OH suppression. 1-D spectra for a pH titration were recorded with a spectral width of 5500 Hz. Double-quantum-filtered COSY spectra, and NOESY spectra with a mixing time of 350 ms were acquired in phase sensitive mode. 1-D spectra were also recorded for the hydrogen exchange experiment and higher resolution 1-D spectra (acquiring 32 K data points) were used for measuring ^{3}J-coupling constants.

The concentrations of mutant and wild-type peptides in the NMR samples were measured using a Uvikon 810P UV spectrophotometer. The calculated relative molecular masses of the peptides were 1796 and 1826 for the wild-type and mutant respectively.

Structure determination

Distance Restraints. Observed NOEs were classified according to strength into three categories—strong, medium and weak, which then corresponded to distance restraints of <0.25, <0.35 and <0.5 nm respectively. Corrections were made for protons with degenerate chemical shifts—methylene, methyl and aromatic H and H (Wüthrich *et al.*, 1982). For non-stereospecifically assigned methylene protons, distance restraints for both protons were set as for the weaker NOE cross peak. Hydrogen bonds were predicted from the presence of slowly exchanging amide protons together with the secondary structure identified from preliminary calculations. Hydrogen bonds were incorporated into the structure calculations as paired restraints of 0.27–0.33 nm for N–O distances and 0.18–0.22 nm for H–O distances.

Calculation. All structure refinement was carried out using the program XPLOR (Brunger, 1990). Initial structures were generated using random ϕ and ψ angles with side chains in an extended conformation, and perfect covalent geometry. Simulated annealing was carried out using a protocol described previously (Brunger, 1990).

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