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Specificity of Membrane Binding of the Neuronal Protein NAP-22

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Abstract. NAP-22, a major protein of neuronal rafts is known to preferentially bind to membranes containing cholesterol. In this work we establish the requirements for membrane binding of NAP-22. We find that other sterols can replace cholesterol to promote binding. In addition, bilayers containing phosphatidylethanolamine bind NAP-22 in the absence of cholesterol. Thus, there is not a specific interaction of NAP-22 with cholesterol that determines its binding to membranes. Addition of a mol fraction of phosphatidylserine of 0.05 to membranes of phosphatidylcholine and cholesterol enhances the membrane binding of NAP-22. The dependence of binding on the mol fraction of phosphatidylserine indicates that NAP-22 binds to membranes with its amino-terminal segment closer to the membrane than the remainder of the protein. We have also determined which segments of NAP-22 are required for membrane binding. A non-myristoylated form binds only weakly to membranes. Truncating the protein from 226 amino acids to the myristoylated amino-terminal 60 amino acids does not prevent binding to membranes in a cholesterol-dependent manner, but this binding is of weaker affinity. However, myristoylation is not sufficient to promote binding to cholesterol-rich domains. An N-terminal 19-amino-acid, myristoylated peptide binds to membranes but without requiring specific lipids. Thus, the remainder of the protein contributes to the lipid specificity of the membrane binding of NAP-22.

Key words: NAP-22 — Neuronal membranes — Rafts — Lipid-protein interaction — Cholesterol — Membrane binding

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Introduction

NAP-22, localized in the synapse (Iino, Kobayashi & Maekawa, 1999), is a major component of the detergent-insoluble, low-density fraction from rat brain, the so-called "raft" fraction. Almost all of the NAP-22 present in the brain is found in the insoluble membrane fraction. The protein can only be solubilized from the membrane with chloroform/methanol or butanol. A protein with a high sequence homology and likely with very similar properties, CAP-23, was first identified by Widmer and Caroni (1990). Along with GAP-43 and MARCKS, CAP-23 is thought to regulate cell cortex actin dynamics (Laux et al., 2000). NAP-22 is enriched in the growth cone, a specialized neurological structure present at the tip of an extended neurite (Maekawa et al., 1993) and is thought to share a unique role with GAP-43 in neurite outgrowth and anatomical plasticity (Frey et al., 2000). The shared roles of GAP-43 and NAP-22 are indicated by the observation that GAP-43 can replace the function of NAP-22 in the knock-out mouse (Frey et al. 2000). Coexpression of GAP-43 and CAP-23 can promote a 60-fold increase in the regeneration of dorsal root ganglion axons in adult mice after spinal cord injury in vivo (Bomze et al., 2001). Thus, NAP-22 has important biological properties as well as being of interest as a major protein component of neuronal rafts.

NAP-22 is a highly acidic protein with a calculated pI of 4.69. The protein is very polar and has no hydrophobic segments, apart from the N-terminal myristoylation (Mosevitsky et al., 1997) that would be expected to insert into a membrane. Although N-terminally acylated proteins are often found in cholesterol and sphingolipid-enriched membrane domains, protein-protein interactions are required to localize a given myristoylated protein to detergent-resistant membranes or caveolin-rich membranes (McCabe & Berthiaume, 2001). However, in lipo-

Table 1. Specificity of membrane binding of NAP-22 to sterol structure

Chemical name	Common name	% NAP-22 Bound[a]
5-Cholesten-3α-ol	Epicholesterol	34 ± 9
5α-Cholestan-3β-ol	Cholestanol	90 ± 16
5,7,22-Cholestatrien-24β-methyl-3β-ol	Ergosterol	87 ± 13
5,22-Cholestadien-24β-ethyl-3β-ol	Stigmasterol	100 ± 12
5α-Pregnan-3β-ol	–	90 ± 15
2,(5α)-Androsten-17β-ol	Androstenol	50 ± 10

[a] The % of protein bound from a mixture of 400:1 lipid to protein molar ratio.

somes devoid of proteins, NAP-22 still binds in a cholesterol-dependent manner (Epand et al., 2001; Maekawa et al., 1999). In the present study we wish to define the requirements for the membrane partitioning of NAP-22, both with regard to the lipid requirements as well as the segments of the protein that facilitate membrane translocation.

Materials and Methods

Materials

NAP-22 was isolated from the brains of two-week old rats, as previously described (Maekawa et al., 1999). A non-myristoylated form of NAP-22 was obtained from an *E. coli* expression system and was purified as previously described (Terashita et al., 2002). A 60-residue, C-terminal deletion mutant of NAP-22 was also obtained from an *E. coli* expression system (Imai et al., 1991). The N-terminal methionine was then cut and a myristate was added to the N-terminal glycine by coexpression with N-myristoyl transferase (Duronio et al., 1990) as previously described (Terashita et al., 2002) to give dNAP60. A synthetic lipopeptide with the structure: Myristoyl-GGKLSKKKKGYNVNDEKAK-amide, corresponding to the 19 amino-terminal residues of NAP-22, was purchased from BioSource International (Hopkinton, MA). Phospholipids and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Other sterols were purchased from Steraloids (Newport, RI).

Incorporation of NAP-22 into Multilamellar Vesicles (MLV)

Lipids were dissolved in chloroform/methanol (2/1) and the lipid deposited on the walls of a glass test tube by solvent evaporation with a stream of nitrogen gas. Last traces of solvent were then removed by evaporation for two hours under vacuum. NAP-22 was incorporated into the membrane by hydrating lipid films with a solution of protein in 10 mM HEPES, 0.14 M NaCl, 1 mM EDTA pH 7.4 buffer. The final protein or peptide concentration was 1–2 μM and that of the lipid adjusted to give a final lipid to protein ratio between about 50 and 1000. The samples were subjected to three cycles of freezing and thawing. All lipid-protein mixtures, as well as the controls, were then incubated for 60 minutes at 37°C. Lipid controls were prepared in the same manner but in the absence of protein.

Centrifugation Assay for Membrane Binding of Peptides and Proteins

Membrane binding of NAP-22 was determined as previously described (Epand et al., 2001). The vesicles with bound protein were pelleted by centrifugation at 200,000 ×*g* for 90 minutes in silanized polycarbonate microcentrifuge tubes at 23°C. The supernatants were removed and assayed for protein and lipid.

Determination of Phospholipid Concentration

The concentration of phospholipid was determined by measuring the amount of inorganic phosphate released after digestion by the method of Ames (1966).

Determination of the Concentration of Protein

The concentration of NAP-22 was determined by the CBQCA assay (Molecular Probes) and by a modified micro-Bradford assay.

Differential Scanning Calorimetry (DSC)

Multilamellar suspensions of lipid at a concentration of 2.3 mg/mL were prepared by hydrating dry lipid films with either pure buffer (20 mM Pipes, 0.14 M NaCl, 1 mM EDTA, 20 mg/L NaN_3) or with a solution of NAP-22 in the same buffer. Suspensions were degassed under vacuum before being loaded into a NanoCal high-sensitivity calorimeter (CSC, American Forks, UT). Both heating and cooling DSC scans were run at a rate of 2°C/min.

Abbreviations

NAP-22, Neuronal axonal membrane protein, also referred to as brain acid soluble protein 1 (BASP1 protein), a 22 kDa myristoylated protein; dNAP60, a myristoylated 60-amino-acid peptide corresponding to NAP-22 truncated from the carboxyl end; DSC, differential scanning calorimetry; MLV, multilamellar vesicles; LUV, large unilamellar vesicles; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PO, 1-palmitoyl-2-oleoyl; DO, dioleoyl; SO, 1-stearoyl-2-oleoyl.

Results

The binding of NAP-22 to membranes of DOPC containing 40% sterol was studied to determine the specificity of the binding to sterol structure. The results are summarized in Table 1. Cholesterol is not unique in being able to bind NAP-22, but there is some specificity with regard to the structure of the sterol. For example, the chemically related isomer, epicholesterol, promotes less binding than cholesterol that binds essentially all of the NAP-22 at this mol fraction (*see* Fig. 2). Androstenol also promotes some

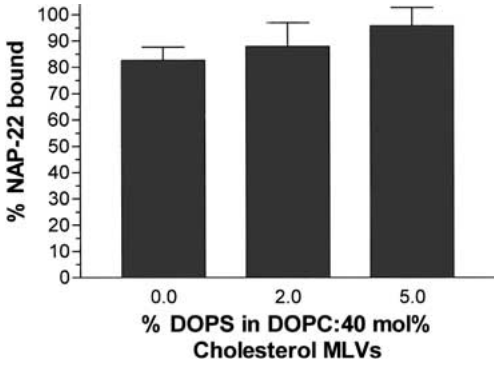

Fig. 1. Binding of NAP-22 to MLV of DOPC with 40% cholesterol containing the indicated mol% of DOPS. NAP-22 concentration 1 μM at a lipid to protein ratio of 125. Error bars are SEM of experiments repeated three times; $p \leq 0.05$ between 0 and 5% DOPS.

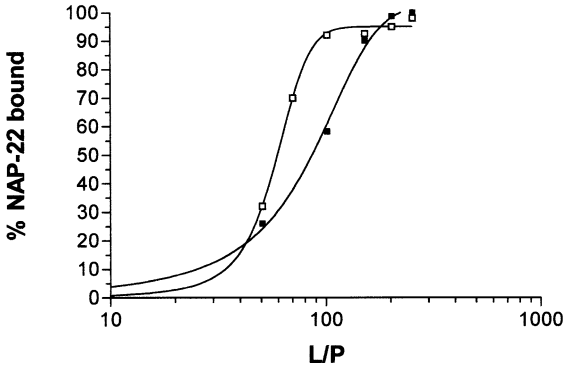

Fig. 2. Binding isotherm for the NAP-22 binding to MLV of DOPC with 40% cholesterol (■) or with the addition of 5 mol% DOPS (□). NAP-22 concentration 1 μM. L/P is lipid to protein molar ratio. Representative binding curves of experiments repeated twice with similar results.

membrane binding of NAP-22 compared with pure DOPC, although it promotes less binding than most of the other sterols. Androstenol is structurally more different from cholesterol than the other sterols and it does not partition into domains in model membranes (Waarts, Bittman & Wilschut, 2002).

This protein does not bind to LUV of PS, even in the presence of high mol fractions of cholesterol (Epand et al., 2001). NAP-22 has a calculated isoelectric point of 4.69. Hence at neutral pH the protein is highly negatively charged and would therefore be repelled from the surface of an anionic lipid such as PS. However, the addition of a mol fraction of 0.05 DOPS to LUV of DOPC with 0.4 mol fraction of cholesterol results in an enhancement of the binding affinity (Fig. 1). The amount of unbound protein was measured in these experiments, so that the differences with and without DOPS were significant and beyond experimental error. This is also shown by the shift of the binding isotherm for NAP-22 to higher affinity binding in the presence of 5% DOPS (Fig. 2).

The other major lipid component of the cytoplasmic leaflet of a mammalian plasma membrane is phosphatidylethanolamine (PE). An equimolar mixture of DOPE with DOPC binds NAP-22 in the absence of cholesterol with an affinity comparable to that of the protein binding to DOPC containing 40% cholesterol (Fig. 3). Therefore, although NAP-22 is found in the raft fraction of neuronal membranes and in vitro this protein binds to DOPC containing 40% cholesterol but not to liposomes of pure DOPC, there is not a specific recognition of cholesterol by this protein. The results suggest that the membrane translocation of NAP-22 is dependent on some physical property of the membrane and not on a specific chemical structure. One possibility is that a membrane with a more hydrophobic interface will facilitate the binding of NAP-22.

We have previously shown that the phase transition behavior of lipid mixtures of PC and cholesterol are affected in a manner that suggests the formation of cholesterol-rich domains (Epand et al., 2001). However, when the DSC of an equimolar mixture of POPE and POPC was measured, we found that the phase transition characteristics were not affected by NAP-22 at a 500:1 lipid to protein ratio (Fig. 4), unlike the case of PC with cholesterol (Epand et al., 2001).

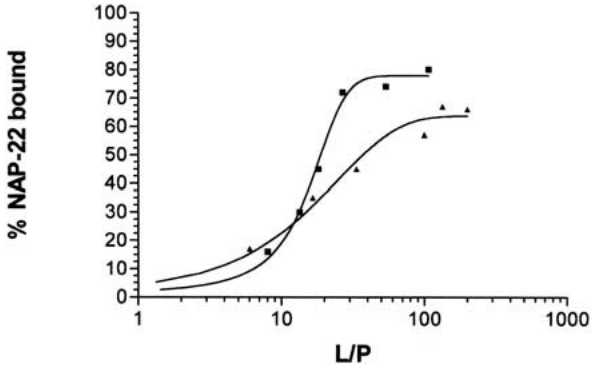

Fig. 3. Binding isotherm for the binding of NAP-22 to MLV of DOPC:DOPE (1:1) (■) or with the addition of 40 mol% cholesterol (▲). NAP-22 concentration 1 μM. L/P is lipid to protein molar ratio. Representative binding curves of experiments repeated twice with similar results.

We also investigated which features of NAP-22 conferred lipid binding properties to the protein. NAP-22 can be produced in *E. coli* in a non-lipidated form that we shall refer to as apo-NAP-22. Apo-NAP-22 binds very weakly to DOPC with a mol fraction of 0.4 cholesterol, but addition of DOPS enhances the binding of apo-NAP-22 (Fig. 5). Thus, the presence of a myristoyl group appears to be important for the partitioning of NAP-22 to a membrane. This is in accord with the estimated energy of membrane binding that correlated well with the

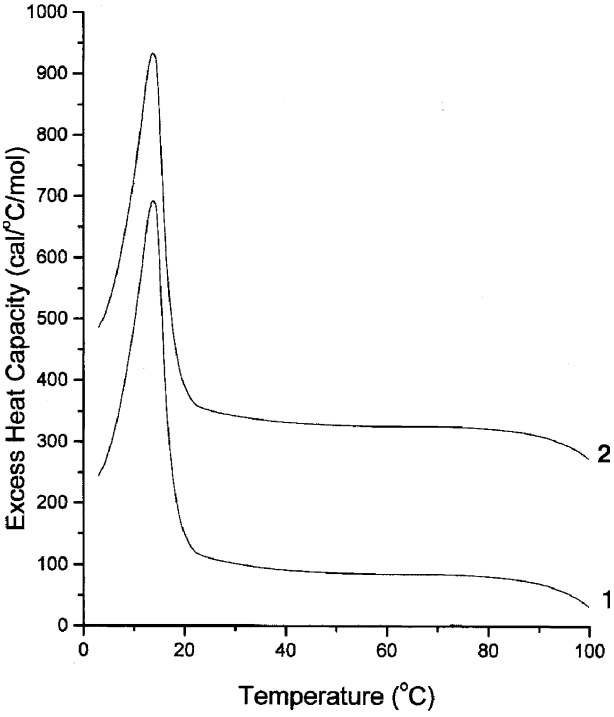

Fig. 4. Differential scanning calorimetry (DSC) of 1:1 POPC:POPE alone (curve *1*) or with the addition of NAP-22 at a lipid to protein molar ratio of 500 (curve *2*). Heat capacity calculated per mole of average phospholipid. Heating scan rate 2°C/min.

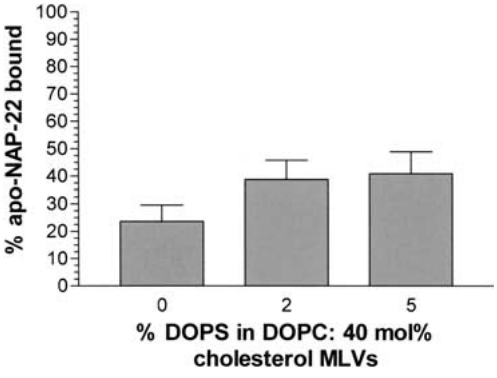

Fig. 5. Effect of DOPS on the binding of apo-NAP-22 to MLV of DOPC with 40% cholesterol. Apo-NAP-22 concentration 1.5 μM at a lipid to protein ratio of 200. Error bars are SEM of experiments repeated three times. $p \leq 0.05$ between 0 and 5% DOPS.

insertion of a myristoyl group into the membrane (Epand et al., 2001). However, other phospholipids, including low mol fractions of PS as well as PE, will also augment the binding of this protein to membranes.

A synthetic myristoylated, 19-amino-acid peptide binds equally well to DOPC as to DOPC with a mol fraction of 0.4 cholesterol (Fig. 6). Even the addition

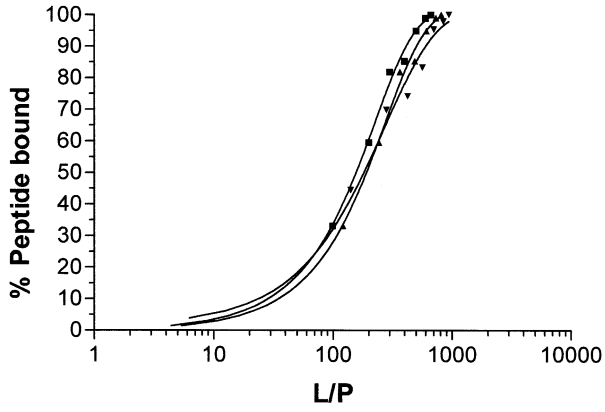

Fig. 6. Binding isotherm for the binding of the N-terminal 19-amino-acid myristoylated peptide to MLV of DOPC (▲); DOPC with the addition of 40 mol% cholesterol (▼); DOPC:DOPS:cholesterol (5:1:4) (■). Peptide concentration, 1.5 μM. L/P is lipid to protein molar ratio. Representative binding curves of experiments repeated twice with similar results.

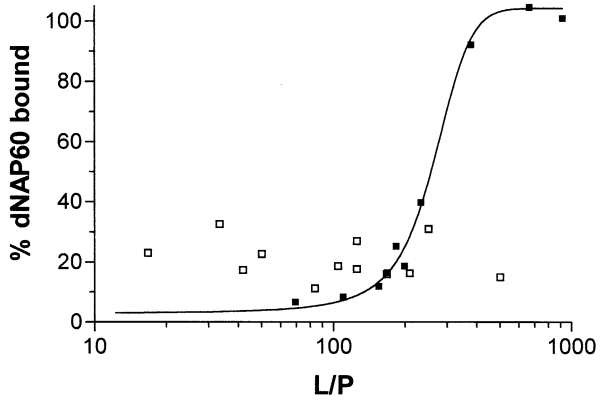

Fig. 7. Binding isotherm for the binding of dNAP60 to MLV of DOPC (□) and DOPC with the addition of 40 mol% cholesterol (■). dNAP60 concentration, 1 μM. L/P is lipid to protein molar ratio. Representative binding curves of experiments repeated twice with similar results.

of DOPS at a mol fraction of 0.1 did not increase the binding of this cationic lipopeptide (Fig. 6). The expected enhancement of binding affinity due to the electrical double layer effects was not detectable. In addition, the 60-residue truncated form of NAP-22 (dNAP60) also partitioned into membranes of DOPC with 40% cholesterol (Fig. 7), but with a lower affinity than the full-length protein. In addition, neither dNAP60 nor the 19-amino-acid myristoylated peptide is capable of sequestering cholesterol to a sufficient extent as to allow the observation of cholesterol transitions in DSC from mixtures of SOPC containing a mol fraction of 0.4 cholesterol (*results not shown*), as has been observed with NAP-22 and this lipid mixture (Epand et al., 2001).

Discussion

NAP-22 is a membrane protein that is found in the detergent-insoluble, low-density fraction from rat brain. It has been shown by fluorescence microscopy that this protein binds to the plasma membrane of COS-7 cells giving a punctate pattern (Terashita et al., 2002). The characteristics of the binding of NAP-22 to cell membranes suggest that it binds to cholesterol-rich domains on the cytoplasmic leaflet of the plasma membrane. NAP-22 is dissociated from the membrane by extraction of cholesterol with methyl-cyclodextrin (Maekawa et al., 1999). It also binds to liposomes in a cholesterol-dependent manner (Epand et al., 2001; Maekawa et al., 1999). However, in this work we show that NAP-22 also binds to membranes containing PE in the absence of cholesterol and anionic lipids augment binding to cholesterol-rich membranes. PE and PS are major lipid components of the cytoplasmic face of the plasma membrane and they may contribute to the partitioning of the protein to a membrane. It has been suggested that single myristoylated proteins require interactions with other membrane proteins in order to be localized in rafts (McCabe & Berthiaume, 2001). Studies of the binding of NAP-22 to model liposomes provide information in a controlled system of limited complexity of the factors and mechanisms involved in the translocation of this protein to membranes. The results are also suggestive of what components of a biological membrane modulate membrane binding of NAP-22, although there are of course possible additional factors in vivo. The present study indicates that in addition to proteins, membrane phospholipid components can also affect the affinity for particular membrane domains. Our results provide additional understanding of the lipid requirements in vitro for the portioning of NAP-22 into membranes. We show that membrane binding can occur in the absence of cholesterol.

An anionic lipid that is found in cholesterol-rich domains of biological membranes is phosphatidylinositol diphosphate (Bodin et al., 2001; Rozelle et al., 2000). This lipid appears to colocalize with NAP-22 in cell membranes (Terashita et al., 2002). Thus, phosphatidylinositol diphosphate may enhance the affinity of NAP-22 for cholesterol-rich domains on the cytoplasmic surface of the plasma membrane. Not only could this electrostatic interaction contribute to the binding of NAP-22, but it will also result in the sequestering of phosphatidylinositol diphosphate into domains with NAP-22 and cholesterol. It has been suggested that the related protein, MARCKS, also sequesters phosphatidylinositol diphosphate into domains that may have important signaling functions (McLaughlin et al., 2002). The promotion of membrane binding of NAP-22 by low mol fractions of anionic lipids together with the observation that this protein is unable to bind to membranes that are composed largely of anionic lipids, suggests that the amino terminus of NAP-22 containing a cluster of cationic residues (Laux et al., 2000), must be juxtaposed close to the membrane interface. This is because the attractive interaction between the peptide cationic cluster and the anionic membrane dominates when the Debye length is short, i.e., at low mol fractions of anionic lipid. This model of membrane binding of NAP-22 fits in well with the requirement for insertion of the myristoyl group into the membrane, which is also at the amino terminus of the protein.

NAP-22 not only sequesters into cholesterol-rich membranes, but there is also evidence that it promotes the segregation of cholesterol into domains (Epand et al., 2001). In contrast, NAP-22 does not alter the thermotropic behavior of lipid mixtures of PC and PE (Fig. 4), indicating that it does not cause lateral phase separation with these lipid mixtures. It is therefore unlikely that PE would be responsible for the punctate pattern of distribution of NAP-22 found in COS-7 cell membranes (Terashita et al., 2002).

The interaction of NAP-22 with membranes containing sterols is not specific for cholesterol. Other sterols can substitute for cholesterol, some with comparable potency to cholesterol itself (Table 1). Even androstenol, which has a significantly different structure, can partially substitute for cholesterol. Androstenol does not partition into cholesterol-rich regions in biological membranes but it does allow for the sterol-dependent fusion of Semliki Forest virus and Sindbis virus (Waarts, Bittman & Wilschut, 2002). This again indicates that there is not a specific interaction between NAP-22 and cholesterol that can explain the binding of the protein only to cholesterol-rich liposomes (Epand et al., 2001; Maekawa et al., 1999).

Myristoylation is required for the partitioning of NAP-22 into membranes and a non-myristoylated form of the protein, apo-NAP-22, binds weakly to membranes either in the presence or in the absence of cholesterol. However, myristoylation by itself does not completely explain the specificity of NAP-22 of binding only to liposomes of certain lipid compositions. Thus, the myristoylated 19-amino-acid peptide, corresponding to the amino terminus of NAP-22, binds to membranes but does not show selectivity for liposomes containing cholesterol (Fig. 6). The 60-amino-acid myristoylated form, dNAP60, exhibits specificity for cholesterol-containing liposomes, but does not bind to membranes with as high an affinity as does NAP-22 (*compare* Figs. 2 and 7). The shorter myristoylated peptides may not exhibit as great a lipid-specificity for binding to liposomes because most of the energy for binding is required to overcome the loss of entropy resulting from transferring the peptide from solution to the smaller volume of the membrane phase. Nevertheless, it is possible that

these lipopeptides, once partitioned into the membrane, would be found at higher concentration in certain domains, such as cholesterol-rich domains. If such a lipid preference exists for the 19-amino-acid peptide, it does not modify the distribution of lipids in a mixed bilayer to an extent sufficient to affect their phase transition properties, as has been observed with the intact NAP-22 (Epand et al., 2001).

In summary, with regard to the molecular mechanism of the partitioning of NAP-22 to membranes, we have shown that this is not a result of a specific binding to cholesterol. NAP-22 can bind to membranes containing certain other sterols with comparable affinity. Furthermore, NAP-22 can sequester to bilayers composed of PC and PE that are devoid of cholesterol. In addition, the enhancement of binding by low mol fractions of anionic lipids may contribute to the partitioning of the protein into the cytoplasmic surface of cell plasma membranes. The dependence of binding on the anionic lipid content of the membrane suggests that NAP-22 binds to the membrane in an orientation in which the amino terminus is juxtaposed closer to the membrane than are other parts of the protein. The myristoyl group at this end of the molecule is required for efficient partitioning into membranes but a 19-amino-acid myristoylated peptide binds to membranes in a manner that is not sensitive to lipid composition, unlike the case of NAP-22. Thus, the lipid specificity of the membrane binding of NAP-22 is a property of a large segment of the protein, while the translocation from aqueous to membrane environments is a property of N-terminal myristoylation.

Several interactions likely contribute to the binding of NAP-22 to the cytosolic surface of the plasma membrane, including the hydrophobic insertion of the myristoyl group, likely interacting with cholesterol, the electrostatic interaction between the cationic cluster of the protein and the anionic lipids on the cytoplasmic surface of the membrane as well as interaction of the protein with PE, which also is enriched on the cytoplasmic side of the plasma membrane. However, since the protein can be extracted from the membrane by the depletion of cholesterol, the interaction of NAP-22 with cholesterol appears to be required for membrane binding.

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