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Biochimica et Biophysica Acta 1375 (1998) 110–116

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Unmyristoylated MARCKS-related protein (MRP) binds to supported planar phosphatidylcholine membranes

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Received 27 May 1998; revised 27 July 1998; accepted 6 August 1998

Abstract

We have recently shown that unmyristoylated MARCKS-related protein (MRP) does not bind to neutral phospholipid vesicles, unless negatively charged phospholipids are present. Similar behaviour has also been reported for MARCKS itself. Here we have compared the binding of MRP to neutral and negatively charged supported planar lipid bilayer membranes (SPLM) using two-mode waveguide spectroscopy. We find appreciable binding of unmyristoylated MRP to neutral SPLM. We propose that hydrophobic residues in the effector domain constitute an additional factor capable of mediating MRP-membrane interaction.

Keywords: Membrane protein; Myristoylation; Phosphorylation; Planar membrane

1. Introduction

Members of the myristoylated alanine-rich C kinase substrate (MARCKS) family are acidic rod-shaped proteins necessary for brain formation [1,2]. Changes in their phosphorylation state, expression and subcellular localization occur concomitantly with major cellular events such as attachment, mitosis, motility, neurosecretion and phagocytosis [3,4]. The putative functions of MARCKS proteins include the regulation of the actin cytoskeleton at the membrane [5], the concentration of free cytosolic calmodulin [4], membrane trafficking [6,7] and phospholipase C activity [8].

Two members of the family are known: MARCKS itself, a ubiquitous 30 kDa protein, and MARCKS-related protein (MRP), a 20 kDa protein highly expressed in brain and reproductive tissues [2,9,10]. Both contain two conserved domains whose properties have been well characterized: the N terminus which is posttranslationally myristoylated by myristoyl CoA:protein *N*-myristoyl transferase [11], and the effector domain (also called the phosphorylation site domain), a highly acidic amphipathic segment comprising 24–25 amino acid residues located in the middle of the primary sequence (murine MRP: KKKKKFSFKKPFKLSGLSFKRNRK), which binds to calmodulin and is phosphorylated by

Abbreviations: MARCKS, myristoylated alanine-rich C kinase substrate; MRP, MARCKS-related protein; myr, myristoylated; OWLS, optical waveguide lightmode spectroscopy; PKM, catalytic subunit of protein kinase C; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol; SPLM, supported planar lipid bilayer membrane; t-b5, tryptic fragment of cytochrome b_5; unmyr, unmyristoylated

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0005-2736/98/$ – see front matter
PII: S0005-2736(98)00141-2

protein kinase C at two and three serine residues in MRP and MARCKS respectively [12–14].

Although their function has not yet been established with certainty, interaction with intracellular membranes, in particular with the plasma membrane, is clearly a key characteristic of these proteins [15]. In agreement with a study at the cellular level [16], experiments with purified proteins or peptides and phospholipid vesicles have shown that both the myristoyl moiety and the effector domain mediate the interaction of MARCKS proteins with membranes (Fig. 1). Partitioning studies with a myristoyl-glycine peptide [17] as well as photolabelling of membrane-bound MARCKS proteins with a photoreactive phospholipid probe [18] have shown that the myristoyl moiety inserts into the bilayer. Partitioning studies with peptides corresponding to the effector domain of MARCKS [19] as well as with intact MARCKS [20] and MRP [18,21] have demonstrated that the positively charged effector domain of MARCKS proteins interacts with the surface of negatively charged phospholipid vesicles via electrostatic interactions. Nevertheless, the partition coefficients for the association of the effector peptide of MARCKS and myristoylated (myr) MARCKS to neutral and negatively charged vesicles show that each of these interactions individually is insufficient to account for the intracellular membrane localization of MARCKS [19,20]. Hence a cooperative model has been proposed, in which the binding of one domain tethers the second domain in close proximity to the membrane surface, thus increasing the chances that the second domain will find the membrane [22]. It provides a rationale for the observation that phosphorylation of MARCKS appears to lead to its removal from the plasma membrane [6,7,12,23–25]. Finally, recent studies have shown that binding of the effector domain of MARCKS to vesicles induces the formation of domains enriched in negatively charged phospholipids [26].

Using the same type of vesicle-based assay previously used to measure the membrane interactions of MARCKS [19,20], we have recently found that the partition coefficient describing the binding of myr MRP to vesicles containing 20% negatively charged phospholipids is at least one order of magnitude lower than for myr MARCKS [18,21], but, as already reported for MARCKS [19,20], we could not detect any binding of unmyristoylated (unmyr) MRP to phosphatidylcholine vesicles in the absence of negatively charged phospholipids.

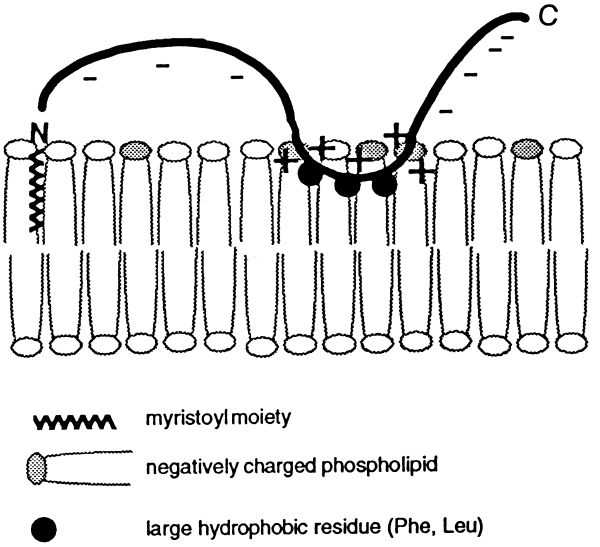

Fig. 1. Model for the membrane topology of MARCKS proteins. The myristoyl moiety at the N terminus inserts into the membrane [17,18]. The effector domain in the middle of MARCKS proteins is highly basic with an average net charge of +0.5/residue. It interacts via electrostatic interactions with the head groups of negatively charged phospholipids [18–21] and sequesters these lipids to form microdomains [26]. Large hydrophobic residues in the effector domain of MARCKS (five Phe; one Leu) and MRP (four Phe; two Leu) also participate in the binding by inserting into the outer leaflet of the membrane [18,36]. Overall, MARCKS proteins are acidic with average net charges of −0.1/residue and −0.2/residue for the amino acid segments on the N-terminal and C-terminal side, respectively, of the effector domain. The average net charge is defined as the number of positively charged residues (Arg, Lys) minus the number of negatively charged residues (Glu, Asp), divided by the total number of residues in the segment of interest.

Here we measure the interaction between MRP and supported planar lipid bilayer membranes (SPLM) deposited on a smooth planar hydrophilic optical waveguide. This type of SPLM has been shown to have properties (fluidity, etc.) closely comparable to unsupported membranes [27]. We use a very sensitive technique, optical waveguide lightmode spectroscopy (OWLS) [28–30] to measure the number of molecules associated with the membrane. From accurate measurements of the phase velocities of two or more guided lightmodes, the amount of bound protein can be determined to a precision of ± 10 pg/mm^2.

We find that the pattern of affinity differs from that observed with vesicles. Although removal of the myristoyl moiety also roughly halves the affinity, binding to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) is closely comparable to the binding to a mixture of 80% POPC and 20% 1-palmitoyl-2-oleoyl-*sn*-gly cero-3-phosphatidylglycerol (POPG) (this lipid mixture will be referred to as POPC:POPG 4:1 throughout the text).

The effect of phosphorylating the serine residues of the effector domain, which should enhance electrostatic repulsion between the protein and negatively charged membranes, was also investigated, and found to be minor.

2. Materials and methods

2.1. Materials

The plasmid pET3dF52M1 containing the *mrp* gene was a gift from Perry Blackshear (Duke University Medical Center, Durham, NC), and the plasmid pBB131NMT containing the gene coding for myristoyl CoA:protein *N*-myristoyl transferase was a gift from Jeffrey Gordon (Washington University School of Medicine, St. Louis, MO). POPC and POPG were from Avanti Polar Lipids (Alabaster, AL). Planar optical waveguides incorporating a grating coupler (type 2400) were obtained from Artificial Sensing Instruments ASI (Zürich, Switzerland).

2.2. Proteins and peptides

The unmyr and myr forms of mouse MRP were expressed in *Escherichia coli* and purified as described previously [18]. MRP could be stoichiometrically myristoylated by coexpressing myristoyl CoA:protein *N*-myristoyl transferase in bacteria, as judged by radioactive labelling with [^{3}H]myristate and by a shift in the migration of the MRP band on SDS-polyacrylamide gels. During purification myr MRP is retained on Phenylsepharose CL-4B (Pharmacia, Uppsala, Sweden) in the presence of 3 M NaCl whereas unmyr MRP is not, ensuring that the myristoylated protein is not contaminated with traces of unmyr MRP [18]. The concentration of MRP was determined by amino acid analysis. The catalytic subunit of protein kinase C (PKM) from rat brain was purchased from Calbiochem (La Jolla, CA). The tryptic fragment of cytochrome b_5 (t-b5) was a gift from R.C. Willson (University of Houston, TX). The proteins were stored in 10 mM MOPS-NaOH, pH 7.4, 0.1 mM EGTA (buffer A) at −80°C. A 25 amino acid peptide corresponding to the effector domain of MARCKS (KKKKKRF-SFKKSFKLSGFSFKKNKK) was synthesized and purified as described previously [31].

2.3. Preparation of the waveguides

Waveguides were soaked overnight in 10 mM MOPS/NaOH, pH 7.4 plus 0.1 mM EGTA and 0.1 M NaCl (buffer B). Bilayers were deposited using a laboratory-built Langmuir trough filled with buffer B. Lipid monolayers were compressed to a surface pressure of 32 mN/m (the so-called bilayer equivalence pressure [32]) and deposited using a combination of vertical (Langmuir-Blodgett) and horizontal (Langmuir-Schaefer) movements as described previously [30]. Only bilayers for which the transfer ratio of each monolayer to the support was 100% were used. As a control of bilayer quality, t-b5, a 17 kDa protein with the same isoelectric point as MRP (4.4) but lacking a transmembrane domain, was used as a probe. It adsorbs to the waveguide in the absence of membranes [28], but does not, however, bind to a POPC:POPG 4:1 membrane (Fig. 2), demonstrating that the bilayer contains no macroscopic defects and perfectly insulates the protein solution from the $Si(Ti)O_2$ surface of the waveguide.

2.4. Measurement of protein binding to SPLM

The membrane-coated waveguides were mounted in the measuring head of an IOS-1 integrated optics scanner (Artificial Sensing Instruments ASI, Zürich, Switzerland). A flow-through cuvette (diameter: 9 mm; height: 0.31 mm) was sealed with an O-ring over the waveguide surface. Typically 1–5 ml of protein or peptide solution in buffer B were flushed through the cuvette over the membrane surface. The flow was controlled by a high precision mechanical syringe pusher delivering 7.7 ml/h. The temperature of the measuring head was maintained at 25.0°C. Binding of MRP molecules on the SPLM

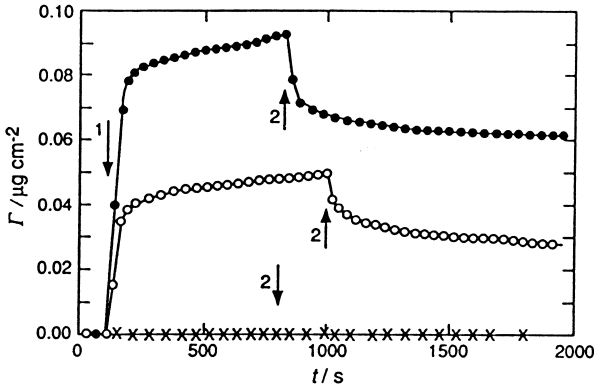

Fig. 2. Effect of myristoylation on the binding of MRP to SPLM. Following equilibration of a POPC:POPG 4:1 SPLM with buffer B, a solution containing 0.6 μM myr (●), or unmyr (○) MRP, or 0.25 μM t-b5 (×) was passed over the membrane (starting at arrow 1). At the times indicated by arrows 2 the protein flow was replaced by buffer B.

results in a protein adlayer whose refractive index n_A is given by:

$$n_A = n_c + c_M dn/dc \quad (1)$$

where n_c is the refractive index of the buffer, c_M is the concentration of MRP within the adlayer and dn/dc is the refractive index increment of the protein, which depends on its polarizability and has a quasi-universal value of 0.18 cm^3/g [33]. The diffraction grating incorporated into the waveguide allows an external He-Ne laser beam to be coupled into the waveguide provided that the angle of incidence α onto the grating satisfies the relation [29,34]:

$$N = n_{air} \sin \alpha + l\lambda/\Lambda \quad (2)$$

where N is the refractive index of the waveguide, n_{air} the refractive index of the ambient air, Λ the grating constant (416.15 nm), l the diffraction order (1), and λ the wavelength of the laser light (632.8 nm). Measurement of the angles α at which the incoupled power is at a maximum allowed the zeroth transverse electric (TE) and transverse magnetic (TM) modes to be determined according to Eq. 2. These modes satisfy their respective mode equations [28,29,34] which can be solved simultaneously to yield n_A and the adlayer thickness d_A, from which the surface concentration Γ of MRP at the membrane is determined according to Eq. 1 and:

$$\Gamma = d_A c_M \quad (3)$$

Dissociation of bound protein was determined by replacing protein flow with buffer B.

2.5. Phosphorylation of unmyr MRP

Unmyr and myr MRP were phosphorylated for 3 h at 30°C in a volume of 400 μl. The solutions contained 3–5 μM MRP, 3 nM PKM, 6 mM $MgCl_2$, 100 mM NaCl, 0.1 mM ATP, 0.2 mM $CaCl_2$, 0.1 mM EGTA and 10 mM MOPS (pH 7.4). The extent of modification was estimated by phosphorylating 20 μl aliquots in the presence of 0.5 μCi [γ-^{32}P]ATP as previously described [35]. The stoichiometry of phosphorylation was determined by phosphorimaging the gels containing phosphorylated MRP together with a nitrocellulose membrane on which known amounts of [γ-^{32}P]ATP (0–200 pmol in 5 μl) had been spotted [35]. The conditions (amount of radioactivity and exposure time) were chosen such that the signals from phosphorylated MRP and from the calibration curve were in the linear range. Although phosphopeptide analysis showed that two serine residues are modified in MRP [14,35] we could only incorporate 1.39 ± 0.15 mol phosphate/mol myr MRP ($n = 4$) and 0.93 ± 0.15 mol phosphate/mol unmyr MRP ($n = 4$). For comparison, a value of 2.5 mol phosphate/mol protein has been reported for unmyr MRP [14]. This value should be reduced to 1.6 mol phosphate/mol protein since amino acid analysis has shown that the Lowry assay overestimates the concentration of MRP by a factor of 1.6 [35]. To follow the binding of phosphorylated MRP to SPLM the solutions were diluted to 0.2 μM MRP with buffer B. A control experiment was performed by omitting PKM from the reaction solution.

3. Results

Following equilibration with buffer B, a solution containing 0.6 μM myr MRP was flushed over a SPLM containing 20% anionic phospholipids (POPC:POPG 4:1), resulting in binding of the protein to the bilayer (Fig. 2, arrow 1). The protein solution was then replaced by buffer B (arrow 2), resulting in release of the protein from the membrane. Fig. 1 also shows that removal of the myristoyl moiety of MRP roughly halves the amount of

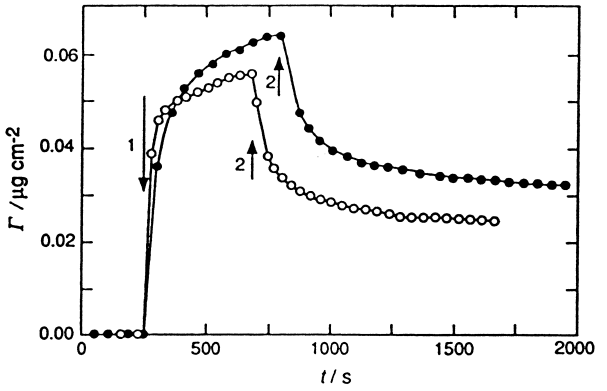

Fig. 3. Effect of anionic phospholipids on the binding of MRP to SPLM. Following equilibration of either a POPC (●) or a POPC:POPG 4:1 (○) SPLM with buffer B, a solution containing 1.8 μM unmyr MRP was passed over the membrane (starting at arrow 1). At the times indicated by arrows 2, the protein flow was replaced by buffer B. Similar observations were made with myr MRP (not shown).

protein binding to POPC:POPG 4:1, in reasonable agreement with previous results from vesicles [18,21].

Fig. 3 shows that comparable amounts of unmyristoylated protein bind to POPC and POPC:POPG 4:1, in marked contrast to the results with vesicles, for which no attachment to POPC could be detected. Replacing POPG by 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine, another acidic phospholipid, gave the same behaviour as POPG (not shown). The only hint of enhanced attraction between the protein and the membrane when negatively charged phospholipids are present is the rapid initial rate of attachment.

Fig. 4 compares the binding of a peptide whose sequence corresponds to the effector domain of MARCKS to POPC and POPC:POPG 4:1. In this case, POPG very significantly enhances the binding, in agreement with results obtained using this peptide in the presence of lipid vesicles [19]. The residual binding of the peptide to POPC membranes, which may reflect insertion of hydrophobic residues (five Phe and one Leu) into the bilayer [18,35], has also been observed with other membrane model systems, including monolayers and black lipid membranes (G. Bähr, A. Diederich, G. Vergères, M. Winterhalter, unpublished work).

Fig. 5 shows that phosphorylation has very little effect on the attachment of unmyr MRP to POPC:POPG 4:1. Again, a hint of electrostatic attraction in the non-phosphorylated case is given by the faster initial binding kinetics. It was verified that the low concentrations of PKM, $CaCl_2$, $MgCl_2$ and ATP present in the solutions did not influence the adsorption kinetics of MRP onto the membranes.

4. Discussion

4.1. Effect of lipid composition

From our observation that the presence of nega-

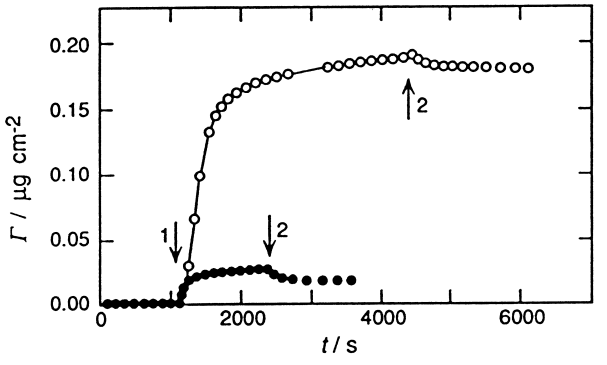

Fig. 4. Effect of anionic phospholipids on the binding of the effector domain of MARCKS to SPLM. Following equilibration of either a POPC (●) or a POPC:POPG 4:1 (○) SPLM with buffer B, a solution containing 0.25 μM peptide corresponding to the effector domain of MARCKS was passed over the membrane (starting at arrow 1). At the times indicated by arrows 2, the protein flow was replaced by buffer B.

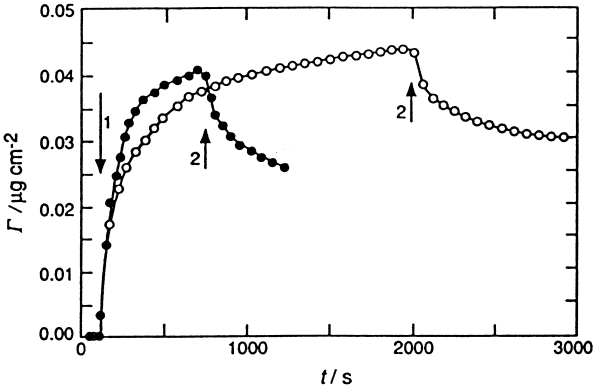

Fig. 5. Effect of phosphorylation on the binding of MRP to SPLM. Following equilibration of a POPC:POPG (4:1) SPLM with buffer B, a solution containing 0.2 μM dephospho (●) or phospho (○) unmyr MRP was passed over the membrane (starting at arrow 1). At the times indicated by arrows 2, protein flow was replaced by buffer B.

tively charged lipids in the membrane does not significantly affect the binding, we infer that electrostatic interactions play a minor role in modulating the interactions of MRP with SPLM at neutral pH and physiological ionic strength. In contrast, binding of a peptide corresponding to the effector domain of MARCKS is strongly increased by the presence of POPG. Hence interactions other than, or additional to, the hydrophobic insertion of the myristoyl group into the bilayer and the electrostatic attraction of positively charged amino acid residues in the effector domain with anionic phospholipids must be involved in the binding of MRP to SPLM.

4.2. Effect of phosphorylation

Numerous in vitro and in vivo studies conclude that phosphorylation could regulate the intracellular localization of MARCKS by decreasing its affinity for phospholipid membranes (see Section 1). We already found that phosphorylation has a barely significant effect on the binding of myr MRP to vesicles [21], and now we find the same using SPLM. Hence PKC-dependent phosphorylation may not significantly regulate the interaction of MRP with lipid membranes, in agreement with several cellular studies [37–39]. This does not exclude the possibility that phosphorylation regulates the interaction of MRP with other membrane-bound proteins, and by this means its intracellular localization.

4.3. Conclusions

What is the origin of the striking differences between MRP binding to vesicles and to SPLM? An obvious difference between the two membrane systems is their curvature, which Honig and coworkers have proposed is coupled to hydrophobicity in protein-membrane interactions [40]. A further difference is that a higher adsorbed protein/lipid ratio was attained with SPLM compared to vesicles; a high ratio favours protein-protein interactions which may promote binding. Note that there is no prima facie case for excluding the possibility that unmyr MRP can bind to pure POPC. Even though electrostatic attraction of the effector domain to the membrane is then absent, other known forces could be involved: the effector domain of MARCKS proteins contains six hydrophobic residues (MRP: four Phe+two Leu; MARCKS: five Phe+one Leu) which should actually contribute to the association of MRP with membranes, and there is already some evidence for it [18,37] (see Fig. 1). These aspects will be discussed in more detail in a subsequent article.

Acknowledgements

We would like to record our gratitude to Christoph Stürzinger for purifying MRP. This research was supported by the Swiss National Science Foundation (grant 3100-042045.94 to G. Schwarz, Department of Biophysical Chemistry, Biozentrum) and the Commission for the Promotion of Scientific Research, Bern (grant 3141.1/3156.2 to JJR).

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