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Biochimica et Biophysica Acta 1778 (2008) 1576–1582

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Review

Proteins and cholesterol-rich domains

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ARTICLE INFO

Article history:
Received 20 November 2007
Received in revised form 19 March 2008
Accepted 24 March 2008
Available online 1 April 2008

Keywords:
Cholesterol
CRAC domain
PtdIns(4,5)P2
gp41
NAP-22

ABSTRACT

Biological membranes are composed of many molecular species of lipids and proteins. These molecules do not mix ideally. In the plane of the membrane components are segregated into domains that are enriched in certain lipids and proteins. Cholesterol is a membrane lipid that is not uniformly distributed in the membrane. Proteins play an important role in determining cholesterol distribution. Certain types of protein lipidation are known to cause the lipoprotein to sequester with cholesterol and to stabilize cholesterol-rich domains. However, proteins that are excluded from such domains also contribute to the redistribution of cholesterol. One of the motifs that favor interaction with cholesterol is the CRAC motif. The role of the CRAC motif of the gp41 fusogenic protein of HIV is discussed. The distribution of the multianionic lipid, phosphatidylinositol(4,5)bis-phosphate (PtnIns(4,5)P_2), is also not uniform in cell membranes. This lipid has several functions in the cell, including a morphological role in determining the sites of attachment of the actin cytoskeleton to the plasma membrane. PtnIns(4,5)P_2 is sequestered by proteins having clusters of cationic residues in their sequence. Certain proteins containing cationic clusters also contain moieties such as myristoylation or a CRAC segment that would also endow them with the ability to sequester to a cholesterol-rich domain. These proteins interact with PtnIns(4,5)P_2 in a cholesterol-dependent manner forming domains that are enriched in both cholesterol and in PtnIns(4,5)P_2 but can also be distinct from liquid-ordered raft-like domains.

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1. General principles

1.1. Chemical composition and organization of biological membranes

Biological membranes are complex mixtures composed primarily of lipids and proteins. The number of individual molecular species is quite large. For the lipids of mammalian membranes there are glycerol-based lipids, sphingolipids and cholesterol. Within each of the first two classes there are many different examples. Furthermore for each type of lipid, such as phosphatidylcholine or sphingomyelin,

Abbreviations: GPI, glycosyl-phosphatidylinositol; PtnIns, phosphatidylinositol; PtnIns(4,5)P_2, phosphatidylinositol(4,5)bis-phosphate; CRAC, Cholesterol Recognition/interaction Amino acid Consensus; PH domain, pleckstrin homology domains; AFM, atomic force microscopy; TIRF, total internal reflectance fluorescence

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doi:10.1016/j.bbamem.2008.03.016

for example, there can be many different molecular species because of variation in the length and degree of unsaturation of the hydrocarbon chains. In addition to the diacylglycerophospholipids, there are lipid species with long chain alcohols in ether linkage to glycerol as well as the variation among sphingolipids in having dihydrosphingosine in place of sphingosine. The specific lipids in a particular membrane will depend on the species, tissue and subcellular location from which the lipid is derived. The sterol in yeast membranes is ergosterol, rather than cholesterol and plants have sitosterol. Most of the sterol is present in the plasma membrane and only small amounts in intracellular membranes. Cardiolipin is a specific lipid marker for mitochondrial membranes. Similarly the nature of proteins present in a membrane will also be dependent on the species and membrane type. The number of different proteins in a particular membrane is large. It is estimated that 20–30% of the genome codes for transmembrane helical integral membrane proteins [1]. In addition there are also integral membrane proteins that are based on a β-barrel structure as well as peripheral membrane proteins. Thus, because of the large number of different molecular species of lipids, as well as the large number of different membrane proteins, there is great complexity in biological membranes on a molecular level.

In contrast to the large variety of chemical species in a membrane, the predominant structural basis of membranes is the bilayer. Both glycerolipids as well as sphingolipids are amphiphilic and most of them will spontaneously form a bilayer structure. In purified form, however, some of the lipids form inverted phases, but large areas of inverted phase are avoided in biological membranes so as to maintain a permeability barrier. Despite this simple common structure, biological membranes are not uniform. They have well documented transmembrane asymmetry, as well as inhomogenieties in the plane of the membrane. These lateral inhomogenieties are a result of preferential interactions of certain molecules. Phospholipid translocases contribute to the asymmetric transmembrane distribution of lipids, while the asymmetric distribution of proteins is determined by their vectorial insertion during membrane biogenesis.

There has been extensive work over the years in defining phase diagrams of lipid mixtures. Gel state immiscibility is common, however liquid–liquid immiscibility is rarer. An example of a lipid mixture that shows liquid–liquid immiscibility is one with equimolar amounts of dioleoylphosphatidylcholine, sphingomyelin and cholesterol [2]. This lipid mixture has been widely studied by many techniques for its ability to form two immiscible liquid domains. One of these domains is enriched in sphingomyelin and cholesterol and is described as being in a liquid-ordered phase (Lo), in contrast to the surrounding domain that is in the liquid-disordered phase (Ld). There has been considerable interest in this phenomenon because it was believed that the liquid-ordered phase resembled the so-called "raft" domains of biological membranes. However, it is now appreciated that raft domains of biological membranes are smaller and more transient. Rafts in biological membranes are considered to be in the range of 10 to 100 nm and too small for visualization by light microscopy. The smaller size of these domains in biological membranes has recently been explained by the presence of integral membrane proteins attached to the cytoskeleton that act as obstacles to limit the size of lipid domains in biological membranes [3]. In addition to lateral domain formation being promoted by interactions among lipids, proteins can also facilitate domain formation by preferential interaction with certain lipids. Cholesterol is a major lipid component of the plasma membrane of mammalian cells. The chemical structure of cholesterol with its four fused rings and small hydroxyl headgroup is quite different from that of polar lipids with flexible hydrocarbon chains and a large polar headgroups. It thus would be expected *a priori* that proteins would not interact to the same extent with both phospholipids and cholesterol. Such preferential interaction is sufficient to promote lateral phase separation of the lipid components.

1.2. Domains by attraction or exclusion

The formation of a domain induced by a protein interacting with particular lipids is one mechanism for this segregation to occur. However, thermodynamically it is required that a protein that avoids interaction with particular lipids will also cause the formation of domains that are enriched in those lipids. The formation of cholesterol-rich domains will be used as an example. If a protein interacts with cholesterol more than with other membrane components, it will promote the formation of a cholesterol-rich domain. This is a direct mechanism for protein-induced domain formation. However, it is also the case that a protein excluded from a cholesterol-rich region of the membrane, will also promote the formation of a cholesterol-rich domain. This would be expected to be a fairly common situation since cholesterol causes the tighter packing of lipid in the liquid-crystalline state and inhibits the insertion of other molecules. The protein will then stabilize the cholesterol-depleted region of the membrane, forcing the cholesterol to concentrate in other regions that then become enriched in cholesterol. Experimental evidence for this type of phenomenon has been presented [4].

2. Cholesterol-rich domains

2.1. Size of domains

Raft domains in biological membranes vary greatly in size and lifetime but in general these domains in biological membranes are smaller and shorter lived than those formed by simple lipid mixtures [5]. However, there are some cases of larger size raft-like domains in biological membranes such as caveolae that can form micron size domains [6]. In addition, it has been demonstrated that giant plasma membrane vesicles or blebs formed from the plasma membranes of cultured mammalian cells can also contain large domains with fluid–fluid immiscibility [7]. However, phase segregation into optically resolvable domains occurs only at temperatures below 25 °C. At 37 °C, the membranes of these vesicles are almost completely optically homogenous.

2.2. Proteins that incorporate into cholesterol-rich domains

It was indicated above that proteins are often excluded from cholesterol-rich domains because the cholesterol induces tighter packing of the bilayer. Nevertheless, certain proteins are found to preferentially partition into such domains. Our understanding of the molecular basis for the partitioning of proteins between raft and non-raft domains is incomplete. There are also methodological difficulties in separating raft and non-raft domains in order to do a proteomics analysis of the constituents of each fraction [8]. There are other complications such as the apparent heterogeneity of membrane rafts as well as the fact that some proteins are well integrated into rafts, while others only associate transiently. Nevertheless, there are some generalizations that can be made regarding the factors that favor the partitioning of proteins into rafts.

2.2.1. Lipidation

Four types of lipidation are found in proteins that associate with raft domains. These are acylation with either myristic acid on the N-terminal amino group or palmitic acid on cysteine residues [9–12], GPI-linkage [13–17] and the covalent attachment of cholesterol [18]. It would be expected that saturated acyl chains and cholesterol moieties that are attached to proteins would partition into raft domains because of their smooth, regular surface. Free cholesterol itself is of course a component of rafts. In addition, lipids with saturated acyl chains are more readily accommodated into raft domains than are lipids with unsaturated acyl chains. In particular, lipids with polyunsaturated acyl groups are excluded from raft domains [19]. The structure of the GPI-linkage is that it has a phosphatidylinositol

moiety that is further glycosylated on the inositol sugar of the PtnIns headgroup. The most abundant form of PtnIns in mammalian membranes is 18:0/20:4. The arachidonoyl chain at the *sn-2* position of glycerol would inhibit partitioning into a raft domain. However, it has recently been found that there is remodeling of the acyl chains of PtnIns when it is converted to the final GPI anchor that is then linked with proteins [20,21]. In its final form of the GPI anchor the arachidonoyl (20:4) chain of PtnIns is replaced by a stearoyl (18:0) group that would be expected to partition well into raft domains. Thus the acyl chain composition of the GPI anchor is uncommon in having a saturated acyl chain at the *sn-2* position. It is thus likely that this acyl chain replacement has some functional significance.

2.2.2. Protein segments

Less is known about how peptide segments of proteins affect the partitioning of the protein between raft and non-raft domains. Some proteins are known to sequester to raft domains [8]. Apart from lipidated proteins discussed above, one can suggest three mechanisms by which proteins will preferentially be concentrated in cholesterol-rich domains. One mechanism is indirect and is a consequence of interaction of the protein with another protein that is a so-called scaffolding protein that itself is in a particular domain. Examples of scaffolding proteins are caveolin [22], present as major components of caveolae, and flotillin [23] a protein present in the raft domain of many cells. The question then becomes why a particular scaffolding protein sequesters into a certain domain. A second mechanism is through the nature of the topology of the inserted hydrophobic segment of the protein. It is indicated above that polyunsaturated acyl chains would be excluded from rafts, so too would helices that do not have a smooth contour. This would be analogous to prenylated proteins that have methyl groups protruding from the hydrocarbon chain and are also excluded from rafts. One example of a hydrophobic peptide segment that has recently been shown to preferentially interact with cholesterol is the hydrophobic segment of the epsilon isoform of diacylglycerol kinase [24]. The partitioning of integral membrane proteins into raft domains is discussed in more detail in the chapter by Gonzalez-Ros et al. 2008 [25]. The third mechanism is by preferential interaction of segments of the protein positioned at the membrane interface with certain lipid components. This mechanism is currently only partly understood. A developing hypothesis regarding the requirements for cholesterol recognition at the membrane interface is by interaction of the sterol with CRAC domains, described below.

2.2.3. CRAC domains

There are also segments of integral membrane proteins that are located at the membrane interface and are thought to facilitate interaction with cholesterol. The membrane interface is the barrier between the membrane and water. It is a complex region with molecules of both lipid headgroups as well as water. The hydroxyl group of cholesterol forms part of the membrane interface and is different in size and hydrophobicity from other major lipid components and hence proteins may preferentially interact with the membrane at sites of cholesterol. The requirements for a protein segment at the membrane interface to recognize cholesterol is poorly understood. An evaluation of the common elements present in several proteins known to interact with cholesterol or that partitioned into cholesterol-rich domains has led to the development of the CRAC motif. A CRAC motif is defined as a sequence pattern -L/V-$(X)_{(1-5)}$-Y-$(X)_{(1-5)}$-R/K-, in which $(\text{-X-})_{(1-5)}$ represents between one and five residues of any amino acid [26]. The loose requirements for a CRAC domain predicts that a large number of sequences would favor interaction with cholesterol [27]. However, if in addition to fulfilling the requirements for a CRAC motif, this segment also is located adjacent to a transmembrane helix, requiring that it be positioned at the membrane interface, and furthermore is a segment of a protein that is known from other criteria to be in a raft domain, then it is more likely that such a CRAC sequence has a role in sequestering the protein to a cholesterol-rich membrane domain. The two scaffolding proteins mentioned above, i.e. flotillin and caveolin, have CRAC domains. Both flotillin 1 and 2 from humans have 4 predicted CRAC domains, but no transmembrane helix. Caveolins have a single transmembrane helix. In the cases of the two more widely expressed forms of caveolin, i.e. caveolins 1 and 2, the putative transmembrane helix comprises residues 104–124 and 86–106, respectively. On the amino terminal side of this putative transmembrane segment there is a CRAC domain at residues 93–100 for caveolin-1 and at 75–85 for caveolin-2. However, in the case of caveolin-3 there is no CRAC domain adjacent to the hydrophobic segment. It is known that caveolin-3 is palmitoylated [28,29] and this may be the factor that promotes partitioning to cholesterol-rich domains. In the case of caveolins it has been found that palmitoylation of the protein is not required for partitioning into caveolae [30,31], suggesting that the presence of CRAC domains are responsible for this localization.

One of the first proteins studied with a CRAC motif was the peripheral-type benzodiazepine receptor [26]. The CRAC segment of the peripheral-type benzodiazepine receptor is also present at the end of a transmembrane helix near the membrane interface. There is evidence that this protein facilitates cholesterol import into the mitochondria. The peripheral-type benzodiazepine receptor can be photolabeled with a sterol and this labeling is prevented by competition with cholesterol [32]. A model peptide containing this CRAC sequence is also labeled by the sterol photolabel and this peptide also inhibits cholesterol uptake [32]. Mutation of the CRAC segment of the peripheral-type benzodiazepine receptor results in the loss of cholesterol uptake [33]. In addition, an NMR study has shown that a peptide comprising this CRAC segment can form a specific complex with cholesterol [33].

Another example of a CRAC segment that has received recent interest is a region in the gp41 fusogenic protein of HIV. This protein has a single transmembrane helical segment. Adjacent to this helix on the amino terminal side, is a segment that is rich in Trp residues that ends with the segment LWYIK. This pentapeptide corresponds to a CRAC domain. It has been shown that when this sequence is added to a maltose binding protein the construct has the ability to bind to cholesteryl-hemisuccinate agarose [34]. In addition, the blocked peptide *N*-acetyl-LWYIK-amide is capable of redistributing cholesterol in mixtures of the sterol with 1-stearoyl-2-oleoyl phosphatidylcholine [35]. We also demonstrated using magic angle spinning NMR that this peptide inserts more deeply into bilayers containing cholesterol than those devoid of cholesterol. The accuracy of the CRAC algorithm in predicting which sequences will interact with cholesterol was studied. Interestingly the sequence IWYIK that is closely related structurally to LWYIK but is not a CRAC motif, exhibits almost no preferential interaction with cholesterol [36]. The first residue of this segment was systematically changed to the other hydrophobic residues Ala or Val [37]. Both AWYIK and VWYIK were weakly active in sequestering cholesterol, somewhat better than IWYIK but much weaker than LWYIK. These results show some of the limitations of the CRAC algorithm. The requirements for the CRAC domain predict that VWYIK would be as active as LWYIK and much more active than AWYIK. This is not observed. It is therefore likely that there are some sequence-specific properties that determine the extent of preferential interaction with cholesterol.

However, what is particularly striking is that introducing these same amino acid substitutions by mutation into the intact gp41 protein of HIV results in changes in the ability of the protein to promote membrane fusion between cells that qualitatively parallel the observations made with the peptides interacting with cholesterol-containing lipid mixtures. The L679I mutation of gp41 has only 42% of the fusogenic activity of the native protein by the criterion of the number of nuclei per syncytia [36]. This is slightly lower than the number of nuclei per syncytia found with the L679A or L679V mutants which is about 47% [37]. There are of course other factors besides interaction with cholesterol that determine the fusogenicity of these

constructs. Nevertheless the finding that relatively minor changes in the protein sequence affects both cholesterol affinity of the isolated segments as well as fusogenicity of the intact protein suggests that cholesterol interaction with the CRAC segment of gp41 contributes to the biological properties of the intact protein.

3. Anionic lipid-rich domains

Several proteins involved in signal transduction pathways contain clusters of cationic amino acid residues in their primary structure. Such a concentration of positive charge has been shown to promote the sequestering of anionic lipids into domains. This phenomenon occurs with many anionic lipids. However, in this review we will focus on the anionic lipid, phosphatidylinositol-4,5-bis-phosphate (PtnIns(4,5)P_2) [38–40]. This example has been chosen both because domains of this lipid may have particular biological functions. In addition we can demonstrate an inter-relationship between formation of domains of PtnIns(4,5)P_2 and the location of cholesterol, thus relating the formation of anionic lipid clusters with the earlier sections of this review.

3.1. PtnIns(4,5)P_2

PtnIns(4,5)P_2 plays a number of import roles in signal transduction. It is the preferred substrate for certain isoforms of phospholipase C that cleaves the lipid into two secondary messengers, the lipid messenger diacyl glycerol and water-soluble inositol triphosphate [41]. In addition, PtnIns(4,5)P_2 has a high affinity for binding to pleckstrin homology (PH) domains in a number of proteins. There is also a large and growing list of ion channels and transporters that exhibit some sensitivity for PtnIns(4,5)P_2 [42]. PtnIns(4,5)P_2 has a charge of approximately −4 at neutral pH. It is thus more prone to recruitment into domains by proteins with cationic clusters, largely through electrostatic interactions [40].

The majority of PtnIns(4,5)P_2 in the cell is in the plasma membrane and accounts for approximately 1% of the membrane lipid. It appears to have an important role in the spatial organization in the cell and is involved in stabilizing interactions between the actin cytoskeleton and the plasma membrane [43–47]. Enzymes involved in PtnIns(4,5)P_2 biosynthesis are enriched at sites of actin polymerization [45,48,49] suggesting that they may play a role in the interactions between the cytoskeleton and the plasma membrane. However, no mechanism has been elucidated to explain the sequestration of PtnIns(4,5)P_2 to these sites once it has been produced [50].

There has also been interest in the relationship of PtnIns(4,5)P_2 localization and raft domains [51]. It has been shown that PtnIns(4,5)P_2 does not spontaneously cluster in fluid bilayers of phosphatidylcholine [52]. In mammalian membranes the most prevalent form of PtnIns(4,5)P_2 is 1-stearoyl-2-arachidonoyl-phosphatidylinositol(4,5) bis-phosphate. It is known that polyunsaturated acyl chains, like the arachidonoyl moiety, disfavor partitioning into raft domains [19]. This is in agreement with the fact that sphingomyelin, a well known raft component, is not colocalized with PtnIns(4,5)P_2 [53]. In addition, atomic force microscopy studies indicate that in bilayers composed of both liquid-ordered and liquid-disordered domains, the PtnIns(4,5)P_2 sequesters into the disordered domain [54]. It is still possible that some PtnIns(4,5)P_2 is localized in raft domains in cells because it binds to proteins that are resident in rafts. There may also be more than one type of PtnIns(4,5)P_2 domain in biological membranes and additionally, that the state of PtnIns(4,5)P_2 in a membrane is cell type dependent. This is suggested by the observations that the lateral mobility of PtnIns(4,5)P_2 is found to be different in different cell types. The mobility is low in atrial myocytes [55] but higher in HEK293 cells [56,57] or in fibroblasts [58].

We suggest, based on our observations in model systems, that some proteins can recruit PtnIns(4,5)P_2 into cholesterol-rich domains that are different from the sphingomyelin-rich, liquid-ordered "raft" domains that have been so well characterized in simple lipid mixtures. One such protein is NAP-22, a 22 kDa mammalian protein (the equivalent protein found in chickens is CAP-23) that is highly expressed in neuronal tissue during development and plays an important role in axonal sprouting and elongation [59]. NAP-22 is an acidic protein with a p*I* of 4.6 and is devoid of stretches of hydrophobic amino acids. The only portion of the protein likely to partition into a membrane bilayer is the N-terminal myristoyl group, added by post-translational modification. The myristoyl group, being a saturated acyl chain, is one of the lipidations that augment the partitioning into raft domains. NAP-22 has been found to bind only to membranes with sufficient cholesterol [60,61]. The protein also sequesters to raft-like liquid-ordered domains in model membranes [62].

A myristoylated, *N*-terminal fragment of NAP-22 corresponding to the structure myristoyl-GGKLSKKKKGYNVNDEKAK-amide was studied in simple lipid mixtures of phosphatidylcholine and cholesterol as well as after addition of PtnIns(4,5)P_2 to the lipids. Amino terminal fragments of NAP-22 are found abundantly *in vivo* [63]. This lipopeptide causes Bodipy-labeled PtnIns(4,5)P_2 to be sequestered into domains in a cholesterol-dependent manner as shown by Bodipy quenching [64]. In the absence of cholesterol the lipopeptide causes little clustering of PtnIns(4,5)P_2. Furthermore, when the Tyr residue at position 11 is replaced with Leu, the ability of the lipopeptide to form domains enriched in PtnIns(4,5)P_2 becomes independent of added cholesterol [64]. There have been suggestions that the flat ring of the phenolic side-chain of Tyr can stack with one of the rings of cholesterol. It should be mentioned that one of the required residues of CRAC domains is Tyr. DSC results also indicate that this lipopeptide can redistribute cholesterol in membranes forming cholesterol-rich and cholesterol-poor domains [64]. Thus, the cluster of cationic residues in this lipopeptide can induce the segregation of PtnIns(4,5)P_2 but the unusual characteristic of this process is that it is cholesterol-dependent because of the presence of the myristoyl group and likely also the Tyr that favor interaction with cholesterol. MAS/NMR showed that cholesterol affected the nature of the incorporation of this lipopeptide into bilayers [64]. The cholesterol dependence of this domain formation was also shown directly using a combination of AFM and TIRF microscopy [65]. Recently a similar system, corresponding to the palmitoylated region of GAP-43 together with its cationic cluster, was shown to sequester PtnIns(4,5)P_2 into cholesterol-rich domains [66]. It has been suggested that there is a relationship between the modes of action of NAP-22 and of GAP-43 as a result of the common nature of their interaction with PtnIns(4,5)P_2 in cholesterol-rich domains [67]. MARCKS has in common with NAP-22 and GAP-43 the fact that all three proteins are found in plasmalemmal rafts, and they all regulate cell cortex actin dynamics [68]. Not surprisingly a model peptide containing the cationic cluster, but devoid of any cholesterol-binding myristoyl group or CRAC segment did not show any sensitivity to the presence of cholesterol in its recruitment of PtnIns(4,5)P_2 [38]. Even the intact MARCKS protein might not exhibit cholesterol-promoted sequestering of PtnIns(4,5)P_2 since the N-terminal myristoyl group is at residue one, while the cationic cluster is much further toward the carboxyl terminus at residues 151–175.

The sequestering of PtnIns(4,5)P_2 by the myristoylated N-terminal segment of NAP-22 was also studied in a lipid mixture of sphingomyelin, phosphatidylcholine and cholesterol that exhibits the formation of raft-like domains with the lipid alone. Addition of the lipopeptide to this mixture caused the recruitment of cholesterol from the liquid-ordered domains into the liquid-disordered region to be colocalized with PtnIns(4,5)P_2 [54]. As a result the liquid-disordered domain became more ordered. Thus lipopeptide-induced domains are formed that are enriched in cholesterol and PtnIns(4,5)P_2 but are distinct from the sphingomyelin-rich domains of systems exhibiting liquid–liquid immiscibility.

Another protein that interacts with PtnIns(4,5)P_2 is the band 4.1 protein. This protein is a major component of the erythrocyte membrane that has an important role in modulating cell morphology and membrane mechanical properties [69]. It does so by interacting with both cytoskeletal proteins such as spectrin and actin, as well as integral membrane proteins such as band 3 and glycophorin. Genetic defects affecting Band 4.1 result in abnormally shaped erythrocytes with decreased mechanical stability [70]. There are also a number of proteins related to Band 4.1 that are found in nucleated cells [71,72]. It has recently been shown that the N-terminal region of the Band 4.1 protein binds to PtnIns(4,5)P_2 resulting in changes in the interactions of Band 4.1 with other proteins [73]. This process will affect cytoskeletal arrangements through the interaction of Band 4.1 with PtnIns(4,5)P_2. As with NAP-22, the interaction of Band 4.1 with PtnIns (4,5)P_2 has been suggested to be a consequence of electrostatic interactions with a cluster of cationic residues on the protein [73]. Charged peripheral membrane proteins can induce domain formation of anionic lipids [74].

Band 4.1 has a segment comprising residues 211–222 with the sequence LLVYKDKLRINR. This segment of 12 residues has 4 cationic amino acids. In addition, it fulfills the requirements of a CRAC motif [26], having a Tyr residue and in this case, a Leu separated by one amino acid on the amino terminal side and by a basic residue separated by two residues on the carboxyl side of the Tyr. Thus, like the NAP-22 lipopeptide, it could interact with both cholesterol and with PtnIns(4,5)P_2. NAP-22 has a cationic cluster of residues that can interact with PtnIns(4,5)P_2 that is close to the myristoyl group that would favor interaction with cholesterol. The Band 4.1 segment also has a cationic cluster that in this case is together with a cholesterol-interacting CRAC domain. Another protein segment that has both a cholesterol-seeking CRAC domain in the same protein segment that has a cationic cluster to recruit PtnIns(4,5)P_2 is present in the scaffolding region of caveolin. A peptide corresponding to this region promotes the sequestrating of both PtnIns(4,5)P_2 and cholesterol [75]. In the case of the 12 residue segment from Band 4.1, the sequence is invariant among members of the family of 4.1 proteins, suggesting its functional importance. The cholesterol dependence of sequestering PtnIns(4,5)P_2 by an *N*-acetyl-peptide-amide of this segment of Band 4.1 was compared with a peptide in which the Tyr was substituted with Ile, thus destroying the CRAC motif that is responsible for its interaction with cholesterol.

The native sequence for the segment of Band 4.1 from residue 211–222, *N*-acetyl-LLVYKDKLRINR-amide, is more efficient in quenching the fluorescence of Bodipy-PtdIns(4,5)P_2 than is the modified sequence: *N*-acetyl-LLVIKDKLRINR-amide in which the Y residue is replaced by I to destroy the CRAC domain (Fig. 1).

When electrostatic interactions predominate, there is sequestering of PtdIns(4,5)P_2, independent of the nature of the surrounding lipid. This is the case with the MARCKS peptide that has 13 positive charges compared to an overall charge of the peptides used in this study of only +3. As a consequence, electrostatic interactions alone will provide a stronger driving force for the MARCKS peptide to sequester PtdIns(4,5)P_2 [38]. Another peptide with an intermediate number of 7 positive charges is the amino terminal segment of the protein NAP-22. This peptide is relatively hydrophilic and has only one aromatic residue, a Tyr, that can insert into the membrane compared with the MARCKS peptide that has 5 Phe residues. The NAP-22 peptide has strong membrane affinity because it is myristoylated at the amino terminus. N-terminal myristoyl groups are known to facilitate the targeting of proteins to cholesterol-rich domains in membranes [76,77]. Regarding the segment of Band 4.1 studied in this work, the amino terminal tetrapeptide is quite hydrophobic with the sequence LLVY or LLVI for the two peptides and would facilitate sequestering the peptide to a membrane. In the former case, for the natural sequence, LLVY forms part of a CRAC sequence that would favor binding of the peptide to cholesterol-rich domains. Interestingly, the

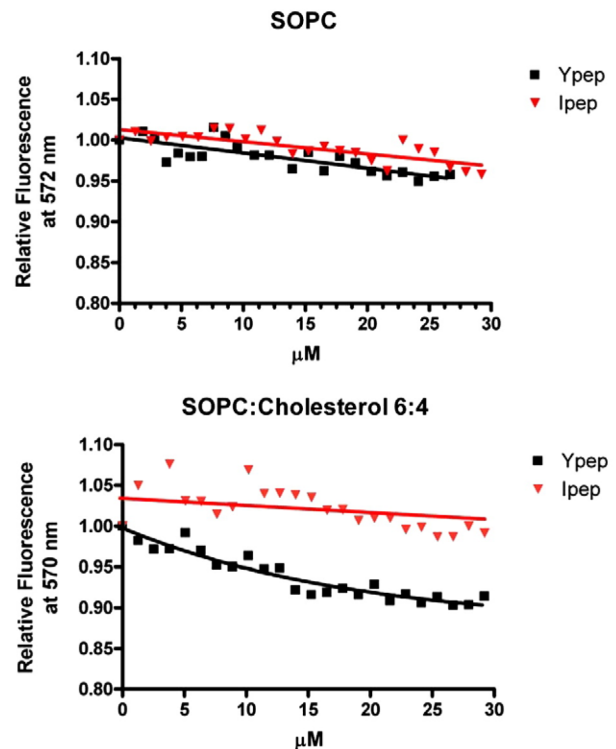

Fig. 1. Quenching of the fluorescence emission by *N*-acetyl-LLVYKDKLRINR-amide (Ypep) or by *N*-acetyl-LLVIKDKLRINR-amide (Ipep) of Bodipy TMR-PtdIns(4,5)P2. LUVs composed of SOPC or SOPC with 40 mol% cholesterol, as indicated. Maximum emission intensity at 572 or 570 nm is plotted against the peptide concentration. LUVs (100 nm diameter) were present in the cuvette at a concentration of 50 μM and the Bodipy-labeled PtdIns (4,5) P2 (GloPIPs Bodipy TMR-PtdIns(4,5)P2-C16, Echelon Biosciences Inc. (Salt Lake City, UT)) were present as 1.0 mol % of the total lipid.

peptide *N*-acetyl-LLVYKDKLRINR-amide sequesters PtnIns(4,5)P_2 in a cholesterol-dependent manner (Fig. 1). This is a consequence of having a segment that interacts with cholesterol as well as several cationic amino acids. This is similar to the case of the NAP-22 peptide that has a cholesterol-targeting myristoyl group along with a cluster of cationic residues and also sequesters PtnIns(4,5)P_2 in a cholesterol-dependent manner [64,65]. Thus, proteins with cationic clusters will tend to sequester PtnIns(4,5)P_2 as a consequence of electrostatic interactions. In addition, in cases such as NAP-22 or Band 4.1 this type of domain formation is dependent on the presence of cholesterol, while for other proteins such as MARCKS it is not. The fact that protein-induced sequestration of PtnIns(4,5)P_2 can be cholesterol-dependent does not necessarily imply their recruitment to "raft" domains in biological membranes. Indeed, studies with the NAP-22 peptide using a combination of AFM and fluorescence microscopy indicated that both PtnIns(4,5)P_2 and cholesterol were sequestered into the liquid-disordered domain [54], thus being located outside of the liquid-ordered domain of a raft. There are several types of cholesterol-rich domains in biological membranes. It is even possible that not all of them are in the liquid-ordered state. The importance of cholesterol in the case of Band 4.1 is that it allows a segment of the protein to recruit PtnIns(4,5)P_2 into a domain. Since cytoskeletal proteins also bind to PtnIns(4,5)P_2, this process will also result in the rearrangement of the cytoskeleton.

Acknowledgements

We wish to thank Dr. Xiuli An of the New York Blood Center for helpful discussions. We are grateful to the Canadian Institutes of

Health Research for financial support for this work (grant MOP-85008). R.M.E. is a Senior Research Investigator of the Canadian Institutes for Health Research.

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