Expression and Membrane Assembly of a Transmembrane Region from Neu†

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ABSTRACT: Transmembrane domains of receptor tyrosine kinases are increasingly seen as key modulatory elements in signaling pathways. The present work addresses problems surrounding expression, isolation, secondary structure recovery, and assembly into membranes, of the relatively large quantities of transmembrane peptides needed to investigate these pathways by NMR spectroscopy. We demonstrate significant correspondence between SDS−PAGE behavior of such peptides and their ^{2}H NMR spectra in lipid bilayer membranes. A 50-residue peptide, Neu_{exp}, containing the transmembrane portion of the receptor tyrosine kinase, Neu, was designed for expression in *Escherichia coli*. The sequence also contained 11−12 amino acids from each side of the transmembrane domain. The common problem of low expressivity of transmembrane peptides was encountered—likely associated with membrane toxicity of the desired gene product. This difficulty was overcome by expressing the peptide as a *TrpE* fusion protein in a pATH vector to target expression products to inclusion bodies, and subsequently removing the *TrpE* portion by cyanogen bromide cleavage. Inclusion bodies offered the additional benefits of reduced proteolytic degradation and simplified purification. The presence of a hexa-His tag allowed excellent recovery of the final peptide, while permitting use of denaturing solvents and avoiding the need for HPLC with its attendant adsorption losses. Isolated expressed peptides were found to be pure, but existed as high oligomers rich in β-structure as evidenced by CD spectroscopy and SDS−PAGE behavior. Dissolution in certain acidic organic solvents led to material with increased α-helix content, which behaved in detergent as mixtures of predominantly monomers and dimers—a situation often considered to exist in cell membranes. For purposes of NMR spectroscopy, peptide alanine residues were deuterated in high yield during expression. The same acidic organic solvents used to dissolve and dissociate expressed transmembrane peptides proved invaluable for their assembly into lipid bilayers. Analogous transmembrane peptides from the human receptor tyrosine kinase, ErbB-2, demonstrated related phenomena.

Protein receptor tyrosine kinases comprise a large family of transmembrane glycoproteins mediating the earliest events in signal transduction across the plasma membranes of higher animal cells. Current models generally hypothesize that receptor physical behavior provides the molecular mechanism of communication. Such thinking underlies modern concepts as to how receptor mutation and overexpression contribute to disease states including cancer (*1*, *2*). It is now widely held that receptor transmembrane segments can form side-to-side associations with one another, and that these associations are critical to signaling pathways (reviewed in refs *1* and *3*−*8*). It is increasingly suggested that subtle structural details of tyrosine kinase transmembrane sequences exert important control over their intermolecular contacts with other copies of the same and different receptors. Hence, it has become desirable to understand the submolecular phenomena underlying transmembrane domain behavior and associations.

Unfortunately, the extreme hydrophobicity of protein transmembrane domains has been a major limitation to their direct physical study. In principle, solid-state NMR spectroscopy has much to offer in this area (reviewed in refs *9*−*16*). However, this technique requires that the molecule examined be obtained in micromolar quantities, labeled with heteronuclear probes, and assembled into a membrane-like environment that supports meaningful noncovalent interactions. Solid-phase synthesis as a route to the necessary material is limited to peptides of modest size, is very costly, and in the case of hydrophobic species often generates products that are extremely difficult to purify in sufficient quantity. We introduce methods for expressing and isolating suitable quantities of transmembrane peptides and for appropriate secondary structure recovery and membrane incorporation. To our knowledge, this is the first report of deuterated transmembrane eucaryotic peptides produced by expression for wide-line NMR.

The large size of receptor tyrosine kinases makes it desirable to dissect them into smaller units for study. Fortunately, transmembrane domains should lend themselves well to this approach. For instance the "two-step" model of membrane protein biogenesis dictates that transmembrane α-helices first insert into the membrane and subsequently associate to form functional multisubunit complexes (*17*). Thus, it has been observed in bacterial rhodopsin that the

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various transmembrane segments which comprise it can spontaneously associate to a functional whole (reviewed in ref *7*). Moreover, numerous chimeric class I receptors have now been produced in which the transmembrane domain is an independently functional transposable cassette. In the present work, a peptide containing the membrane-spanning stretch of the rat class I receptor tyrosine kinase, Neu,[1] was expressed in *Escherichia coli*, isolated, and assembled into membranes to demonstrate feasibility. Receptors of this class are composed of a single chain of amino acids; with an external glycosylated portion, a hydrophobic stretch of sufficient length to cross the membrane only once, and an intracellular portion (*1*, *3*−*5*). The intracellular portion exhibits phosphorylation sites, docking sites, and protein kinase activity. The expressed transmembrane 50-mer, designated Neu_{exp}, included some 12 amino acids from the contiguous cytoplasmic and extracellular domains. Findings were further tested in a comparable peptide from a corresponding human receptor, ErbB-2.

MATERIALS AND METHODS

Sources. 1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) was obtained from Avanti Polar Lipids (Birmingham, AL) and was used without further purification. Deuterium-depleted water and deuteromethyl L-alanine were from Cambridge Isotope Laboratories (Andover, MA). 2,2,2-Trifluoroethanol, NMR grade, bp 77−80 °C was from Aldrich (Milwaukee, WI). Empigen (*n*-dodecyl-*N*,*N*-dimethylglycine) was from Calbiochem (San Diego, CA). Peptides made by solid-phase synthesis were produced as described previously (*18*, *19*). Low molecular weight standards for SDS−PAGE were bacitracin, insulin b chain, aprotinin, α-lactalbumin, myoglobin, and triosephosphate isomerase (Bio-Rad, CA).

Construction of the TrpE-Neu Chimeric Protein. A chimeric protein of anthranilate synthase, with the desired transmembrane sequence from the *Neu* proto-oncogene fused to the C-terminal end, was generated as follows. General techniques for molecular biology were performed according to standard protocols (*20*). The plasmid, pSV2neuN (*21*) encoding the cDNA of the *Neu* proto-oncogene was kindly supplied by Dr. R. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA). PCR was used to amplify the desired segment of the *Neu* sequence: the recombinant protein was initiated at position Ala^{649} and included the putative transmembrane domain. The upstream PCR oligonucleotide was GGAATTCCATATGCACCAC-CACCACCACCACGCAGAGCAGAGAGCCA, which includes an *Eco*RI site (GAATTC), an *Nde*I site (CATATG), a hexa-His tag, and the initial amino acids of the Neu sequence. The downstream oligonucleotide primer used was CATGCCATGGCCTCCACTAACTCAGTTTCCT, which runs to Glu^{703} in Neu and includes sites for *Nco*I and *Pst*I. Since the PCR products proved difficult to digest, they were ligated into a pGEM-T-vector (Promega). After verification by sequencing, it was double digested with *Eco*RI and *Pst*I and the fragment isolated from a 2% SeaPlaque GTG agarose (FMC) gel according to the manufacturer's instructions. This fragment was then ligated into the pATH11 vector (*22*) which had been similarly digested and purified. The construct was confirmed by sequencing, and designated "pATH-*Neu*". All cloning steps were performed in *E. coli* strain JM101.

Expression of the TrpE-Neu Fusion Protein. Recombinant proteins were expressed following the method of Koerner et al. (*22*). Several different strains of *E. coli* were tested for expression of the chimeric protein, including JM101, BL21, DH5α, and JM109. Cells were grown to an OD_{600} of 0.4 in M9 media supplemented with 5 mg/mL Bacto-casamino acids (Difco), 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% glucose, 0.01 mg/mL thiamin B1, 0.02 mg/mL tryptophan, and 100 μg/mL carbenicillin (Sigma). This culture was diluted 10-fold into the same media lacking Trp and incubated at 37 °C for 2 h. Indoleacrylic acid (Sigma) was then added to a final concentration of 9 μg/mL, and the suspension incubated for an additional 4 h. To achieve alanine labeling, the bacteria were "force fed" deuterated alanine: the casamino acids were omitted, and the M9 media was supplemented with all amino acids (including deuterated alanine), except for Trp, at a concentration of 100−200 μg/mL (*23*).

Isolation of the Expressed Neu Peptide, Neu_{exp}. TrpE fusion proteins, including TrpE-Neu of the present work, target to inclusion bodies, which were isolated in the following manner. Cells were harvested, resuspended in 3 mL of lysis buffer containing 0.15 mM PMSF/gram cell pellet (*20*), subjected to two French press cycles, and centrifuged at 12000*g*. Pelleted inclusion bodies were washed twice with lysis buffer containing 0.1% TritonX-100, and total protein was determined using the *DC* Protein Assay (Bio-Rad). The pellet was then treated with cyanogen bromide (CNBr), which cleaves at Met residues, to separate the peptide, Neu_{exp}, from the fusion protein. For this purpose, the pellet was dissolved in 20 mL of 70% formic acid, and 3 mg of CNBr was added per mg total protein. The mixture was incubated at 42 °C for 4 h, and then lyophilized after adding an equal volume of water. The resultant dry powder was suspended in 20 mL of 9% Empigen (*24*) and relyophilized to remove any residual CNBr. This step was repeated with 20 mL of water.

Purification of Neu Recombinant Peptide. The peptide, Neu_{exp}, (with attached hexa-His tag) was purified by nickel-chelate chromatography (*25*), after cleavage from the fusion protein as above. The CNBr cleavage products were dissolved in 6 M GuHCl, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 8.0, over a period of up to 6 h with periodic brief bath sonication. The resultant solution was centrifuged at low speed to remove particulate matter and then incubated overnight with nickel-NTA resin (Qiagen) at room temperature on a rocker table. The quantity of resin used was according to the manufacturer's recommended binding capacity, assuming that all the isolated protein corresponded to the protein of interest. After incubation, the resin was

[1] Abbreviations: Neu, a class I receptor tyrosine kinase of rat origin; Neu_{exp}, a 50-residue expressed transmembrane peptide corresponding to Ala^{649} to Met^{692} of Neu, with a hexa-His tag at the N-terminus; Neu_{syn}, a 38-residue synthetic transmembrane peptide corresponding to Pro^{655} to Thr^{691} of Neu, with a Lys residue at the N-terminus; EGF, epidermal growth factor; ErbB-1, a human class I receptor tyrosine kinase (the human EGF receptor); $ErbB\text{-}1_{syn}$, a 34-residue synthetic transmembrane peptide corresponding to Ile^{622} to Thr^{654} of ErbB-1; $ErbB\text{-}2_{exp}$, a 50-residue expressed transmembrane peptide comparable to Neu_{exp} but corresponding to Ala^{648} to Met^{691} of the human protein homologous to Neu; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; FACE, formic acid/acetic acid/chloroform/ethanol {1:1:2:1 ratio by volume}; FACT, FACE with trifluoroethanol replacing ethanol.

loaded onto a 0.9 × 15 cm Pharmacia column and washed with 10 bed volumes of 6 M GuHCl, 0.1 M NaH_2PO_4, and 0.01 M Tris-Cl, pH 8.0. This was followed by a series of 8 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris-Cl washes in which the pH was progressively lowered in steps from 8.0 to 6.3, 5.9, and 4.5. At each step, the column was washed with 10 bed volumes of each solution. The Neu_{exp} peptide was finally eluted by washing the column with 10 bed volumes of 6 M guanidine hydrochloride containing 0.2 M acetic acid. Yield of pure peptide was typically 4−5 mg/L of growth medium: the only significant product by mass spectroscopy had the expected target M_r. Peaks corresponding to dimers or higher oligomers were not found by mass spectrometry.

Gel Electrophoresis and Western Blots. SDS−PAGE was performed using a mini-gel system (Bio-Rad). Larger proteins were typically run on 12% gels as described by Laemmli (*26*). Small proteins were run on the 16.5% tris-tricine gels described by Schägger (*27*). Samples were dissolved in standard loading buffer (*20*) and incubated for 30 min at 42 °C prior to loading. Gels were stained with Coomassie Brilliant Blue. Following gel transfer to a PVDF membrane (Gelman Sciences), Western blots were performed to detect the hexa-His tag using a HisProbe Western Blotting kit (Pierce). This kit utilizes Ni-activated horseradish peroxidase to detect polyhistidine proteins. The manufacturer's procedure was followed except that blots were blocked overnight with 4% casein in TBST. Samples for electrophoresis were usually pretreated with organic acid as described in Results. Such pretreatment typically involved dissolution and incubation for 1 h in formic acid (90%)/glacial acetic acid/chloroform/ethanol (1:1:2:1 ratio by volume) ["FACE" (*28*)] or in the same solvent with ethanol replaced by trifluoroethanol ("FACT"), prior to drying under a stream of nitrogen followed by vacuum desiccation for 16 h. Samples were heated in loading buffer for 30 min at 42 °C prior to loading onto the gel.

Preparation of Samples for NMR Spectroscopy. Except where noted otherwise, liposome generation was according to the following protocol. Acidic organic solvent mixtures, particularly FACT, were employed to prepare solutions of lipid plus peptide that could be dried to form thin films for subsequent hydration with sample buffer. Typically 4 mL of solvent was added to dry peptide (10 mg) and appropriate amounts of dry lipid, with warming to 45 °C. Samples were incubated at this temperature for at least 30 min after visually apparent complete dissolution. Solvent was then rapidly removed under reduced pressure at 45 °C on a rotary evaporator to leave thin films in 50 mL round-bottom flasks. These were subsequently vacuum desiccated for 18 h at 23 °C under high vacuum with continuous evacuation. Even prolonged exposure to the acid solvents used did not give rise to detectable lipid degradation as measured on heavily overloaded thin-layer chromatography plates. Hydration was with 30 mM HEPES with 20 mM NaCl and 5 mM EDTA, pH 7.1−7.3, made up in deuterium-depleted water (vortexing was avoided to minimize production of small vesicles). ^2H NMR spectra were acquired at 76.7 MHz on a Varian Unity 500 spectrometer using a single-tuned Doty 5 mm solenoid probe with temperature regulation to ±0.1 °C. A quadrupolar echo sequence (*10*) ("SSECHO" from the Varian pulse library) was employed with full phase cycling and $\pi/2$ pulse length of 5−6 μs. Pulse spacing was typically 20 μs, and sweep width was 100 kHz.

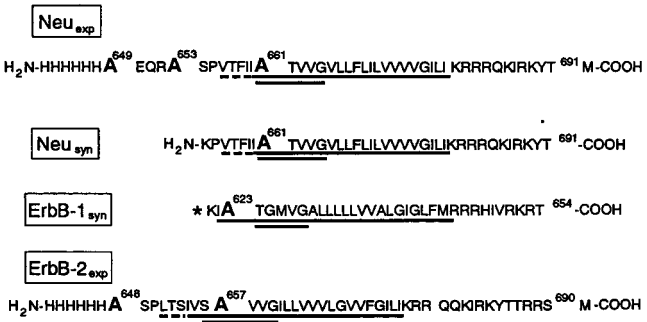

FIGURE 1: Peptide sequences. From top to bottom: Neu_{exp}, A^{649} to M^{692} of rat Neu with His tag, made by expression and subsequent cyanogen bromide cleavage. Neu_{syn}, KP^{655} to T^{691} of rat Neu. Made by solid-phase synthesis. ErbB-1_{syn}, I^{622} to T^{654} of human ErbB-1. Made by solid-phase synthesis. A biotinylated lysine (*) replaces S^{621} of the natural sequence. ErbB-2_{exp} A^{648} to M^{691} of human ErbB-2 with His tag. Made by expression and subsequent cyanogen bromide cleavage. In each case, the natural cytoplasmic end is to the right. Putative transmembrane domains (*44*) have been single underlined. Residue numbering corresponds to the intact native protein. The short broken line indicates variability in the point at which the transmembrane domain is predicted to emerge from the membrane hydrophobic interior [result of four different algorithms (*44*−*47*)]. Expressed peptides were designed to extend well clear of both membrane surfaces while not being large enough to form extensive denatured domains. Deuterated alanine residues are indicated in bold print. Double solid underlining indicates the predicted "motif" region for dimer formation (*29*).

Table 1: Summary of Bacterial Expression Systems Investigated

vector system	Neu sequence expressed[a]	*E. coli* strain	level of expression
pGEX-KG	fusion with GST and $A^{649}-K^{721}$	BL21(DE3)pLysS	very low
pET-21a	$A^{649}-T^{691}$	BL21(DE3) and	low
	$A^{649}-E^{700}$	BL21(DE3)pLysS	low
pATH11	fusion with anthranilate synthase and $A^{649}-L^{696}$	JM101	high

[a] The putative transmembrane domain of Neu extends from I^{660} to I^{680} (*44*).

RESULTS

Figure 1 shows the amino acid sequence and putative transmembrane domain of the expressed 50-mer, Neu_{exp}, containing amino acid residues Ala^{649} to Met^{692} of rat Neu, plus an N-terminal hexa-His tag. Two shorter transmembrane peptides, Neu_{syn} and ErbB-1_{syn} prepared by solid-phase synthesis, are also shown, as is another expressed 50-mer, ErbB-2_{exp}. Neu_{syn} is a synthetic transmembrane peptide with very little extension beyond the membrane surface at the N-terminus, and ErbB-1_{syn} is a similar short synthetic peptide from the human EGF receptor (ErbB-1). ErbB-2_{exp} is the corresponding transmembrane peptide from the *human* protein homologous to Neu. In each case, the putative transmembrane domain is underlined, and the 5-amino acid motif suggested to be involved in dimer formation (*29*) is double underlined.

A variety of expression techniques were attempted to produce Neu transmembrane sequences of similar length to Neu_{exp} (see Table 1). Expression as a GST-fusion protein, or directly under the control of the strong T7 promoter in

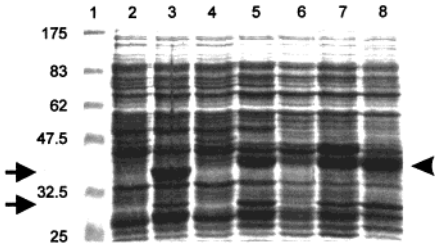

FIGURE 2: SDS−PAGE demonstration of desired fusion protein induction. Four cell lines were tested for ability to efficiently express the 40 kDa fusion protein containing the product of the *TrpE* gene fused to the 5.7 kDa peptide, Neu_{exp}. Shown is a Coomassie blue-stained 12% SDS−polyacrylamide gel having the following numbered lanes: (1) prestained M_r markers (sizes in kilodaltons listed to their left), (2) control of *un*induced JM101 cells transformed with the unaltered pATH11 vector, (3) control of induced JM101 cells transformed with the unaltered pATH11 vector [left-hand arrows indicate the products of the *TrpE* gene (anthranilate synthase, upper arrow 35 kDa) and β-lactamase (lower arrow, 26 kDa)], (4) *un*induced JM101 cells transformed with the pATH11 vector after fusion to the Neu_{exp} construct (pATH-*Neu*), (5) induced JM101 cells transformed with pATH-*Neu* (right-hand arrowhead indicates the 40 kDa protein containing anthranilate synthase fused to the 5.7 kDa Neu_{exp} peptide), (6) induced JM109 cells transformed with pATH-*Neu*, (7) induced DH5α cells transformed with pATH-*Neu*, (8) induced BL21 cells transformed with pATH-*Neu*. The equivalent of an $OD_{600} = 0.1$ was loaded per lane.

pET-21a, failed to yield sufficient product to be detectable on SDS−PAGE by Coomassie blue staining. The desired protein could, however, generally be detected by the more sensitive Western blot technique. Several measures were taken to improve peptide yield from the vectors, including varying the temperature and duration of induction. In the case of the pET expression system, two different bacterial strains were tried: BL21(DE3) and BL21(DE3) pLysS.

It appeared likely that the source of difficulty in achieving adequate levels of expression was membrane toxicity of the hydrophobic peptide product, as manifest by failure to grow and by cell lysis during growth. It has been observed by previous workers that such toxicity is less marked in systems which direct the expressed peptide to inclusion bodies within the bacteria. To force this outcome, cDNA corresponding to Neu_{exp} was fused to the *TrpE* gene, which codes for anthranilate synthase. PCR primers were designed such that they included an upstream *Eco*RI site and produced an N-terminal hexa-His tag in the desired transmembrane peptide. The PCR product was ligated into a pGEM-T vector and subsequently digested with *Eco*RI and *Pst*I to produce a fragment which could be ligated into a pATH11 expression vector to form pATH-*Neu*. pATH-*Neu* was transformed into JM101 bacteria and induced using standard protocols (*22*).

In optimizing expression of the pATH-*Neu* chimeric protein, the plasmid was transformed into a variety of bacterial strains including JM101, JM109, BL21(DE3), and DH5α. As a control, the pATH11 plasmid without any insert was transformed into JM101 cells. All strains were induced under identical conditions, and equal aliquots at cell density of $OD_{600} = 0.1$ were loaded onto denaturing 12% gels. Typical results are displayed in Figure 2. The desired product band at 40 kDa was readily apparent on Coomassie-blue-stained gels (arrowhead to the right of lane 8). The fusion protein was identified based on its abundance, its absence in uninduced control cultures, and comparison with the gel migration pattern of the control unfused anthranilate synthase protein. Consistent with the results of Koerner et al. (*22*), control induction of anthranilate synthase on its own in JM101 cells led to the appearance of two bands, one near 35 kDa and another near 26 kDa (Figure 2 lane 3, arrows to the left of markers). The lower band has been suggested to be β-lactamase while the upper band corresponds to anthranilate synthase. Induction of the pATH-*Neu* construct in JM101 cells also gave two bands: the lower one remained unchanged near 26 kDa, while the upper band had shifted to about 40 kDa. This is the expected result for successful induction, since the fusion protein mass should increase by the 5.7 kDa mass of Neu_{exp} over that of the anthranilate synthase protein on its own. BL21(DE3) and DH5α gave the same banding pattern as JM101, with expression levels in JM101 being generally the highest of the cell lines tested. The JM109 cells gave the lowest levels of expression.

Isolation of inclusion bodies by lysis and differential centrifugation of the cells, provided an initial fraction enriched in the expressed fusion protein. The preferred subsequent workup in our hands involved cyanogen bromide cleavage of the inclusion body isolate. This avoided the problems of peptide insolubility and accessibility of the cleavage site, which would be associated with enzymatic methods, since the cyanogen bromide reaction is performed in 70% formic acid. Purification of the desired product, Neu_{exp}, took advantage of the fact that binding and release of hexa-His tagged peptides on nickel chelate columns occurs efficiently even in the strong denaturing solvents that are often required to dissolve hydrophobic peptides. Thus, the products of cyanogen bromide cleavage were dried and dissolved in 6 M guanidine hydrochloride: from this solution the hexa-His tagged Neu_{exp} peptide was selectively retained on nickel columns. Elution with acidic guanidine hydrochloride led to highly pure peptide as judged by mass spectrometry (see Materials and Methods). A number of attempts were made to improve upon this protocol (see also ref *24*). In particular, increasing the number of pH steps in the graded series of acid eluent failed to improve purity or yield, and left the peptide in inconveniently large volumes. Using imidazole instead of acidic guanidine hydrochloride to release the product from the nickel column produced inferior yields and lower purity. This was also true when urea and guanidine were replaced with detergents such as dodecyltrimethylammonium bromide or Empigen at either 3% or 9% concentration. Chloroform/methanol organic extraction of the cyanogen bromide cleavage products in an attempt to selectively enrich the hydrophobic transmembrane peptide also proved ineffective.

Removal of guanidine hydrochloride from the column fractions containing pure Neu_{exp} typically led to 4−5 mg of peptide/L of original growth medium. This material ran on SDS−PAGE as a broad and diffuse band of very high M_r (Figure 3, panel A, lane 2). CD spectra of the same SDS solutions demonstrated an intense negative peak at 218 nm (spectra not shown). These findings suggest the presence of mainly unstructured very high oligomers. Freeze-dried peptide dissolved readily in the strong organic acids, FACE (*28*) and FACT: subsequent complete solvent removal, followed by dissolution in SDS loading buffer, produced a gel banding pattern indicating that the oligomeric complexes had been dissociated into a population of species with well-defined secondary structure, migrating primarily as apparent dimers

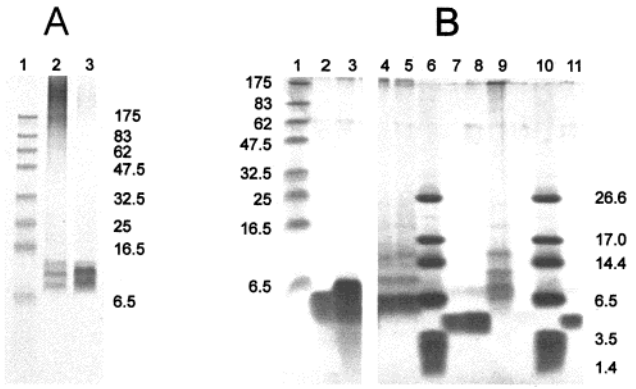

FIGURE 3: SDS−PAGE behavior of purified peptides in detergent micelles; and effects of treatment with strong organic acid. Panels A and B display Coomassie blue-stained 16.5% SDS-polyacrylamide gels [panel A corresponds to a mini-gel (6 × 8 cm) run for 80 min at 100 V; panel B corresponds to a full size gel (14 × 16 cm) run for 4 h at 0.02 amps]. (A) SDS−polyacrylamide gel has the following numbered lanes: (1) prestained M_r markers (sizes in kilodaltons listed to the right of panel); (2) pure Neu_{exp} (as in lane 3) *prior* to renaturation in organic acid; (3) pure Neu_{exp} (as in lane 2) *after* treatment with the strong organic acid mixture FACT, followed by drying and dissolution in SDS gel loading buffer. (B) SDS−polyacrylamide gel has the following numbered lanes: (1) prestained M_r markers (sizes in kilodaltons listed to the left of panel); (2) Neu_{syn}, a synthetic 38-mer containing the Neu transmembrane domain plus a dozen amino acids of the cytoplasmic domain; (3) Neu_{syn} (twice as much loaded); (4, 5) ErbB-2_{exp}, an expressed 50-mer very similar to Neu_{exp} and produced, isolated, and treated identically, but from the corresponding region of the homologous human receptor ErbB-2; (6) nonprestained M_r markers (sizes in kilodaltons listed to the right of panel); (7) ErbB-1_{syn}, a synthetic 34-mer containing the transmembrane domain of another RTK (ErbB-1, the human EGF receptor) plus a dozen amino acids of the cytoplasmic domain; (8) ErbB-1_{syn}, (twice as much loaded); (9) Neu_{exp}; (10) M_r markers (nonprestained); (11) ErbB-1_{syn} (half as much loaded as lane 7).

(Figure 3, panel A, lane 3—described in more detail below). CD spectroscopy was once again consistent with this interpretation in that there were now two negative minima, at 208 and 222 nm, indicating α-helix as a major structural feature. Simpler solvents such as methanol, hexafluoro-2-propanol, and trifluoroethanol achieved relatively poor dissolution of the expressed peptides. FACT was devised for the present work because trifluoroethanol is widely viewed as an optimal "lipomimetic" solvent that induces α-helix formation in peptides to which this structure is native (e.g. ref *30*).

The monomer M_r of Neu_{exp} is 5785 Da, and this was the only significant product found by mass spectrometry in the pooled fractions. SDS−PAGE banding patterns of this material were measured for six separate peptide lots relative to molecular weight markers. Typical results are shown for comparison with related peptides in Figure 3. Renatured Neu_{exp} migrated as several distinct bands, with predominant intensity in two bands—one at 1.5−1.8× monomer M_r and the other at 2× monomer M_r (panel A, lane 3). A similar result is seen in panel B under conditions that expand the low molecular weight region of the gel: in addition to the major bands at 1.5× M_r and 2× M_r, in this case, there is also faint intensity in bands corresponding to 1× monomer M_r and at 3× M_r (lane 9). Usually SDS−PAGE of the final renatured product displayed a minor diffuse band near the junction between the stacking gel and the running gel: this band was higher than the 175 kDa marker and presumably reflects some highly oligomerized material as described above (Figure 3, panel A). Given the propensity of transmembrane peptides to retain α-helicity of their membrane-spanning portions in SDS (*7*, *31*−*33*) and their tendency to run somewhat "too fast" on SDS−polyacrylamide gels (*34*), it seems very likely that the predominant Neu_{exp} species in SDS micelles is a tightly coiled dimer. In general, when performing SDS−PAGE, we preincubated samples only to 42 °C in loading buffer, since it has been observed that heating hydrophobic peptides to 100 °C in SDS can induce irreversible aggregation (*24*, *35*). In a limited number of cases, we heated SDS samples at 100 °C for 5 min, observing that this did indeed have a tendency to induce greater formation of very high molecular weight oligomers in SDS−polyacrylamide gels of renatured Neu_{exp}: such aggregation was, however, reversible by freeze-drying the samples and retreating with the solvents FACE or FACT.

In attempting to interpret these findings regarding the behavior of Neu_{exp} in SDS micelles, results were compared to those obtained with shorter related peptides produced by solid-phase synthesis. The transmembrane 38-mer, Neu_{syn} (M_r, 4378 Da), which was essentially the same as Neu_{exp} but lacking 11 amino acids at the N-terminus (Figure 1), ran on SDS−polyacrylamide gels as only a single very broad band that extended from 0.8× to 1.6× the monomer M_r (Figure 3, panel B, lanes 2 and 3). A closely related synthetic 23-mer from Neu has been noted by others to run in a similar fashion on SDS−polyacrylamide gels—a pattern that was interpreted by the investigators as reflecting a rapid monomer/dimer equilibrium (*36*). In our hands, a similar synthetic peptide, ErbB-1_{syn} (Figure 1), which comprises a comparable transmembrane domain from the human EGF receptor (another class I RTK), ran on gels as a single relatively sharp band centered at 0.9× monomer M_r of 4000 (Figure 3, panel B, lanes 7, 8, and 11). The latter result demonstrates that ErbB-1_{syn} is a predominant monomer in SDS under these conditions. On balance, these results confirm that the longer transmembrane peptide, Neu_{exp}, has a relatively high tendency to form dimers and other low oligomers which are stable on the long time scale of SDS−PAGE. Comparison with another (long) expressed peptide, ErbB-2_{exp}, supported the above interpretation. ErbB-2_{exp} was a transmembrane 50-mer from ErbB-2 (the human homologue of Neu), which corresponded extremely closely to Neu_{exp} and which was expressed and isolated in the same fashion (Sharpe et al., unpublished material). After organic acid renaturation, this peptide ran as a series of bands (Figure 3, panel B, lanes 4 and 5) qualitatively similar to those described for Neu_{exp} (Figure 3, panel A, lane 3, and Figure 3, panel B, lane 9). However, there was a significant quantitative difference between these two homologous 50-mers: the predominant intensity of ErbB-2_{exp} was in a band at 0.9× monomer M_r (monomer M_r 5643 Da)—which corresponds well with the NMR spectral differences described below.

A key requirement for NMR structural analysis of proteins is the ability to incorporate heteronuclear probes. Relatively high yields of expressed peptides (5−10 mg of purified peptide/L) were obtained while supplying deuterated alanine in the growth medium, and similar yields have been obtained using media that supply ^{15}N as NH_4Cl (unpublished). Figure 4 displays ^2H NMR spectra of the peptides discussed here,

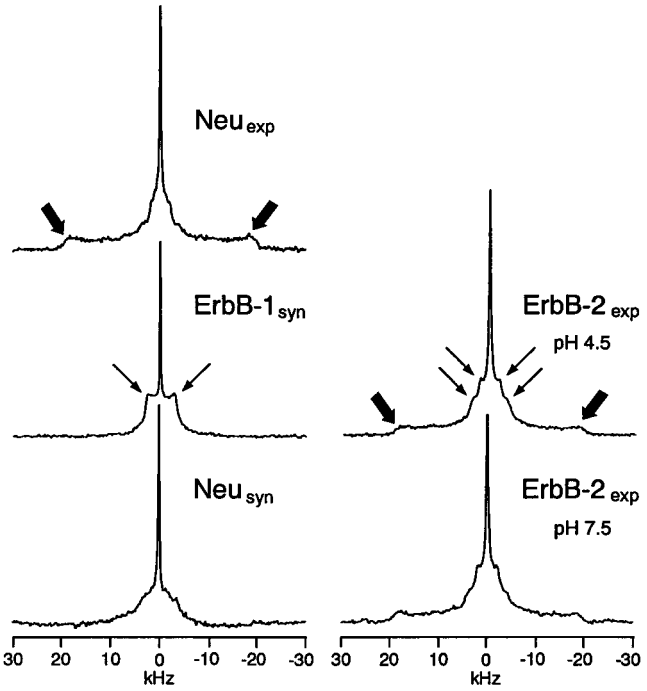

FIGURE 4: ^{2}H NMR spectra of deuterated peptides in fluid POPC bilayers at 35 °C. Each peptide contained one or more alanine residues with perdeuterated side chains ($-CD_3$). Key spectral features referred to in the manuscript are indicated by arrows. Peptide/phospholipid mol ratio was 6 mol %. The number of accumulated transients represented by each spectrum ranged from 100 000 to 1 300 000. Expressed peptide spectra have been normalized to constant area. The top spectrum in the left column corresponds to the expressed 50-mer, Neu_{exp}, deuterated at all three alanine sites. Below it for comparison are typical spectra of the shorter (synthetic) peptides, ErbB-1_{syn} deuterated at Ala^{623} and Neu_{syn} deuterated at Ala^{661}. Thick arrows in spectra of Neu_{exp} indicate the limiting 40 kHz splitting expected of alanines in peptides that are immobilized by lateral associations. *Thin* arrows in the case of ErbB-1_{syn} indicate the narrowed Pake doublet characteristic of rapid symmetrical peptide rotation. The right-hand column shows spectra of the 50-amino acid expressed transmembrane peptide, ErbB-2_{exp}, for samples in which the hexa-His tag carries a charge of +6 (upper) and zero (lower). Thin arrows in the upper ErbB-2_{exp} spectrum indicate the two narrowed Pake doublets (from the two deuterated alanines) associated with a rapidly rotating fraction of ErbB-2_{exp}; while the thick arrows indicate wider features associated with peptide molecules involved in longer term lateral associations.

assembled at 6 mol % in fluid bilayers of POPC. These demonstrate correlation with behavior of the same peptides on SDS−PAGE gels.

Solid-state ^{2}H NMR spectroscopy provides a sensitive measure of peptide dynamic behavior. Elongated amphiphiles dispersed in fluid membranes tend to undergo rapid symmetric rotation about axes perpendicular to the bilayer. For molecules containing deuterium nuclei, spectral splittings are related to orientation and motional characteristics. Few examples exist of ^{2}H NMR spectra from peptides having the long hydrophobic transmembrane segments characteristic of receptor tyrosine kinases, and detailed interpretation of such spectra in terms of peptide−peptide interaction will require considerable study. In an immobilized peptide, all alanine CD_3 groups give rise to a single Pake doublet of splitting about 40 kHz. For Neu_{exp} under the conditions described, this is a dominant spectral feature (Figure 4, thick arrows). Spectral intensity shifted from these 40 kHz peaks into narrower spectral features when peptide concentration in the membrane was reduced (not shown here). Thus, it would appear that Neu_{exp} under these conditions of high peptide concentration is quite immobilized in the membrane, consistent with its highly self-associated behavior in SDS micelles. A very sharp peak in the middle of each spectrum arises from residual deuterated water, and from the presence of some vesicles with high curvature for which the quadrupole splittings are motionally averaged to zero. Probe nuclei undergoing asymmetric motion [e.g., 2-fold rotational jumps and 3 (or higher)-fold jumps with unequal population weighting of available conformers] can also give rise to intensity in the spectral center (*9*, *10*, *15*).

Rotational diffusion of transmembrane peptides in fluid bilayers leads to progressive reduction of the alanine CD_3 group spectral splitting to less than the 40 kHz width seen for immobilized peptides. We have demonstrated previously that a single Pake doublet having splitting much less than 40 kHz is seen for each alanine CD_3 group in ErbB-1_{syn} (e.g., 6 kHz when the deuteration site is Ala^{623}—Figure 4) (*18*, *37*). We interpreted this as indicating that ErbB-1_{syn} is undergoing very rapid axial rotation as a predominant monomer in fluid bilayer membranes (*18*, *19*). In keeping with this, SDS−PAGE of ErbB-1_{syn} produced essentially only a monomer band as described above. Spectra of the synthetic 38-mer, Neu_{syn}, deuterated at a comparable single site, Ala^{661}, also display narrow spectra (*19*), but typically of a more "pyramidal" shape, as seen in Figure 4. Once again, the narrow width indicates the presence of rapid rotational diffusion, although in this case the shape suggests axial asymmetry of peptide motion. Our interpretation has been that Neu_{syn} is in rapid monomer/dimer equilibrium, such that its rotational diffusion is no longer smoothly symmetric (*19*). The latter is consistent with behavior of Neu_{syn} on SDS−PAGE gels (Figure 3). It can be seen that features similar in width to those of the short peptides can also be found in the spectrum of Neu_{exp}, but as a minor component.

A second expressed 50-mer, ErbB-2_{exp}, while being the same length as Neu_{exp} and from the same region of the corresponding *human* receptor, had a quantitatively different spectrum (Figure 4). ErbB-2_{exp} migrated as predominant monomers in SDS−PAGE. Its dominant spectral feature in bilayer membranes is a pair of narrowed Pake doublets (splittings 4 and 8 kHz, thin arrows, one doublet for each of the two deuterated alanines in ErbB-2_{exp}), suggesting that the predominant fraction is undergoing rapid rotational diffusion. In this case, the proportion of more immobilized peptide, indicated by intensity out to 40 kHz splitting (thick arrows), is reduced. Thus spectra of ErbB-2_{exp} might be seen as intermediate between those of Neu_{exp} and the short synthetic peptides. This is consistent with the observation that ErbB-2_{exp} has a quantitatively lower tendency to self-associate in SDS micelles than does Neu_{exp} (Figure 3), while having a greater such tendency than do the short synthetic peptides.

It has been reported that an N-terminal poly-His tag on a transmembrane bacterial protein of M_r 13 200 was observed to have only a passive role on bilayer interactions (*24*). The high sensitivity of ^{2}H NMR spectra to peptide orientation and dynamics permitted consideration of possible behavioral contributions from the hexa-His tag in the present experiments. All peptides studied possessed overall (+) charges

at each membrane surface under all conditions. However, the charge on the hexa-His tag could be selectively altered from zero at neutral pH to +6 at acid pH since the pK_a of histidine under comparable physical conditions is in the range 6.0−6.4 (e.g., ref *38*). This manipulation had no significant effect on observed splittings or spectral features for Neu_{exp} and ErbB-2_{exp}. A typical such result is shown for ErbB-2_{exp}, whose spectra have clearly identifiable features (Figure 4).

DISCUSSION

We have been attempting to adapt the technique of wide-line ^{2}H NMR spectroscopy to studies of receptor tyrosine kinases and their role in transmembrane signaling. Advantages of this technique include high sensitivity to dynamic and associative behavior of molecules in fluid, fully hydrated lipid bilayer membranes. Our initial experiments involved synthetic peptides corresponding to transmembrane segments from the class I receptor tyrosine kinases, ErbB-1 and Neu (*18*, *19*, *37*). Here, we have laid a groundwork for extending such experiments to (longer) expressed peptides, whose production in micromole quantities and membrane assembly can be limiting problems. The approach developed is a general one; although, if a methionine residue occurs within the desired peptide sequence, it would be necessary to replace this residue with some other during PCR primer design if one wishes to use cyanogen bromide for fusion protein cleavage. To our knowledge, this represents the first report of wide-line ^{2}H NMR experiments on expressed transmembrane proteins from higher animals.

The methods described here have several attractive features. Notably they address the serious problems that arise from the fact that transmembrane peptides are generally water-insoluble. Membrane-localized peptides are often toxic to bacterial cells: this problem was circumvented by utilizing a vector that dependably targets its fusion protein product to inclusion bodies. Peptide hydrophobicity also often precludes the use of standard isolation procedures at several levels. For instance, fusion proteins containing such highly hydrophobic peptides tend to be insoluble in the nondenaturing solvents needed for their isolation and subsequent enzymatic cleavage. Denaturing solvents cannot be used with affinity columns for GST fusion proteins or in enzymatic cleavage protocols for target peptide recovery. Also hydrophobicity typically leads to unacceptable losses during HPLC purification. In contrast, the hexa-His tag method of purification works well in denaturing solvents that dissolve a very wide range of difficult species. In addition, cyanogen bromide cleavage operates efficiently in a solvent which will dissolve virtually all peptides. The approach does require "renaturation" of the peptides isolated from inclusion bodies. Fortunately transmembrane sequences have a strong tendency to form α-helical structures in appropriate acidic organic solvents, and α-helicity is considered to be the key aspect of transmembrane domain secondary structure (*7*, *31*−*33*).

SDS detergent micelles are thought to provide important characteristics of a membrane environment. A straightforward explanation of the behavior of the (short) synthetic peptides is that ErbB-1_{syn} exists in SDS micelles as a predominant monomer, while Neu_{syn} exists as a mixture comprising significant fractions of monomers and dimers in rapid equilibrium on the time scale of SDS−PAGE. Following the same logic, the longer species, Neu_{exp}, existed in SDS micelles as a mixture of predominantly long-lived homodimers and oligomers. Analogous behavior has been recorded for other transmembrane species. For instance the intact transmembrane protein, glycophorin A, has been observed to form homodimers that are long-lived on the SDS−PAGE time scale, and isolated transmembrane peptides from bacteriorhodopsin and glycophorin A are capable of stable dimer formation in SDS (reviewed in ref *7*). Li et al. examined a short 23-mer peptide containing key amino acid sequence features of the transmembrane domain of Neu (*36*) and observed that it ran on SDS−polyacrylamide gels as a broad band corresponding to a rapid monomer/dimer equilibrium. Smith and Bormann (*39*) have measured NMR intensity transfer resulting from dimerization of the transmembrane domain of glycophorin in gel phase phospholipid bilayers. Millar and Shore have measured dimerization of a transmembrane mitochondrial protein by chemical cross-linking in *Saccharomyces cerevisiae* (*40*).

An important concept relating to class I receptors is that their single-α-helix transmembrane portions may form side-to-side homodimers/oligomers (*6*, *29*, *31*, *41*, *42*). Interestingly, full-length Neu and ErbB receptor glycoproteins exhibit only monomer bands on SDS−PAGE of extracted membrane proteins (*7*). Thus, our results might be seen as evidence that full-length extramembranous portions inhibit spontaneous dimer/oligomer formation (at least when these very large domains are unfolded in SDS). It has been shown that the extracellular domain of RTKs can be inhibitory of their activity (*43*). The differences in behavior on SDS−PAGE between Neu_{exp} and Neu_{syn} are presumably a further reflection of structural influence on side-to-side association (discussed below with regard to NMR spectroscopy). The fact that dimers of Neu_{syn} appear to be less stable than those of Neu_{exp} on the time scales of SDS−PAGE might be rationalized by considering that the "motif" region in this short synthetic peptide is close to the peptide N-terminus, which as a result, should be subject to unravelling (i.e., be less capable of stable ridges-into-grooves secondary structure fit with a neighboring peptide). Neu_{syn} also has a relatively poor N-cap (lysine), that would reduce helix stability. ErbB-1_{syn} shared these features with Neu_{syn}.

Use of acidic solvents to dissolve intractable peptides for analysis is well-known in peptide chemistry (*28*). Without the complex solvent mixtures FACE and FACT, experiments with the expressed peptides would have been impossible. FACE has been described previously for isolating membrane proteins from cells (*28*), but has not to our knowledge been used for the purposes described here: peptide secondary structure recovery and membrane assembly. Dissolution in the strong organic acid, FACT, appeared to permit spontaneous "recovery" of the secondary structure considered to be the native thermodynamically stable one for transmembrane peptides in a hydrophobic membrane environment. We have previously used trifluoroethanol to dissolve transmembrane peptides (along with phospholipids) for bilayer preparation (*18*, *19*, *37*). However, transmembrane peptides are often insoluble in simple organic solvents, and in the present work, a stronger solvent was found necessary to dissolve the products of bacterial expression.

^{2}H NMR spectroscopy of transmembrane peptides has developed around bacterial and model peptides (reviewed

in refs *9*, *10*, *15*, and *16*), and relatively few examples exist for higher animal proteins. It seems likely that transmembrane domains of class I receptor tyrosine kinases will demonstrate spectral complexities relating to their very long transmembrane portions and their likely tendencies to self-associate. The ^{2}H NMR spectra in fluid bilayers bore qualitative correspondence to peptide behavior in SDS micelles. Thus, ErbB-1_{syn} demonstrated rapid and symmetric rotational diffusion, while Neu_{syn} displayed some evidence of self-association. Spectra of the expressed peptide, Neu_{exp}, which had a high tendency to exist as predominantly homodimers/oligomers in SDS, indicated only a very small rapidly rotating fraction by ^{2}H NMR. ErbB-2_{exp}, which was predominantly monomeric in SDS micelles while having significant tendency to self-association, gave a spectrum which indicated that the predominant fraction was rotating rapidly and symmetrically, while there also existed a fraction with restricted mobility.

The approach described here for peptide expression leaves a hexa-His tag at the N-terminus. Although it was not possible to completely rule out some influence of this structure, conferring a charge of +6 vs zero on the tag had little impact on peptide ^{2}H NMR spectra. Such a drastic change in tag properties, with little spectral effect, suggests that the hexa-His tag does not introduce unacceptable bias on peptide behavior or association under the conditions of our NMR spectroscopy experiments, which included the presence of EDTA. In agreement with this conclusion, other workers have noted that a hexa-His tag on a small (13.2 kDa) transmembrane bacterial protein (diacylglycerol kinase) was mobile, did not prevent its membrane insertion in vivo, and was passive in terms of perturbing protein-bilayer interactions (*24*).

CONCLUSIONS

A general approach is reported for expression, isolation, and lipid bilayer assembly of transmembrane peptides containing heteronuclei for solid-state NMR spectroscopy. Expression as a *TrpE* fusion protein using the pATH vector led to high yields, with the product being directed to inclusion bodies. Deliberate placement of a methionine residue at the desired C-terminus (and avoidance of this residue elsewhere in the desired final peptide sequence) made it possible to use cyanogen bromide to remove the fusion protein—an approach that works with high efficiency even for insoluble species. Incorporation of a hexa-His tag permitted easy isolation and purification in the strongly denaturing solvents often needed to dissolve hydrophobic peptides. Strongly acidic organic solvents were important for solubilization, renaturation, and membrane assembly of the peptide product. Organic acid mixtures such as FACE or FACT are likely to be generally useful, if not critical, in such studies. NMR spectra of the transmembrane peptides studied suggested that their behavior in fluid phospholipid bilayers was related to that seen by SDS−PAGE.

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REFERENCES

1. Hynes, N. E., and Stern, D. F. (1994) *Biochim. Biophys. Acta 1198*, 165−184.
2. Yu, D., and Hung, M.-C. (1998) *Cancer Metastasis Rev. 17*, 195−202.
3. Fantl, W. J., Johnson, D. E., and Williams, L. T. (1993) *Annu. Rev. Biochem. 62*, 453−481.
4. van der Geer, P., Hunter, T., and Lindberg, R. A. (1994) *Annu. Rev. Cell Biol. 10*, 251−337.
5. Heldin, C.-H. (1995) *Cell 80*, 213−223.
6. Alroy, I., and Yarden, Y. (1997) *FEBS Lett. 410*, 83−86.
7. Lemmon, M. A., MacKenzie, K. R., Arkin, I. T., and Engelman, D. M. (1997) in *Membrane protein assembly* (von Heijne, G., Ed.) R. G. Landes Co.
8. Weiss, A., and Schlessinger, J. (1998) *Cell 94*, 277−280.
9. Opella, S. J., and Stewart, P. L. (1989) *Methods Enzymol. 176*, 242−275.
10. Davis, J. H. (1991) in *Isotopes in Physical and Biomedical Science* (Buncel, E., and Jones, J. R., Eds.) Vol. 2, Elsevier, Amsterdam.
11. Smith, S. O., and Peersen, O. B. (1992) *Annu. Rev. Biophys. Biomol. Struct. 21*, 25−47.
12. Cross, T. A., and Opella, S. J. (1994) *Curr. Opin. Struct. Biol. 4*, 574−581.
13. Henry, G. D., and Sykes, B. D. (1994) *Methods Enzymol. 239*, 525−535.
14. Opella, S. J. (1997) *Nat. Struct. Biol. NMR Suppl.* 845−848.
15. Siminovitch, D. J. (1998) *Biochem. Cell Biol. 76*, 411−422.
16. Watts, A. (1998) *Biochim. Biophys. Acta Rev. Biomembr. 1376*, 297−318.
17. Popot, J.-L., and Engelman, D. M. (1990) *Biochemistry 29*, 4031−4037.
18. Rigby, A. C., Barber, K. R., Shaw, G. S., and Grant, C. W. M. (1996) *Biochemistry 35*, 12591−12601.
19. Jones, D. H., Barber, K. R., and Grant, C. W. M. (1998) *Biochim. Biophys. Acta 1371*, 199−212.
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular cloning: a laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
21. Bargmann, C. I., Hung, M. C., and Weinberg, R. A. (1986) *Cell 45*, 649−657.
22. Koerner, T. J., Hill, J. E., Myers, A. M., and Tzagoloff, A. (1991) *Methods Enzymol. 194*, 477−490.
23. Muchmore, D. C., McIntosh, L. P., Russell, C. B., Anderson, D. E., and Dahlquist, F. W. (1989) *Methods Enzymol. 177*, 44−73.
24. Sanders II, C. R., Czerski, L., Vinogradova, O., Badola, P., Song, D., and Smith, S. O. (1996) *Biochemistry 35*, 8610−8618.
25. Janknecht, R., de Martynoff, G., Lou, J., Hipskind, R. A., Nordheim, A., and Stunnenberg, H. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8972−8976.
26. Laemmli, U.K. (1970) *Nature 227*, 680−685.
27. Schägger, H. (1994) in *A practical guide to membrane protein purification* (Schägger, H., and Von Jagow, G., Eds.) Chapter 3, Academic Press, San Diego.
28. Findlay, J. B. C. (1990) in *Protein purification applications, a practical approach* (Harris, E. L. V., and Angal, S., Eds.) IRL Press, Oxford.
29. Sternberg, M. J. E., and Gullick, W. J. (1989) *Nature 339*, 587.
30. Sönnichsen, F. D., Van Eyk, J. E., Hodges, R. S., and Sykes, B. D. (1992) *Biochemistry 31*, 8790−8798.
31. Gullick, W. J., Bottomley, A. C., Lofts, F. J., Doak, D. G., Mulvey, D., Newman, R., Crumpton, M. J., Sternberg, M. J. E., and Campbell, I. D. (1992) *EMBO J. 11*, 43−48.
32. Smith, S. O., Smith, C. S., and Bormann, B. J. (1996) *Nat. Struct. Biol. 3*, 252−258.
33. Li, S.-C., and Deber, C. M. (1993) *J. Biol. Chem. 268*, 22975−22978.
34. Grisshammer, R., and Tate, C. G. (1995) *Q. Rev. Biophys. 28*, 315−422.
35. Deber, C. M., Khan, A. R., Li, Z., Joensson, C., Glibowicka, M., and Wang, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11648−11652.

36. Li, S.-C., Deber, C. M., and Shoelson, S. E. (1994) in *Peptides: Chemistry, Structure and Biology* (Hodges, R. S., and Smith, J. A., Eds.), ESCOM, Leiden.
37. Jones, D. H., Barber, K. R., VanDerLoo, E. W., and Grant, C. W. M. (1998) *Biochemistry 37*, 16780−16787.
38. Andersson, H., Bakker, E., and von Heijne, G. (1992) *J. Biol. Chem. 267*, 1491−1495.
39. Smith, S. O., and Bormann, B. J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 488−491.
40. Millar, D. G., and Shore, G. C. (1994) *J. Biol. Chem. 269*, 12229−12232.
41. Brandt-Rauf, P. W., Rackovsky, S., and Pincus, M. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8660−8664.
42. Sternberg, M. J. E., and Gullick, W. J. (1990) *Protein Eng. 3*, 245−248.
43. Rodrigues, G. A., and Park, M. (1994) *Curr. Opin. Genet. Dev. 4*, 15−24.
44. Rost, B. (1996) *Methods Enzymol. 266*, 525−539.
45. Yanagihara, N., Suwa, M., and Mitaku, S. (1989) *Biophys. Chem. 34*, 69−77.
46. Hofmann, K., and Stoffel, W. (1993) *Biol. Hoppe-Seyler 347*, 166.
47. Cserzo, M., Wallin, E., Simon, I., von Heijne, G., and Elofsson, A. (1997) *Protein Eng. 10*, 673−676.

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