Membrane Interaction of Small N-Myristoylated Peptides: Implications for Membrane Anchoring and Protein-Protein Association

Mantripragada B. Sankaram
Department of Biochemistry, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908 USA

ABSTRACT The effect of the covalent attachment of a myristoyl moiety to the N-terminal glycine residue in proteins, N-myristoylation, on lipid-protein interactions was investigated in a model system using magnetic resonance spectroscopic methods. Two peptides with sequences conserved among known N-myristoylated proteins were chosen for this study. Using two-dimensional nuclear magnetic resonance techniques, it was shown that N-myristoylation results in an aggregation of both peptides in solution, although they lack well defined folded conformations in solution either when chemically N-myristoylated or when nonacylated. The interaction of the acylated peptides with lipid bilayers was investigated using spin label electron spin resonance and ^2H NMR techniques. The results show that when bound to membranes, the covalently linked myristoyl chain of one of the peptides is directly inserted into or anchored to the lipid bilayer. The binding of the other peptide with membranes is effected by interactions between amino acid residues and the phospholipid headgroups. In this case, the covalently linked myristoyl moiety is most likely not in direct contact with the acyl chains of the host lipid bilayer. Rather, the N-myristoyl chains stabilize the peptide aggregate by forming a hydrophobic core. Measurements of peptide binding to membranes showed that N-myristoylation affects both the lipid:peptide stoichiometry at saturation and the equilibrium binding constant, in a manner that is consistent with the structural information obtained by magnetic resonance methods.

INTRODUCTION

A novel type of protein modification discovered in recent years, N-myristoylation, involves the covalent attachment of myristic acid to N-terminal amino groups of glycine residues in soluble proteins (Schultz et al., 1988; Towler et al., 1988; Schmidt, 1989; Gordon et al., 1991). N-myristoylated proteins exist in a wide variety of organisms including viruses, fungi, yeast, angiosperms, sea urchin, vertebrates, and mammals. Many eukaryotic and viral proteins related to regulation of cell growth and/or signal transduction, e.g., enzymes, α-subunits of G-proteins, immunoglobulins, and growth factors are N-myristoylated. N-myristoylation is catalyzed by *N*-myristoyl transferase (Towler et al., 1987). Interestingly, not all proteins with an N-terminal glycine are myristoylated in vivo (Buss et al., 1987), although synthetic peptides of their N-terminal regions can be myristoylated in vitro (Towler et al., 1988).

An examination of the primary sequences of N-myristoylated proteins reveals a consensus sequence for the N-terminal region of the proteins (Schultz et al., 1988). An N-terminal glycine is an absolute requirement for N-myristoylation. The second and fifth positions may be occupied by most amino acids except proline and those with bulky side chains. No specific amino acid requirement was found for the third and fourth positions. A majority of N-myristoylated proteins contain serine as the fifth residue, whereas threonine is the next most highly conserved residue at the fifth position (Gordon et al., 1991). Although it is generally assumed that N-myristoylation helps to anchor a protein to the membrane, clear biochemical evidence for this assumption has not been forthcoming.

One way to study the structural, functional, and mechanistic consequences of N-myristoylation is to study its effect on a well characterized system. In this paper, the solution conformations and interactions with lipid bilayers of two peptides, whose sequences correspond to the sequence similarity features mentioned above, in both acylated and deacylated forms are examined. One of the peptides has the primary sequence Gly-Gly-Asp-Ala-Ser-Gly-Glu (SerPep). This peptide was chosen because it contains the obligatory glycine residue at the N-terminal, a serine at the fifth position (hence, the abbreviation SerPep) and a non-bulky residue, glycine, at the second position. This sequence resembles closely the N-terminal region in proteins such as the α-subunit of the heterotrimeric G-protein transducin (Tanaba et al., 1985), the polypeptide VP4 from polio virus (Hughes et al., 1986), $p60^{src}$ (Pellman et al., 1985). The other peptide has a primary sequence Gly-Arg-Gly-Asp-Thr-Pro (ThrPep). This peptide was chosen for two reasons. First, this peptide contains the obligatory glycine residue at the N-terminal, and the second most abundant residue, threonine, at the fifth position (hence, the abbreviation ThrPep). Second, it corresponds closely to the N-terminal region of proteins

Received for publication 26 August 1993 and in final form 29 March 1994.

Abbreviations used: DQF-COSY, double-quantum-filtered proton-proton correlation spectroscopy; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPC-d_{54}, 1,2-di(perdeuteriomyristoyl)-*sn*-glycero-3-phosphocholine; ESR, electron spin resonance; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; Myr_{27}-SerPep, *N*-perdeuteriomyristoyl-SerPep; Myr_{27}-ThrPep, *N*-perdeuteriomyristoyl-ThrPep; NOESY, proton-proton nuclear Overhauser spectroscopy; 16-PCSL, 1-stearoyl-2-[16-(4,4-dimethyloxazolidine-*N*-oxyl)]-stearoyl-*sn*-glycero-3-phosphocholine; 16-SASL, 16-(4, 4-dimethyloxazolidine-*N*-oxyl) stearic acid; SerPep, Gly-Gly-Asp-Ala-Ser-Gly-Glu; 16-SteSL-SerPep, *N*-(16-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl-SerPep; 16-SteSL-ThrPep, *N*-(16-(4,4-dimethyloxazolidine-*N*-oxyl)stearoyl-ThrPep; ThrPep, Gly-Arg-Gly-Asp-Thr-Pro; Tris, tris(hydroxymethyl)aminomethane.

0006-3495/94/07/105/08 $2.00

such as the Moloney murine leukemia virus p15^{gag} protein (van Beveren et al., 1981) and the polyprotein Pr65^{gag}, which contain an N-terminal glycine and a threonine at position 5 (Schultz et al., 1988).

MATERIALS AND METHODS

Materials

DMPC was purchased from Avanti Chemical Company (Alabaster, AL). The peptide SerPep was purchased from Calbiochem Corporation (La Jolla, CA), and the peptide ThrPep was from Bachem Feinchemikalien AG (Bubendorf, Switzerland). 1-Hydroxybenzotriazole and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 16-SASL, and perdeuterated myristic acid were purchased from Sigma Chemical Co. (St. Louis, MO). The phospholipid spin label, 16-PCSL, was synthesized as described before (Sankaram and Thompson, 1990a).

N-acylation of the peptides

Both of the peptides were better than 98.8% pure as determined by the supplier using high pressure liquid chromatography (HPLC) and were used without further purification. The N-myristoylated derivatives of SerPep and ThrPep were synthesized by a covalent linkage of myristic acid to the N-terminal amino group of the glycine residue. To do so, first the free carboxyl groups of the peptide were methyl-esterified. N-myristoylation was then carried out using standard carbodiimide/1-hydroxybenzotriazole-mediated coupling reaction between the fatty acid and the protected peptide (Konig and Geiger, 1970), followed by base hydrolysis to deprotect the peptides. The peptides were purified by HPLC on a Vydac C18 reverse-phase column employing an acetonitrile-water or methanol-water (each with 0.1% trifluoroacetic acid) linear gradient elution mode with a flow rate of 2 ml/min and detection at 230 nm. The deuterated peptides, Myr-d_{27}-SerPep and Myr-d_{27}-ThrPep, and the spin-labeled peptides, 16-SteSL-SerPep and 16-SteSl-ThePep, were synthesized and purified by the methods described above using perdeuterated myristic acid or the spin-labeled fatty acid 16-SASL. After purification by HPLC, the peptides were lyophilized and stored at −70°C.

The acylated peptides were characterized by two-dimensional (2D) proton NMR (see below), the results of which are summarized in Table 1. Because the method used to synthesize the spin-labeled and deuterated acylated peptides was the same as that for unlabeled acylated peptides, the isotopically labeled acylated peptides most likely have the same structure.

Lipid-peptide binding studies

Large unilamellar vesicles (LUVs) of 1 μm diameter from DMPC were formed in a Tris buffer (10 mM Tris, pH 7.4) at a lipid concentration of 13 mM using standard methods (Hope et al., 1985). A 1-ml solution in Tris buffer of peptide, in the concentration range 0.1 and 4 mM as required, was added to LUVs. The mixture was incubated at 40°C for 24 h. The peptide-bound LUVs were repeatedly freeze-thawed to form multilamellar vesicles (MLVs). After several freeze-thaw cycles, the MLVs were separated into a pellet and a supernatant fraction using bench-top centrifugation. The pellets and supernatants were assayed for lipid and

TABLE 1 ^{1}H chemical shifts of the amino acid and fatty acid residues in SerPep, ThrPep, MyrSerPep, and MyrThrPep

Peptide	Amino acid/ fatty acid residue	Chemical shifts (ppm)			
		NH	αH	βH	Side-chain protons/fatty acid protons[a]
SerPep	Gly 1	–	3.92	–	
SerPep	Gly 2	8.38	3.96	–	–
SerPep	Asp 3	8.44	4.75	2.83, 2.72	–
SerPep	Ala 4	8.76	4.27	1.18	–
SerPep	Ser 5	8.21	4.61	3.81, 3.79	–
SerPep	Gly 6	8.33	3.93		–
SerPep	Glu 7	8.60	4.71	2.10, 1.99	γCH$_2$ 2.52
MyrSerPep	CH$_3$ 14	–	–	–	0.89
MyrSerPep	(CH$_2$)$_{10}$ 4–13	–	–	–	1.23
MyrSerPep	CH$_2$ 3	–	–	–	1.62
MyrSerPep	CH$_2$ 2	–	–	–	2.28
MyrSerPep	Gly 1	8.45	3.99	–	–
MyrSerPep	Gly 2	8.42	3.98	–	–
MyrSerPep	Asp 3	8.46	4.75	2.83, 2.72	–
MyrSerPep	Ala 4	8.76	4.27	1.18	–
MyrSerPep	Ser 5	8.20	4.62	3.81, 3.79	–
MyrSerPep	Gly 6	8.33	3.93	–	–
MyrSerPep	Glu 7	8.59	4.70	2.10, 1.99	γCH$_2$ 2.52
ThrPep	Gly 1	–	3.93	–	
ThrPep	Arg 2	8.29	4.37	1.89, 1.78	γCH$_2$ 1.70; δCH$_2$ 3.32; NH 7.17, 6.61
ThrPep	Gly 3	8.38	3.92	–	–
ThrPep	Asp 4	8.43	4.78	2.84, 2.75	–
ThrPep	Thr 5	8.29	4.32	4.22	γCH$_3$ 1.23
ThrPep	Pro 6	–	4.42	2.28, 2.01	γCH$_2$ 2.03; δCH$_2$ 3.68, 3.63
MyrThrPep	CH$_3$ 14	–	–	–	0.90
MyrThrPep	(CH$_2$)$_{10}$ 4–13	–	–	–	1.23
MyrThrPep	CH$_2$ 3	–	–	–	1.63
MyrThrPep	CH$_2$ 2	–	–	–	2.31
MyrThrPep	Gly 1	8.44	3.95	–	
MyrThrPep	Arg 2	8.31	3.37	1.89, 1.78	γCH$_2$ 1.79; δCH$_2$ 3.32; NH 7.17, 6.61
MyrThrPep	Gly 3	8.41	3.93	–	
MyrThrPep	Asp 4	8.43	4.78	2.84, 2.75	–
MyrThrPep	Thr 5	8.31	4.31	4.22	γCH$_3$ 1.23
MyrThrPep	Pro 6	–	4.42	2.28, 2.01	γCH$_2$ 2.03; δCH$_2$ 3.68, 3.63

[a] The myristic acid moiety is numbered from 1–14 starting at the carbonyl group covalently linked to the N-terminal glycine.

peptide content using standard procedures (Eibl and Lands, 1969; Lowry et al., 1951). No lipid was found in the supernatant fractions. The lipid: peptide ratios throughout the manuscript are bound lipid:peptide mole ratios, unless otherwise indicated.

ESR spectroscopy

ESR spectra were recorded on a Varian E-Line 9 GHz spectrometer equipped with a temperature controller, employing the sample preparation procedures described before (Sankaram and Thompson, 1990a). A modulation amplitude of 1 G and spectral width of 100 G were used.

2D NMR spectroscopy

NMR samples were prepared at a concentration of about 10 mM in 90% H_2O/10% D_2O at pH 7.4. The pH meter reading was used without any correction for the deuterium isotope effect. NMR experiments were performed at 25°C on a GN-Omega spectrometer operating at 500 MHz for protons. The 2D experiments, double-quantum-filtered correlation spectroscopy (DQF-COSY), and phase-sensitive nuclear Overhauser spectroscopy (NOESY), were performed according to standard procedures (Wüthrich, 1986) using quadrature detection in both dimensions. The carrier frequency was centered on the water signal with sweep widths of 4,000 Hz in both dimensions. The solvent signal was suppressed by a solvent suppression sequence (Bax, 1985). The 2D spectra were obtained using 2,048 points for each t1 value. For NOESY experiments, 512 t1 values were used, and for DQF-COSY experiments, 800 t1 values were acquired using 4,096 points in t2. Before Fourier transform, both time domain data were multiplied by $\pi/8$- and $\pi/4$-shifted sine bell functions for t2 and t1 domains, respectively.

^{2}H NMR spectroscopy

^{2}H NMR spectra were recorded on a Nicolet NT360B spectrometer, running at 45 MHz for the ^{2}H nucleus (Sankaram and Thompson, 1990b). A fixed-frequency high-power variable-temperature probe, HP-50 from Cryomagnet Systems, Inc. (Indianapolis, IN), with a 5-mm solenoid was used for the ^{2}H NMR experiments. The sample temperature was controlled by a Nicolet variable-temperature accessory. The quadrupole echo pulse sequence (Davis et al., 1976) was used with a 90° pulse with of 2.4 μs. The spectral width was 125 kHz. The interpulse delay was optimized for each sample to obtain a digitized point at the top of a symmetric echo and to minimize the imaginary part of the magnetization. The delay values were generally between 30 and 60 μs. A relaxation delay of 300 ms was used.

RESULTS

DQF-COSY and phase-sensitive NOESY of acylated and nonacylated peptides

To facilitate the comparison between the two peptides in nonacylated form, SerPep and ThrPep, and in N-myristoylated form, MyrSerPep and MyrThrPep, the NMR study was performed under the same pH (7.4), temperature (25°C), and concentration (10 mM) conditions. Sequence-specific assignment of the ^{1}H NMR spectra was carried out by well established methods (Wüthrich, 1986). The different spin systems were identified using DQF-COSY techniques. The connectivities between the adjacent spin systems were established using NOESY spectra. Most of the protons were assigned, and the results are reported in Table 1. The vicinal coupling constants between the amide protons of residue i and the CαH protons of $i + 1$ residue, $^3J_{\alpha H}$, were greater than 8 Hz for all four peptides.

The observed sequential and short-range NOEs are presented in Fig. 1. The NOEs between CαH and NH, NH and NH, and CβH and NH protons of the i and $i + 1$ residue, $d_{\alpha N}$, d_{NN}, and $d_{\beta N}$ respectively, are shown as lines spanning the i and the $i + 1$ residue in Fig. 1. When the diagonal peaks are positive and the cross-peaks are negative, the NOEs are shown as thin lines. When both the diagonal peaks and the cross-peaks are positive, they are shown as thick lines.

The same short-range, sequential NOEs were observed for the N-myristoylated peptides, MyrSerPep and MyrThrPep, as for the nonacylated peptides, SerPep and ThrPep, but with the opposite sign. When the diagonal peaks were positive, the cross-peaks were negative for the nonacylated peptides, SerPep and ThrPep, and positive for the N-myristoylated peptides, MyrSerPep and MyrThrPep. In addition to $d_{\alpha N}$, d_{NN}, and $d_{\beta N}$, two additional sequential NOEs between protons of the myristoylated chain and protons of the amino acid residues were observed for both MyrSerPep and MyrThrPep (see Fig. 1). One of them is an NOE between the methylene protons adjacent to the carbonyl group in the myristoyl moiety and the NH protons of the amide bond between myristic acid and N-Gly, d_{2N}. The other NOE is between the methylene protons of the myristoyl chain at positions 2 and 3, d_{23}.

No long-range NOEs were observed either for the nonacylated peptides, SerPep and ThrPep, or for the N-myristoylated peptide, MyrSerPep. However, for MyrThrPep, a set of long-range NOEs were observed between the combined resonance arising from the unresolved methylene groups at positions 4–13 in the myristoyl group and the side-chain β protons of Asp, Thr, and Pro, $d_{4-13\beta}$. As mentioned above, the cross-peaks were positive.

Peptide binding

SerPep, MyrSerPep, ThrPep, and MyrThrPep bind to DMPC bilayers with different affinities. Binding curves for SerPep and MyrSerPep are shown in Fig. 2 *A*, and those for ThrPep and MyrThrPep are shown in Fig. 2 *B*. The mole ratio of the bound peptide, P, to lipid, L_t, is plotted as a function of the mole ratio of the initially added peptide, P_t, to lipid, L_t. The data were fit to simple mass action binding to determine the lipid:peptide stoichiometry at saturation, n, and the equilibrium binding constant, K. At saturation, 1 mol of SerPep was bound to 29.3 ± 0.6 mol of DMPC, 1 mol of MyrSerPep was bound to 34.7 ± 0.2 mol DMPC, 1 mol of ThrPep was bound to 20.0 ± 0.4 mol of DMPC, and 1 mol of MyrThrPep was bound to 7.3 ± 0.2 mol of DMPC. The binding constants were 3058 ± 327 M^{-1} for SerPep, 30200 ± 5408 M^{-1} for MyrSerPep, 4715 ±

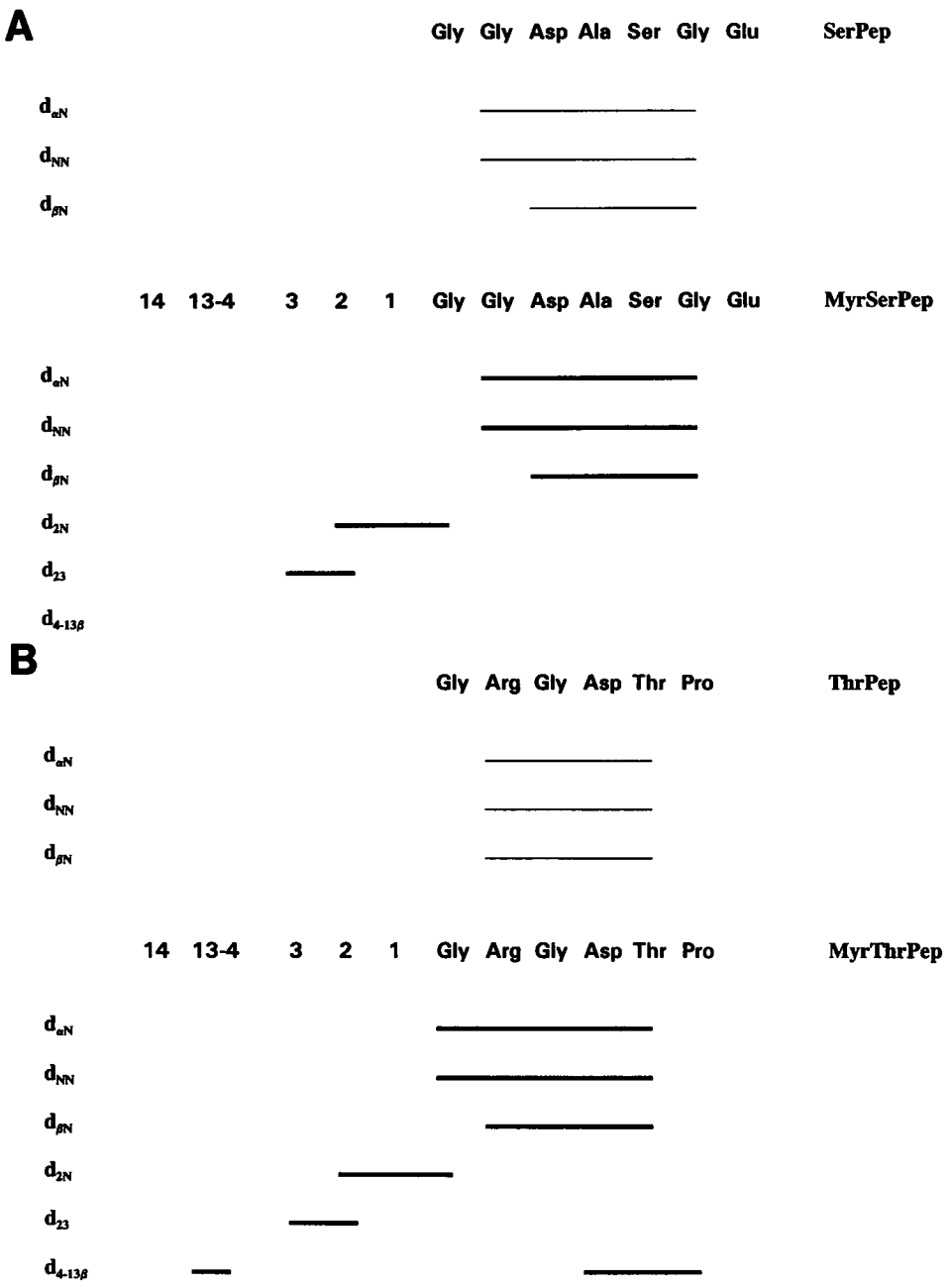

FIGURE 1 Amino acid sequence of (*A*) SerPep and MyrSerPep, and (*B*) ThrPep and MyrThrPep with summary of NOESY cross-peaks. NOESY cross-peaks are indicated by lines connecting the amino acid/amino acid or fatty acid/amino acid residues. Both short-range sequential NOEs ($d_{\alpha N}$, d_{NN}, $d_{\beta N}$, d_{2N}, d_{23}) and the long-range NOE, $d_{4-13\beta}$ are shown. The thin lines correspond to negative cross-peak NOE intensities, and the thick lines correspond to positive cross-peak NOE intensities, both with positive diagonal peaks. See Table 1 for ^1H chemical shifts. The peptide concentration was 10 mM in 90% H_2O/10% D_2O at pH 7.4. The NMR spectra were recorded at 25°C.

50 M^{-1} for ThrPep, and 4012 ± 289 M^{-1} for MyrThrPep. The values for the stoichiometry and binding constant given above include the mean and the SD from the curve-fitting procedure.

ESR spectra of acylated and nonacylated peptides

The interaction of SerPep and ThrPep in N-myristoylated form and nonacylated form with phospholipid bilayers was investigated by ESR spectroscopy. ESR spectra at 30°C and at a 1 mol% concentration of a phospholipid spin label, 16-PCSL, and of a fatty acid spin label, 16-SASL, in DMPC bilayers in the presence and absence of the N-myristoylated peptides, MyrSerPep and MyrThrPep, are shown in Fig. 3, *a*, *b*, *c*, and *e*, respectively. The ESR spectra are characteristic of a nitroxide spin label located in the hydrophobic region of membranes in the liquid crystalline phase.

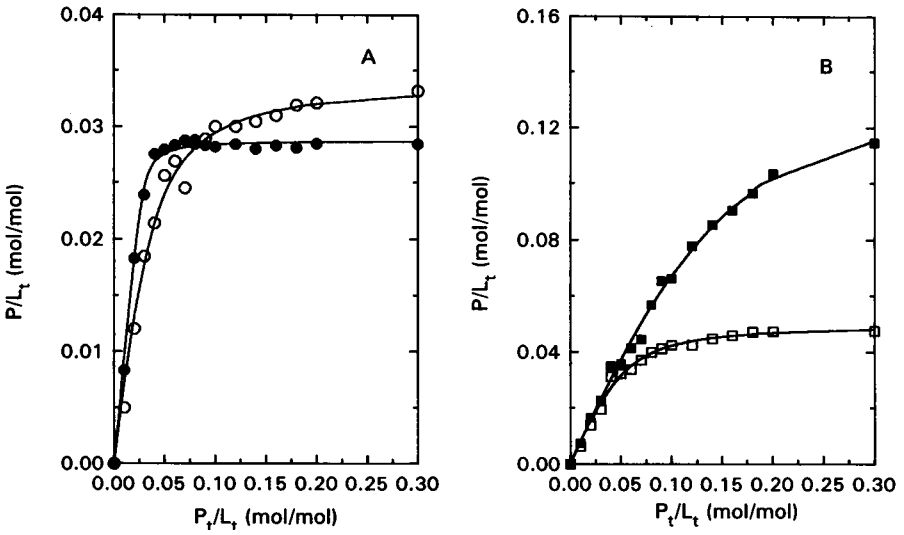

FIGURE 2 Binding of (*A*) SerPep, MyrSerPep, and (*B*) ThrPep and MyrThrPep to DMPC bilayers. The bound peptide:lipid mole ratio, P/L_t, is plotted against the initial peptide:lipid mole ratio, P_t/L_t, for SerPep (○), MyrSerPep (●), ThrPep (□), and MyrThrPep (■). The lines represent simple mass action binding curves. The lipid concentration, L_t, was 13 mM.

ESR spectra at 30°C and at a 1 mol% concentration of the spin-labeled acylated peptides, 16-SteSL-SerPep and 16-SteSL-ThrPep, in DMPC bilayers are also shown in Fig. 3, *d* and *f*, respectively. The spectrum of 16-SteSL-SerPep is similar to that obtained with 16-PCSL or with 16-SASL in DMPC bilayers with and without MyrSerPep and MyrThrPep. In contrast, the spectrum of 16-SteSL-ThrPep is extensively spin-spin-broadened.

^{2}H NMR spectroscopy

Lipid-peptide interactions between DMPC and MyrSerPep or MyrThrPep were also investigated using ^{2}H NMR. The ^{2}H NMR spectrum at 30°C obtained from perdeuterated DMPC, DMPC-d_{54}, bilayers is shown in Fig. 4 *a*. This spectrum is an axially symmetric powder pattern characteristic of lipid bilayers in the liquid crystalline phase. The ^{2}H NMR spectra at 30°C of DMPC-d_{54} in the presence of the N-myristoylated peptides, MyrSerPep and MyrThrPep, are similar in shape and in magnitude of quadrupole splittings to those of peptide-free DMPC-d_{54} bilayers (spectra not shown).

The ^{2}H NMR spectra at 30°C of DMPC bilayers containing the acylated peptides with perdeuterated acyl chains, Myr-d_{27}-SerPep and Myr-d_{27}-Thr-Pep, at a DMPC:peptide mole ratio of ~30:1 are shown in Fig. 4, *b* and *c*, respectively. The spectrum of the DMPC−Myr-d_{27}-SerPep system resembles that of DMPC-d_{54}, indicating that the orientational order of the covalently linked fatty acid in Myr-d_{27}-SerPep is similar to that of the myristoyl chains of the host bilayer lipid, DMPC. The quadrupole splittings of the perdeuterated myristoyl chain in Myr-d_{27}-SerPep (Fig. 4 *b*) are somewhat smaller (up to 20%) than those of the perdeuterated myristoyl chains of DMPC-d_{54}. This might be because the peptide-linked fatty acyl chains are more disordered than those of the host lipids. In contrast, the spectrum of the DMPC-Myr-d_{27}-ThrPep has a complex line shape that may correspond either to the no-motion limit such as would be observed for lipids in a gel phase (Sankaram and Thompson, 1992), or to multiple components.

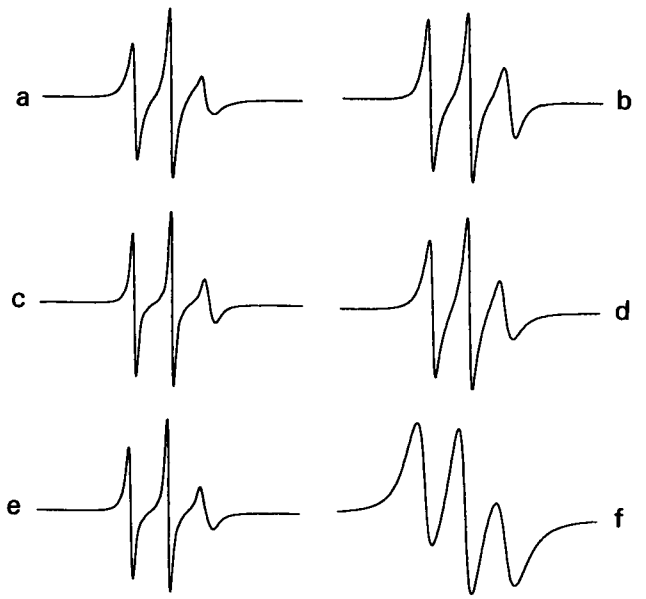

FIGURE 3 ESR spectra of acylated peptides reconstituted into DMPC bilayers. In each case the spin label concentration was 1 mol%. All of the spectra were recorded at 30°C with a modulation amplitude of 1 G and scan range of 100 G. (*a*) 16-PCSL in DMPC bilayers (*b*) 16-SASL in DMPC bilayers, (*c*) 16-PCSL in DMPC bilayers containing MyrSerPep, (*d*) the spin-labeled peptide, 16SteSL-SerPep, in DMPC bilayers, (*e*) 16-PCSL in DMPC bilayers containing MyrThrPep, and (*f*) the spin-labeled peptide, 16SteSL-ThrPep, in DMPC bilayers. The bound lipid:peptide mole ratio in all cases was 100:1.

DISCUSSION

Lipid binding of myristoylated and nonacylated peptides

The analysis of the thermodynamics of peptide binding to membranes must take into account the fact that binding of charged peptides to membranes leads to a negative cooperativity of binding (Cevc and Marsh, 1987; Kim et al., 1991; Mosior and McLaughlin, 1991; Mosior and McLaughlin,

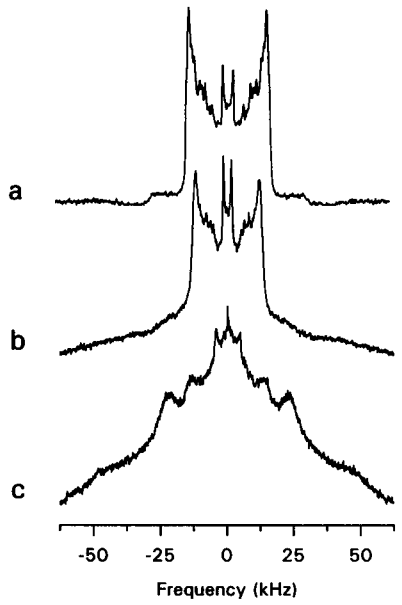

FIGURE 4 ^{2}H NMR spectra of acylated peptides reconstituted into DMPC bilayers. All of the spectra were recorded at 30°C. The bound lipid: peptide mole ratio was ~30:1. (*a*) DMPC-d_{54} bilayers; (*b*) DMPC bilayers containing the SerPep with a covalently linked perdeuterated myristoyl chain; Myr-d_{27}-SerPep; and (*c*) DMPC bilayers containing the ThrPep with a covalently linked perdeuterated myristoyl chain, Myr-d_{27}-ThrPep.

1992; Sankaram and Marsh, 1993). As more peptide is bound, the binding becomes weaker as given by

$$K = K_0 \exp\left(\frac{-Ze\phi}{kT}\right), \quad (1)$$

where K is the effective binding constant, K_0 is the intrinsic binding constant, Z_e is the net effective charge on the peptide, Φ is the surface potential, k is the Boltzmann constant, and T is the absolute temperature. This is because, as more negatively charged peptide is bound to the zwitterionic DMPC bilayers, further binding of the peptide is attenuated by an electrostatic repulsion between the negatively charged peptide-bound membranes and the negatively charged peptide. SerPep, ThrPep, MyrSerPep, and MyrThrPep have a net charge of -2, 0, -3, and -1, respectively. According to Eq. 1, binding of the peptides with a net negative charge to the zwitterionic DMPC bilayers results in a value for K smaller than that for K_0. If the lipid:peptide interactions are entirely electrostatic in nature for the four peptides, then the ratios of ln K for the peptides must correspond to the ratios of the net charges of them (Sankaram and Marsh, 1993). This is not the case because the ln K values are in the ratio (SerPep:ThrPep:MyrSerPep:MyrThrPep) 0.78:0.82:0.80:1.00, whereas the values of Z are in the ratio 2:0:3:1. The fact that the ln K values are nearly the same for SerPep ($Z = -2$) and ThrPep ($Z = 0$) renders invalid the assumption that the intrinsic binding constant, K_0, is the same for the two peptides.

If it is assumed that Eq. 1 holds for all the four peptides, then the observation that the ratios of the ln K values do not follow the ratios of Z for the four peptides suggests either that the intrinsic binding constant, K_0, is not the same for the four peptides or that the interaction among SerPep, ThrPep, MyrSerPep, and MyrThrPep and lipid bilayers is not entirely electrostatic in nature. It is also possible that the acylated and nonacylated peptides all have different K_0 values and that the interaction with the membrane contains nonelectrostatic components. The nonelectrostatic component might arise for the acylated peptides from interaction of the covalently linked myristoyl moiety with DMPC bilayers and/or with the peptides. An examination of both the lipid:peptide stoichiometry at saturation binding, n, measured for the peptides and the calculated binding constants, K, strongly argues in favor of this hypothesis. SerPep has a value for n of 20 and a binding constant of 3058 M^{-1}. The value of n for the N-myristoylated peptide, MyrSerPep, is 34, whereas the binding constant is increased to 30,200 M^{-1}. The increase in K upon myristoylation is most likely due to the interaction of the covalently linked myristoyl chain with the myristoyl chains of DMPC, whereas the increase in n is due to an increase in the net negative charge. In contrast, the binding constant, K, of 4715 M^{-1} for ThrPep is decreased to 4012 M^{-1} upon myristoylation, whereas n is decreased from a value of 20 for ThrPep to 7 for MyrThrPep. The small decrease in K is probably due to the increased net negative charge upon myristoylation. Because the binding constant is not increased upon myristoylation of the ThrPep, it is unlikely that the covalently linked myristoyl chains interact with the DMPC host membrane. The concomitant decrease in n indicates that the MyrThrPep may be bound to DMPC bilayers in an aggregated form.

Solution conformations of N-myristoylated and nonacylated peptides

The results of the NOESY experiments shown in Fig. 1 reveal three different effects of N-myristoylation on peptide structure and conformation in solution. First, values for $^3J_{N\alpha}$ larger than 8 Hz indicate that the peptides, whether N-myristoylated or not, do not have a stable folded conformation. This result is expected because the peptides are too short to adopt an α-helical or other well defined secondary structure. Second, the sign of the NOE is changed upon N-myristoylation of both SerPep and ThrPep. For SerPep and ThrPep, the NOE cross-peaks are negative with positive diagonal peaks. This result reflects the small size of the peptides. In this case, the relaxation is in the extreme motional narrowing regime, and the condition that $\omega_0\tau_c \ll 1$ (ω_0 is spectrometer frequency that at 500 MHz is $3 \cdot 10^{-11}$ s^{-1}; τ_c is the correlation time) is satisfied. However, for MyrSerPep and MyrThrPep the cross-peaks are positive with positive diagonal peaks. Thus, N-myristoylation results in an aggregation of both peptides. The third effect of N-myristoylation is on the relative orientation of the myristoyl chain with respect to the amino acid residues in MyrSerPep and MyrThrPep. For MyrSerPep, no long-range NOEs were observed. As seen in Fig. 1 for MyrThrPep, NOEs were observed between the methylene protons of the fatty acid and the side-chain

protons of Thr and Pro. The average distance between the sets of protons between which the long-range NOEs were observed is known to be less than about 5 Å (Wüthrich, 1986).

The 2D NMR results suggest that the nonacylated peptides, SerPep and ThrPep, are unstructured monomers in solution, whereas the acylated peptides MyrSerPep and MyrThrPep are aggregated in solution. Although both MyrSerPep and MyrThrPep are aggregated in solution, the structures for the aggregates of the two peptides must be different. No NOE evidence for the presence of local structure was found for MyrSerPep. A possible structure for the aggregate, shown in Fig. 5 *a*, is similar to that of a micelle. On the other hand, the individual MyrThrPep molecules in the soluble aggregate have a local structure in which the acyl chain is close to the amino acid residues. As shown in Fig. 5 *b* for MyrThrPep, a possible result of placing the myristoyl chain within a 5-Å distance of the uncharged residues Thr and Pro is that the side chains of the charged residues Arg and Asp are located away from the hydrophobic myristoyl chain. This arrangement leads to an amphipathic structure for the aggregate that is stabilized by a hydrophobic core formed by the myristoyl chains (see Fig. 5 *b*).

Lipid-peptide interactions with acylated peptides

The ESR and ^{2}H NMR results presented in Figs. 3 and 4 show that N-myristoylation leads to different modes of lipid-peptide interactions for the two peptides. The ESR spectra of nitroxide-derivatives of phospholipids and fatty acids in DMPC membranes in the presence and absence of 16-SteSL-SerPep are characteristic of freely diffusing probes in membranes. The ^{2}H NMR results obtained with Myr-d_{27}-SerPep (Fig. 4 *b*) confirm these results. The spectrum of this peptide is axially symmetric, indicating that a fluid environment similar to the host lipid bilayer. The quadrupole splittings for this peptide are about 20% smaller than those for the host lipids at the same temperature, presumably because of a deeper vertical bilayer location of the covalently linked fatty acid than that of the fatty acid chains of the host lipid. These observations suggest that N-myristoylation most likely results in membrane binding of MyrSerPep by an "anchoring" mechanism, in which the covalently linked fatty acid is directly inserted into the lipid bilayer (see Fig. 5 *c*).

For 16-SteSL-ThrPep, the ESR spectral line-broadening suggests the formation of peptide aggregates that bind to membranes. The ^{2}H NMR data on Myr-d_{27}-ThrPep (Fig. 4 *c*) show that the covalently linked myristoyl chain in this peptide does not undergo the gel-fluid phase transition at the same temperature as the myristoyl chains of the host lipid, DMPC, membranes. The covalently linked perdeuterated myristoyl chain of Myr-d_{27}-ThrPep, when bound to DMPC membranes, exhibits a gel-fluid transition at 35°C, whereas in DMPC-d_{54}-MyrThrPep complexes, the perdeuterated myristoyl chains of DMPC-d_{54} melt at 23°C (results not shown).

These observations indicate that membrane binding in this case occurs between an aggregate of the peptide and is mediated by a direct contact between the amino acid residues and phospholipid headgroups. In this model, shown in Fig. 5 *d*, the covalently linked myristoyl chains are not in direct contact with the host lipid myristoyl chains. Thus, membrane binding is not accompanied by an anchoring of the fatty acyl chains. The size of the complexes, i.e., the number of interacting monomeric units, and the geometrical arrangement of the units shown in Fig. 5 is not obtainable from the present work. The number of peptides per aggregate and the shape of the aggregate used to draw this figure are only for illustrative purposes. It should be emphasized that these are simple models that are consistent with the spectroscopic data.

The aggregation state of amphipathic molecules, such as MyrSerPep and MyrThrPep, is expected to depend on pep-

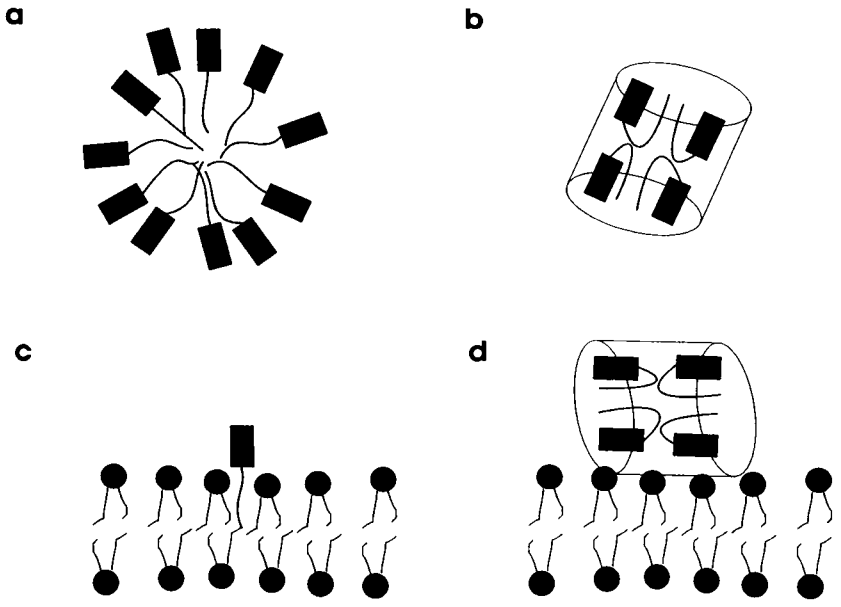

FIGURE 5 A schematic illustration of the possible structures for SerPep, ThrPep, MyrSerPep, and MyrThrPep in solution and when bound to membranes. (*a*) MyrSerPep in solution; (*b*) MyrThrPep in solution; (*c*) MyrSerPep bound to membranes by a direct insertion of the myristoyl chain into the lipid bilayer; and (*d*) MyrThrPep bound to membranes by lipid-protein interaction. The number of peptides per aggregate and the shape of the aggregate used to draw this figure are only for illustrative purposes.

tide concentration. Because the peptide concentration was 10 mM for 2D NMR experiments, MyrSerPep and MyrThrPep must be aggregated at concentrations above 10 mM. However, because the binding curves obtained for MyrSerPep and MyrThrPep (at a phospholipid concentration of 13 mM and in the peptide concentration range 0.1–4 mM) are not biphasic, it is likely that these peptides are aggregated at concentrations above 0.13 mM.

Based on these results, the origin of the effect of N-myristoylation on peptide aggregation in solution and on membrane binding cannot be attributed to the presence of Ser at position 5. Thus, this highly conserved residue is probably required for recognition by N-myristoyl transferase and not for regulating the biological function of N-myristoylated proteins.

It is demonstrated here that binding of N-myristoylated peptides to membranes is not always by a membrane-anchoring mechanism. The present data provide evidence for membrane-anchoring (direct insertion of the covalently linked fatty acid into the lipid bilayer) and for peptide-peptide association (no direct contact between the covalently linked fatty acid and the acyl chains of the host lipids). Specifically, these results suggest that in biological systems N-myristoylation can lead to protein-protein association if the N-terminal sequence adopts a conformation in which the covalently linked myristic acid lies close to the neutral or hydrophobic face of the structure with the charged residues residing on the apposing face. If N-myristoylation of a cytosolic protein does not result in protein-protein association, the protein is most likely to be anchored to a membrane. If protein-protein association does occur, membrane-binding is expected to be determined by the energetics of solvation versus lipid-protein interactions of the protein aggregate. It should be mentioned that the choice of short peptides for this study has proven useful to differentiate between the two modes of interaction, while ignoring contributions from protein conformational changes. The logical step now is to carry out this study systematically with proteins or larger peptides with the aim of determining the effect of N-myristoylation on secondary structure.

I am grateful to Professor Thomas E. Thompson for his encouragement and support in carrying out this work. In addition, I gratefully acknowledge the expert technical assistance with the NMR experiments by Dr. Jeff Ellena and Dr. Laurie Kelsh, and the access to the magnetic resonance equipment provided by the Department of Chemistry, University of Virginia. I would also like to thank Professor Stuart McLaughlin for helpful discussions on peptide-lipid binding.

REFERENCES

Bax, A. 1985. A spatially selective composite 90° radiofrequency pulse. *J. Magn. Res.* 65:142–148.

Buss, J. E., S. M. Mumby, P. J. Casey, A. G. Gilman, and B. M. Sefton. 1987. Myristoylated alpha subunits of guanine nucleotide-binding regulatory proteins. *Proc. Natl. Acad. Sci. USA.* 84:7493–7497.

Cevc, G., and D. Marsh. 1987. Phospholipid Bilayers: Physical Principles and Models. Wiley-Interscience, New York. 14–42.

Davis, J. H., K. R. Jeffrey, M. Bloom, M. I. Valic, and T. P. Higgs. 1976. Quadrupolar echo deuteron magnetic resonance spectroscopy in ordered hydrocarbon chains. *Chem. Phys. Lett.* 42:390–394.

Eibl, H., and W. E. M. Lands. 1969. A new, sensitive determination of phosphate. *Anal. Biochem.* 30:51–57.

Gordon, J. I., R. J. Duronio, D. A. Rudnick, S. P. Adams, and G. W. Gokel. 1991. Protein N-myristoylation. *J. Biol. Chem.* 266:8647–8650.

Hope, M. J., M. B. Baley, G. Webb, and P. R. Cullis. 1985. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta.* 812:55–65.

Hughes, P. J., D. M. Evans, P. D. Minor, G. C. Schild, J. W. Almond, and G. Stanway. 1986. The nucleotide sequence of a type 3 poliovirus isolated during a recent outbreak of poliomyelitis in Finland. *J. Gen. Virol.* 67: 2093–2102.

Kim, J., M. Mosior, L. A. Chung, H. Wu, and S. McLaughlin. 1991. Binding of peptides with basic residues to membranes containing acidic phospholipids. *Biophys. J.* 60:135–148.

Konig, W., and R. Geiger. 1970. Eine neue Methode zur Synthese von Peptiden: Aktivierung der Carboxylgruppe mit Dicyclohexylcarbodiimid unter Zusatz von 1-Hydroxy-benzotriazolen. *Chem. Ber.* 103:788–798.

Lowry, O. H., N. J. Rosebrough, A. C. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

Mosior, M., and S. McLaughlin. 1991. Peptides that mimic the pseudosubstrate region of protein kinase C bind to acidic lipids in membranes. *Biophys. J.* 60:149–159.

Mosior, M., and S. McLaughlin. 1992. Binding of basic peptides to acidic lipids in membranes: effects of inserting alanine(s) between the basic residues. *Biochemistry.* 31:1767–1773.

Pellman, D., E. A. Garber, F. R. Cross, and H. Hanafusa. 1985. Fine structural mapping of a critical NH_2-terminal region of $p60^{src}$. *Proc. Natl. Acad. Sci. USA.* 82:1623–1627.

Sankaram, M. B., and D. Marsh. 1993. Protein-lipid interactions with peripheral membrane proteins. *In* Protein-Lipid Interactions. A. Watts, editor. Elsevier, Amsterdam. 127–162.

Sankaram, M. B., and T. E. Thompson. 1990a. Interaction of cholesterol with glycerophospholipids and sphingomyelin. *Biochemistry.* 29: 10670–10675.

Sankaram, M. B., and T. E. Thompson. 1990b. Modulation of phospholipid acyl chain order by cholesterol. A solid-state ^2H NMR study. *Biochemistry.* 29:10676–10684.

Sankaram, M. B., and T. E. Thompson. 1992. Deuterium magnetic resonance study of phase equilibria and membrane thickness in binary phospholipid mixed bilayers. *Biochemistry.* 31:8258–8268.

Schmidt, M. F. G. 1989. Fatty acylation of proteins. *Biochim. Biophys. Acta.* 988:411–426.

Schultz, A. M., L. E. Henderson, and S. Oroszlan. 1988. Fatty acylation of proteins. *Annu. Rev. Cell Biol.* 4:611–647.

Tanaba, T., T. Nukada, Y. Nishikawa, K. Sugimoto, H. Suzuki, H. Takahashi, M. Noda, T. Haga, A. Ichiyama, K. Kangawa, N. Minamino, H. Matsuo, and S. Numa. 1985. Primary structure of the A-subunit of transducin and its relationship to ras proteins. *Nature.* 315:242–245.

Towler, D. A., S. P. Adams, S. R. Eubanks, D. S. Towery, E. Jackson-Machelski, L. Glaser, and J. I. Gordon. 1988. Myristoyl CoA: protein N-myristoyltransferase activities from rat liver and yeast possess overlapping yet distinct peptide substrate specificities. *J. Biol. Chem.* 263: 1784–1790.

Towler, D. A., S. R. Eubanks, D. S. Towery, S. P. Adams, and L. Glaser. 1987. Amino-terminal processing of proteins by N-myristoylation. Substrate specificity of N-myristoyl transferase. *J. Biol. Chem.* 262: 1030–1036.

Towler, D. A., J. I. Gordon, S. P. Adams, and L. Glaser. 1988. The biology and enzymology of eukaryotic protein acylation. *Annu. Rev. Biochem.* 57:69–99.

van Beveren, C., F. van Straaten, J. A. Galleshaw, and I. M. Verma. 1981. Nucleotide sequence of the genome of a murine sarcoma virus. *Cell.* 27:97–108.

Wüthrich, K. 1986. NMR of Proteins and Nucleic Acids. Wiley-Interscience, New York. 1–447.