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The Structure of Myristoylated Mason-Pfizer Monkey Virus Matrix Protein and the Role of Phosphatidylinositol-(4,5)-Bisphosphate in Its Membrane Binding

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Abstract

We determined the solution structure of myristoylated Mason-Pfizer monkey virus matrix protein by NMR spectroscopy. The myristoyl group is buried inside the protein and causes a slight reorientation of the helices. This reorientation leads to the creation of a binding site for phosphatidylinositols. The interaction between the matrix protein and phosphatidylinositols carrying C₈ fatty acid chains was monitored by observation of concentration-dependent chemical shift changes of the affected amino acid residues, a saturation transfer difference experiment and changes in ³¹P chemical shifts. No differences in the binding mode or affinity were observed with differently phosphorylated phosphatidylinositols. The structure of the matrix protein–phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P₂] complex was then calculated with HADDOCK software based on the intermolecular nuclear Overhauser enhancement contacts between the ligand and the matrix protein obtained from a ¹³C-filtered/¹³C-edited nuclear Overhauser enhancement spectroscopy experiment. PI(4,5)P₂ binding was not strong enough for triggering of the myristoyl-switch. The structural changes of the myristoylated matrix protein were also found to result in a drop in the oligomerization capacity of the protein.

Keywords

retrovirus; M-PMV; myristoylated; protein structure; phosphatidylinositol

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Supplementary Data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jmb.2012.07.021>

Introduction

Mason-Pfizer monkey virus (M-PMV) belongs to a family of betaretroviruses that form immature virus-like particles (VLPs) in the periplasmic region of an infected host cell. These particles are subsequently transported to and bound by the plasma membrane (PM) prior to their release from the cell.¹ Gag polyprotein is the precursor of all viral structural proteins.² The N-terminal domain of Gag, a matrix protein (MA), is responsible for both the transport of VLPs and their binding to the PM. Most retroviral MAs are N-terminally myristoylated, which is important for both transport and binding of VLPs to the PM.³ Binding of an MA to the PM is facilitated not only by the myristoyl moiety but also by a patch of basic amino acid residues.⁴ Binding must be tight enough to enable budding of the host cell through the PM but must also be reversible because the MA must dissociate from the viral membrane during the early phase of the viral life cycle.⁵ To satisfy these requirements, the MA exists in two conformational states: a myr-sequestered state and a myr-released state. During the transport of Gag to the PM, the myristoyl group is sequestered inside the protein core. When the MA reaches the PM, the myristoyl group is released from the protein core and anchors the MA to the PM.⁶⁻⁸ This change is called the “myristoyl-switch” and was also observed for other myristoylated proteins, such as recoverin⁹ and c-Abl tyrosine kinase.¹⁰

The molecular basis for the interaction between Gag and the PM was described thoroughly for HIV-1^{11,12} and HIV-2¹³ [human immunodeficiency virus (HIV)]. Both HIV-1 and HIV-2 belong to the lentiviridae family, which differs from the betaretroviridae family in the site of VLP assembly. Lentiviral Gag proteins are transported individually to the PM where they assemble to form VLPs prior to the budding.¹⁴ Saad *et al.* demonstrated that the primary trigger for the myristoyl-switch is binding to phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P₂], a phospholipid present only in the PM. PI(4,5)P₂ binds to MA with its inositol head and one of its fatty acid residues, thereby causing conformational changes that lead to myristate exposure.¹² PI(4,5)P₂ also facilitates binding between MA and the membrane. It serves as an additional membrane anchor for MA because one of its fatty acid residues interacts with the protein, while the other remains in the membrane.¹² Both HIV-1 and HIV-2 MAs interact specifically with PI(4,5)P₂ and more weakly with other phosphoinositols¹³; however, other factors may also affect the myristoyl-switch, for example, oligomerization of MAs¹¹ or an interaction between MAs and calmodulin.¹⁵ Nevertheless, the interaction with PI(4,5)P₂ seems to be the most biologically relevant and has been observed with other retroviruses, including equine infectious anemia virus¹⁶ and Moloney murine leukemia virus (MMLV). MMLV MA interacts specifically with PI(4,5)P₂ but only in the presence of phosphatidyl-serine (PS). In the absence of PS, the MA does not discriminate between differently phosphorylated phosphatidylinositol phosphates (PIPs) and binds all of them with the same affinity.¹⁷

Direct interaction between M-PMV MA and PI(4,5)P₂ has not been detected yet, but Stansell *et al.* reported that depletion of PI(4,5)P₂ from the PM led to a dramatic (90%) decrease in VLP release from the host cell.¹⁸ They also found that mutations in lysine residues influence the transport of VLPs and, most importantly, their binding to the PM. VLPs bearing a K16A or K20A point mutation budded into intracellular vesicles. The R10A, R22A, K27A, K33A or K39A mutations caused accumulation near the PM, and the K25A mutation interfered with early phases of VLP transport by causing VLPs to be randomly distributed in the cytoplasm. Other mutations in M-PMV MA disrupt its binding to the PM, including T41I/T78I,¹⁹ Y11F/Y28F and Y28F/Y67F.²⁰ Single mutations slowed down the release of VLPs but were not able to fully arrest it (except the T41I mutation, which resulted in wild-type levels of virus release).

Myristoylation also influences oligomerization of MAs. Tang *et al.* found that myristoylated HIV-1 MA forms trimers in solution, while the non-myristoylated protein remains monomeric.¹¹ Trimerization of HIV-1 MA was reported to trigger myristate exposure. Interestingly, HIV-2 MA, which is very similar to HIV-1 MA, remains monomeric both in the myristoylated form and in the non-myristoylated form.¹³ However, we previously found that non-myristoylated M-PMV MA forms oligomers (mostly dimers and trimers) very readily in solution.^{21,22}

Although myristoylation is essential for the late phase of the viral life cycle, only two structures of myristoylated retroviral MAs have been determined so far, both of which belong to the lentiviridae genera (HIV-1 and HIV-2). The MA-PIP interaction has been studied only with the abovementioned MAs.^{11,13} In this paper, we report the structure of a non-lentiviral myristoylated MA and provide a detailed insight into the molecular basis of its interaction with the PM.

Results

Structure of myristoylated M-PMV MA

We have determined the three-dimensional structure of myristoylated M-PMV MA (myrMA) with a C-terminal 18-amino-acid extension of phosphoprotein (pp24) and a his-tag (further referred exclusively as myrMA). Protein preparation was described previously when we demonstrated that the C-terminal extension of MA does not affect its structure.²³ To determine the structure of myrMA, we used high-resolution multidimensional NMR spectroscopy in combination with computational methods. Myristoylation caused large changes in the ¹H/¹⁵N chemical shifts when compared to the spectra of the non-myristoylated analogue; therefore, we assigned all protein resonances *de novo*. To this end, we used a standard set of three-dimensional experiments. The resonance assignment was deposited into the Research Collaboratory for Structural Bioinformatics (RCSB) database (RCSB ID: RCSB102686). Structure calculations were based primarily on distance constraints and backbone dihedral angle restrictions calculated with TALOS software.²⁴ The exact position of the myristoyl group was determined based on inter-residue contacts between the myristic acid and the rest of the protein determined by three-dimensional ¹³C-filtered/¹³C-edited nuclear Overhauser enhancement spectroscopy (NOESY) experiments.²⁵

The 19 best structures of M-PMV myrMA were chosen out of 100 generated based on the quality of their structural parameters (Fig. 1) and deposited to the Protein Data Bank (PDB) database (PDB ID: 2LPY). The positions of the backbone atoms are well defined: the average rmsd relative to the mean atom positions is 0.91±0.17Å (Supplementary Table 1). The global structure of the myrMA is similar to the structure of the non-myristoylated analogue (rmsd of the backbone atoms is 2.16Å) but also reveals some interesting differences. Both structural motifs are composed of four α-helices (Fig. 2). The myrMA construct contains additional 18 amino acids from pp24 and a his-tag (PPHis) at the C-terminus, which are absent in the (myr-)MA. We previously reported that the PPHis segment does not influence the structure of the (myr-)MA.²³ The N-terminus of the myrMA is fixed by the myristoyl group so that the first helix is located closer to the rest of the protein. Additionally, the orientation of the second helix is slightly different between the two forms. The N-terminus is shifted away from the third and fourth helices in the myrMA when compared to the (myr-) MA, which is observed as the difference between the corresponding inter-helical angles between the second and third helices. While the structure of the (myr-)MA shows that both helices are almost perpendicular (the angle is 88°), the angle between the two helices in the myrMA structure is 67° (Supplementary Table 2). The largest changes are located in the area surrounding the sequestered myristoyl. The myristoyl itself is in an extended conformation and points toward the fourth helix. A hydrophobic pocket, in

which the myristoyl is buried, is formed by the second helix on one side and the C-terminus of the MA together with the loop between the second and third helices on the other side. In contrast to the (myr-)MA, where the C-terminus is located behind the fourth helix and is quite flexible, the C-terminus of the myrMA interacts with the myristoyl group so that it is brought closer and fixed to the rest of the protein. Several nuclear Overhauser enhancement (NOE) contacts indicate that the C-terminal residues of the MA together with the N-terminus of pp24 appended after them (amino acids 97–105) form a short α -helix that is bound to the rest of the protein. The absolute C-terminus of myrMA is unstructured and does not interact with the rest of the protein.

Unlike myristoylated HIV-1 MA, in which about 40% of the fatty acid remained solvent accessible,¹¹ most of the myristoyl group is buried inside the hydrophobic cavity of M-PMV myrMA. We found NOE contacts between the myristoyl group and the side chains of L5, L15, Q48, T50, I51, I86, I90, V95, Q98, A101 and A102. All of these residues, with the exception of glutamines 48 and 98, form the hydrophobic cavity in the interior of the protein; therefore, they are located in close proximity to the myristoyl group.

We have previously found that M-PMV (myr-) MA exists in a concentration-dependent monomer–dimer–trimer equilibrium.²¹ This was discovered by monitoring chemical shift changes in the amino acid residues that form the oligomerization interface. However, upon comparison between the $^1\text{H}/^{15}\text{N}$ heteronuclear single quantum coherence (HSQC) spectra for the (myr-)MA with those of the myrMA at various concentrations ranging from 0.01mM to 0.5mM, we observed much smaller chemical shift changes (Fig. 3). The changes were so small that it was not possible to determine the exact K_d . For a better understanding of the oligomerization state of the myrMA, the chemical cross-linking agent 3,3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP) was used. The results (Fig. 1 in Supplementary Materials) suggest that the prevailing form is a monomer, but some dimers and trimers might also be present. The myrMA exhibits a certain capacity to oligomerize that is not enhanced by myristoylation. In contrast, the NMR data indicate that the myrMA forms oligomers less readily than the (myr-)MA. No evidence that oligomerization might affect the myristoyl-switch was found, as was described previously for the HIV-1 MA.¹¹

Interaction of M-PMV MA with PIPs

Native PI(4,5)P₂ containing stearic and arachidonic acids is not soluble in water but forms micelles instead. Therefore, it is not suitable for NMR measurements in a liquid state because the presence of micelles in solution leads to severe line broadening. Saad *et al.* previously used a more soluble form of PI(4,5)P₂ with shorter fatty acid chains (C₄ and C₈).^{12,13} They found a strong interaction between C₄-PI(4,5)P₂ and HIV-1 and HIV-2 MAs, while C₈-PI(4,5)P₂ caused a precipitation of the MAs even at lower concentrations.

We studied the interaction of PI(4,5)P₂ with both myristoylated and non-myristoylated MAs. The interaction was monitored by observation of the chemical shift changes in the MA amino groups by $^1\text{H}/^{15}\text{N}$ HSQC. The exact binding site of PI(4,5)P₂ on the MA was determined from the NOE contacts between both molecules obtained from three-dimensional ^{13}C -filtered/ ^{13}C -edited NOESY experiments. A saturation transfer difference (STD) experiment together with observation of changes in the ^{31}P chemical shifts provided additional information about the interaction between the PI(4,5)P₂ and the MA.

Titration of the (myr-)MA with C₄-PI(4,5)P₂ did not lead to any significant chemical shift changes; therefore, we concluded that no interaction occurred. We observed chemical shift changes in certain residues with C₈-PI(4,5)P₂, namely, L31, L32, F34, D36, T41, I51, D61, Q64, Y66, Y67, V75, T78, A79 and F80. However, the chemical shift changes were rather small, indicating a weak and likely nonspecific interaction. The affected residues did not

form a contiguous patch on the surface of the MA molecule (Supplementary Fig. 2). Therefore, it was concluded that PIP molecules bind nonspecifically to several places on the MA. PIP:MA ratios greater than 2:1 (molar) led to severe line broadening of the MA signals, which is likely due to the formation of micelles or other high molecular aggregates; therefore, 2:1 was the maximal ratio observed by NMR.

Neither the (myr-)MA nor the myrMA was found to interact with C_4 -PI(4,5) P_2 , but the interaction of C_8 -PI(4,5) P_2 with the myrMA was different from the interaction with the non-myristoylated analogue. The chemical shift changes were approximately 1 order of magnitude larger (Fig. 4), and it was possible to titrate up to the molar ratio of 4:1 (PIP:MA) without a substantial decrease in resolution. The largest changes were observed for residues Q3, L5, L19, G23, V24, K27, L31, F34 and F80 (Fig. 5). These residues are either in close proximity to the myristoyl group or located in or around the loop between the first and second helices. A similar loop is also a part of the PIP binding site in HIV-1 and HIV-2 MAs. ^{13}C -filtered/ ^{13}C -edited NOESY was measured for a mixture of the myrMA and PI(4,5) P_2 at a molar ratio of 1:2 (MA:PIP). NOE contacts between the side chains of K16, L19, V24, V26, L32 and A79 and aliphatic protons of the PIP were detected. Moreover, NOE contacts between the myristoyl group and the rest of the protein were observed. These contacts were identical with those found previously in the ^{13}C -filtered/ ^{13}C -edited NOESY spectrum of the MA without the PIP. The results show that PI(4,5) P_2 binds to a similar site on M-PMV MA as on HIV-1 and HIV-2 MAs. Although the interaction between the PIP and the myrMA is specific, it is not strong enough to trigger the myristoyl-switch and to force the myristoyl group to change into the extended conformational state.

By fitting the combined 1H and ^{15}N chemical shift changes, we calculated the dissociation constant (K_d) of the PI(4,5) P_2 and myristoylated MA complex to be $92.8 \pm 7.5 \mu M$, which is comparable to the K_d of the interaction between HIV-1 and HIV-2 MAs and C_4 -PI(4,5) P_2 . We did not determine the K_d for the non-myristoylated MA due to large errors in the readings of the small chemical shift changes.

To determine which part of the PIP interacts with the MA, we measured STD spectra. In the STD experiment, a selected resonance of the receptor molecule (MA) is irradiated, and the resulting magnetization is transferred through chemical exchange to the ligand where it can be observed in the form of enhanced resonances. The signals of the ligand atoms (primarily protons) directly interacting with the receptor are enhanced more than the remaining atoms. In the STD spectra collected, strong signals from the fatty acid hydrogen atoms were clearly visible, but signals from the rest of the PIP, primarily the inositol head, were missing (Fig. 6). Thus, at least one of the fatty acid residues is in close contact with the MA (both fatty acids are equivalent), but the glycerol and inositol groups may not interact directly with the protein. STD provides no information about the phosphates of the PIP because their hydrogen atoms exchange rapidly with water. Because the PIP binding site on the MA consists of several basic amino acid residues and the importance of the phosphates for the interaction between the PIP and HIV MA was shown previously, ^{31}P spectra of PI(4,5) P_2 with and without the MA were measured. The signals of all three phosphorus atoms were shifted upon binding of the PIP to the myrMA. The largest chemical shift change was detected for phosphorus 4 (0.16ppm) followed by phosphorous 1 and phosphorous 5 (both 0.07ppm, at a 1:1 MA:PIP ratio) (Supplementary Fig. 3). These results prove that PIPs interact with MA not only through their fatty acid chains but also through their phosphate groups.

We also tested the interaction between the MA and differently phosphorylated PIPs, namely, PI(3)P, PI(4)P, PI(5)P, PI(3,4) P_2 , PI(3,5) P_2 and PI(3,4,5) P_3 . No differences were found

between the different PIPs based on the chemical shift changes and STD data (Supplementary Fig. 4).

Structure of myrMA–PI(4,5)P₂ complex

All of the collected experimental data were used to determine the structure of the myrMA–PI(4,5)P₂ complex using the HADDOCK program. Inputs for the program were the structures of the myrMA and PI(4,5)P₂ and a list of amino acid residues with ¹H/¹⁵N combined chemical shift difference (CCSD) greater than 1SD above the average and residues with NOE contacts with PI(4,5)P₂. Calculations were performed for both myristoylated and non-myristoylated MAs.

Out of the 200 calculated structures for the myrMA–PI(4,5)P₂ complex, 129 fell into the most favorable cluster. In each of these structures, PI(4,5)P₂ is located close to the loop between the first and the second helices and the beginning of the fourth helix. One of the fatty acid chains is buried deep into the protein core along the fourth helix (Fig. 7). The inositol group is located on the surface of the protein, and its phosphates interact with residues K25, K27 and K33 (Fig. 8). This structure corresponds well with the experimental data because PI(4,5)P₂ is located in close proximity to the residues with the largest chemical shift changes and interacts with the myrMA primarily through its phosphates and fatty acid chains. The final structure is similar to the structure of PI(4,5)P₂ bound to HIV-1 MA.

We did not obtain one dominant cluster of structures for the (myr-)MA–PI(4,5)P₂ complex but, rather, observed multiple clusters of 20–30 structures. These data correspond with the aforementioned results and prove that binding of PIPs to the (myr-)MA is not specific.

Discussion

We found that the structure of M-PMV myrMA is very similar to the structure of (myr-)MA. The largest differences between the two structures were observed near the myristoyl at the N-terminus. The first helix of myrMA was determined to be parallel with the third and fourth helices as evidenced from the inter-helical angles (Supplementary Table 2). Furthermore, in the structure of the (myr-)MA, the loop between the second and third helices is very flexible; however, in the myrMA structure, it is more tightly bound by the protein body primarily due to the interaction between residue I51 and the myristoyl group.

It was expected that the myristoyl group would be sequestered in the middle of the molecule between the first and second helices.²⁰ In our structure, it is located closer to the C-terminus of the MA and underneath the first helix rather than between the first and second helices. Rhee and Hunter and Stansell *et al.* reported several MA mutants that were unable to interact with the PM and be released from infected cells.^{19,20} They hypothesized that, due to the increased hydrophobicity of the protein core, the VLPs could not expose the myristoyl group. When considering the positions of the mutated amino acid residues (Y11, T41, Y28, Y67 and T78) in the structure, it can be concluded that only mutation at Y11 would be in agreement with this hypothesis because Y11 forms part of the myristoyl group binding site. T41 might also affect myristoyl group binding; however, it is rather distant from the myristoyl, and sole T41I mutation did not prevent budding.¹⁹ However, we found that the remaining mutated residues, Y28, Y67 and T78, are located on the opposite side of the molecule; thus, direct interaction with the myristoyl group would only be possible if the mutation(s) significantly changed the protein structure, which is not the case. These residues are located very close to the PIP binding site; thus, they may significantly contribute to the interaction between the MA and PI(4,5)P₂, and their mutation may affect this interaction rather than cause stronger binding of the myristoyl group. Final conclusions could be drawn

following determination of the structures of the myristoylated mutants (T41I/T78I and/or Y28F/Y67F) and characterization of their binding to both myristoyl and PI(4,5)P₂.

We have recently published the structures of non-myristoylated wild-type MA and an R55F mutant, and we clearly demonstrated that exposure of the CTRS sequence (P43–G60) mediates the interaction of Gag with Tctex-1, which is a cargo-binding component of the dynein molecular motor.²⁶ While the CTRS was exposed on the surface of the wild-type molecule, it was partially hidden in the structure of the R55F mutant. Therefore, the Gag precursor carrying this mutation could not interact with dynein or be transported to the original assembly site within the cytoplasm. Because myristoylated MA is the naturally occurring N-terminal domain of Gag, it was interesting to determine whether the CTRS was exposed in the myrMA structure. The loop between the second and third helices is more rigid due to its interaction with the C-terminus of the myrMA. However, similar to the non-myristoylated MA, the entire CTRS sequence is exposed on the surface of the myrMA molecule (Supplementary Fig. 5), where it can be easily accessed by Tctex-1. This is consistent with our previously published data showing that both non-myristoylated MA produced in bacteria and myristoylated MA produced in COS-1 cells interact with Tctex-1.²⁶

It was previously reported that myristoylated HIV-1 MA readily forms trimers, which is not true for its non-myristoylated analogue.¹¹ Interaction with the PM is energetically more favorable for the trimer when compared to the monomer.^{11,27} Our recent results concerning the non-myristoylated M-PMV MA also demonstrated a certain capacity for the formation of oligomers.²¹ Surprisingly, negligible chemical shift changes were observed for M-PMV myrMA upon increasing its concentration, which indicates limited oligomerization ability. Comparison of the (myr-)MA and myrMA structures suggests that the structural changes caused by burying the myristoyl into the hydrophobic cavity of the MA affected the oligomerization interface on the surface of the molecule. An originally continuous and well-exposed stretch of amino acid residues (T41, W44, F45, D61, C62, D65, Y66, Y67, T69 and F70) became partially hidden and the binding interface segregated into two separate regions (Supplementary Fig. 6). Similar structural changes to the oligomerization interface were described previously for the R55F mutant, which also led to a loss of its oligomerization capacity.²¹ Because these structural changes are caused by the interaction of the myristoyl with the rest of the protein, we can speculate that, upon binding of the MA to the PM, the myristoyl is released from the protein core, which partially restores the oligomerization capacity of the MA. M-PMV virions carrying the R55W(F,Y) MA mutations are noninfectious presumably due to the loss of their ability to incorporate Env proteins.²⁸ Forster *et al.* suggested that the oligomeric arrangement of HIV-1 MA favors Env incorporation.²⁹ Alfadhli *et al.* confirmed that HIV-1 MA is oligomeric when bound to the PM and also suggested that oligomerization of the MA may contribute to Env incorporation.³⁰ Although the precise mechanism of Env incorporation into virus particles has not been clarified, we believe that the trimerization ability of the MA may contribute to Env incorporation into the PM as suggested previously.^{29,30}

We found that the interaction between M-PMV myrMA and PIPs depends strongly on the presence of the myristoyl group and the length of the PIP fatty acid chains. A stronger interaction with C₈-PIPs compared to C₄-PIPs was also reported for HIV-1 and HIV-2 MAs.^{12,13} However, we observed only minor changes in the chemical shifts of M-PMV MA upon interaction with C₄-PIPs, while chemical shift changes comparable to those described for the interaction between C₄-PIPs and HIV-1 MA or HIV-2 MA were observed upon interaction with C₈-PIPs. These results indicate that the interaction between M-PMV MA and PI(4,5)P₂ is weaker than that with HIV-1 or HIV-2 MA. Furthermore, for both HIV-1 and HIV-2 MA, there were no differences in PI(4,5)P₂ binding affinity between myrMA and

(myr-)MA. This observation is slightly contradictory to what we found with M-PMV MA where only the myrMA interacts with PIPs. In other words, myristoylation is a necessary prerequisite for the interaction of PIPs with M-PMV MA. The PIP interaction pocket of the myrMA becomes opened (Fig. 9) upon reorientation of the first and second helices, while no such binding cavity exists on the surface of the (myr-)MA.

The observed changes in the chemical shifts of amino acid residues in close proximity to the myristoyl group upon PI(4,5)P₂ binding suggest a shift of the myristoyl group within the hydrophobic cavity. However, no substantial changes in NOE contacts between the myristoyl group and the rest of the protein were observed, which indicates that the myristoyl group remained in the cavity in the sequestered position. We speculate that if C₈-PI(4,5)P is not long enough to trigger the myristoyl-switch, then the C₁₈ and C₂₀ fatty acids of natural PIPs may be sufficient for this process. In contrast to C₈ fatty acids, either stearoyl or arachidonoyl will insert even further into the MA molecule and might cause release of the myristoyl group to facilitate interaction with the PM.

Both HIV-1 and HIV-2 MAs interact specifically with PI(4,5)P₂, whereas their interactions with other PIPs were reported to be significantly weaker.^{12,13} We did not observe such behavior for M-PMV MA, as all of the tested PIPs caused similar chemical shift changes. Uniform interaction between differently phosphorylated PIPs and the matrix protein was also previously reported for MMLV MA.¹⁷ The interaction of MMLV MA with PIPs was studied by observation of the matrix protein binding to liposomes containing different lipids. Liposomes composed of various PIPs bound the matrix protein with the same affinity. However, liposomes with a mixed lipid composition that contained PS in addition to PI(4,5)P₂ bound the MA more tightly than all the other PIPs. A patch of basic amino acid residues on the MA surface forms a suitable environment for this interaction. Therefore, specific binding of PI(4,5)P₂ may require other phospholipids. In addition, PIPs with unnaturally short fatty acid residues can behave differently than naturally occurring PIPs. M-PMV MA may also interact with other PIPs *in vivo* because it has been shown that M-PMV Gag protein interacts with Env in the endosomal membrane during transport to the PM.³¹ However, interaction between the MA and PI(4,5)P₂ is essential for virus contact with the PM because depletion of PI(4,5)P₂ from the membrane or mutation of the lysine or arginine residues in the PIP binding site blocks virus budding.²⁰

These data show that despite some structural differences between M-PMV and HIV MA, as well as other retroviruses, M-PMV myrMA interacts with PI(4,5)P₂ similarly to the other retroviral MAs. M-PMV myrMA is more similar to HIV-2 MA than HIV-1 MA because its myristoyl group is buried deeper into the protein core and C₈-PIP binding itself is not sufficient to trigger exposure of the myristoyl group.

Materials and Methods

Sample preparation

M-PMV myrMA and (myr-)MA were prepared as previously described.^{23,26} All phosphoinositides were obtained from Echelon Biosciences Incorporated (Salt Lake City, UT, USA) and used without further purification. Samples for NMR experiments were dissolved in buffer containing 100mM phosphate (pH6), 300mM NaCl and 5mM DTT.

NMR spectroscopy

All NMR data were measured on a Bruker Avance^{III} 600-MHz NMR spectrometer equipped with a cryoprobe with the exception of the ³¹P measurements, which were acquired on a Bruker DRX 500 Avance NMR spectrometer (Bruker BioSpin, GmbH, Germany) at the working frequency of 202.4MHz. The backbone atoms of myrMA were assigned using the

standard set of triple-resonance experiments. The distance restraints used for calculation of the structure of the myrMA were determined based on ^{13}C - and ^{15}N -edited NOESY spectra. The positions of the myristoyl group and PI(4,5) P_2 were determined using the ^{13}C -filtered/ ^{13}C -edited NOESY data.²⁵ Chemical shift changes were determined based on $^1\text{H}/^{15}\text{N}$ HSQC spectra. Interaction of PIPs with the MA was also monitored by a saturation difference experiment (STD) and ^{31}P experiments. The data were processed either by TopSpin (Bruker BioSpin GmbH, version 2.1) or NMRPipe³² and further analyzed using Sparky³³ and CcpNmr analysis.³⁴

Structure calculation

The calculation of the myrMA structure was based primarily on pair-wise inter-proton distance restraints obtained from NOESY spectra, the dihedral angles Φ and Ψ and a regular hydrogen bond network. An estimate of the backbone dihedral angles Φ and Ψ was performed with the TALOS program²⁴ and was based on the $^1\text{H}^{\text{N}}$, ^{13}CO , $^{13}\text{C}^{\alpha}$, $^{13}\text{C}^{\beta}$ and $^{15}\text{N}^{\text{H}}$ chemical shifts. Hydrogen bond restraints were used for α -helical segments identified in the later stages of the computational process. Distance restraints were calculated from NOE contacts using r^{-6} distance summation with CcpNmr analysis.³⁴ The structures were calculated with Xplor-NIH software using standard protocols for simulated annealing with torsion angle dynamics.³⁵ The myristoyl group was parameterized using PRODRG2³⁶ and HIC-UP³⁷ Web servers. A final set of 19 out of 100 calculated structures were chosen for further analysis. Structures were visualized with the PyMOL³⁸ and VMD³⁹ programs and validated using the iCING Web server.⁴⁰ The inter-helical angles and distances were calculated with the UCSF Chimera 1.6.1 program.⁴¹

Determination of the oligomerization state

A chemical cross-linking study was performed using DTSSP. We allowed (myr-)MA and myrMA samples with a concentration of 0.18mM protein to react with DTSSP at final concentrations of 0.1, 0.2, 0.5 and 1mM for 1h. The reactions were quenched using 1.5M Tris and analyzed by Tris-*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine SDS-PAGE followed by staining with Coomassie brilliant blue G-250. Chemical shift changes were observed by measuring the $^1\text{H}/^{15}\text{N}$ HSQC spectra of myrMA at concentrations ranging from 0.05mM to 0.5mM.

Interaction with phosphatidylinositols

Both (myr-)MA and myrMA, at a concentration of 0.1mM, were mixed with PIPs [either C_4 or C_8 , PI(3)P, PI(3,5) P_2 , PI(3,4,5) P_3 , PI(4)P, PI(4,5) P_2 and PI(5)P] at MA:PIP molar ratios from 10:1 to 1:5 in 100mM Hepes, 300mM NaCl, 5mM DTT (pH6) and 10% D_2O . $^1\text{H}/^{15}\text{N}$ HSQC spectra were measured and chemical shift changes were read by Sparky software.³³ The $^1\text{H}/^{15}\text{N}$ combined chemical shift (CCSD) changes were calculated using following formula:

$$\delta_{\text{CCSD}} = \sqrt{\delta_{\text{H}}^2 + \frac{\delta_{\text{N}}^2}{25}}$$

The threshold for the CCSDs of active residues was set to 1SD above the average CCSD of all residues. The equilibrium dissociation constants (K_{d} values) were determined by nonlinear minimization performed by in-house routines written in MATLAB,⁴² using the following formula:

$$\delta_{\text{CCSD}}^{\text{obs}} = \delta^{\text{MAX}} \frac{(K_d + [L] + [P]) - \sqrt{(K_d + [L] + [P])^2 - 4[P][L]}}{2[P]}$$

where δ^{MAX} and K_d are two fitted parameters, and [P] and [L] are the protein and ligand concentrations, respectively.⁴³

To determine the PIP binding site on the MA, we measured ^{13}C -filtered/ ^{13}C -edited NOESY²⁵ on an equimolar mixture of myrMA and PI(4,5)P₂ at a concentration of 0.5mM. The structure of the MA–PIP complex was then calculated by HADDOCK⁴⁴ with ambiguous interaction restraints derived from chemical shift changes and NOEs. The active myrMA residues were L15, A18, L19, R22, G23, V24, K25, V26, K27, L31, L32, F34, A79 and F80, and the active (myr-)MA residues were L15, V24, L31, D36, I51, D52, I53, D56, T78 and A79. Passive residues were determined automatically. The PDB structure of PI(4,5)P₂ was generated using the PRODRG2 Web server.³⁶

STD experiments were measured on a mixture of 0.1mM PIP and 0.02mM MA, and ^{31}P data were collected from an equimolar mixture of 0.1mM PIP and MA. Both samples were dissolved in 100mM phosphate (pH6), 300mM NaCl, 5mM DTT and D₂O (99.99% deuterium) buffer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

M-PMV	Mason-Pfizer monkey virus
VLP	virus-like particle
PM	plasma membrane
HIV	human immunodeficiency virus
PIP	phosphatidylinositol phosphate
PI(4,5)P₂	phosphatidylinositol-(4,5)-bisphosphate
MMLV	Moloney murine leukemia virus
PS	phosphatidylserine
NOESY	nuclear Overhauser enhancement spectroscopy
NOE	nuclear Overhauser enhancement
HSQC	heteronuclear single quantum coherence
DTSSP	3,3-dithiobis(sulfosuccinimidyl propionate)
STD	saturation transfer difference
RCSB	Research Collaboratory for Structural Bioinformatics

PDB	Protein Data Bank
CCSD	combined chemical shift difference

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Fig. 1. Superposition of the 19 best structures of myristoylated M-PMV MA. The helices are marked by roman numbers, and the myristate is shown in red.

Fig. 2. Comparison of the myristoylated MA (green) and non-myristoylated MA (red). The myristoyl group is represented as sticks. The structures were superimposed with the PyMOL program.³⁸

Fig. 3. ^1H and ^{15}N CCSD histograms of myristoylated and non-myristoylated MA residues calculated from the $^1\text{H}/^{15}\text{N}$ HSQC spectra of the MAs at a concentration range of c_0 to $c_0/10$.

Fig. 4. Dependence of the MA L31 residue ^1H and ^{15}N CCSD on the PIP:MA molar ratio. Myristoylated MA (blue diamonds) and non-myristoylated MA (red squares).

Fig. 5. Structure of the myristoylated MA with the residues significantly affected by interaction with C₈-PI(4,5)P₂. ¹H/¹⁵N CCSDs greater than 1SD above the average CCSD are highlighted. The myristoyl group is shown in blue, and the affected residues are shown in red and pink (the CCSDs of the red residues were greater than 1SD above the average CCSD in all steps of the titration, whereas the CCSDs of the pink residues were greater in at least four points).

Fig. 6. ¹H STD spectrum of a C₈-PI(4,5)P₂:MA 50:1 (molar) mixture (black) together with the assigned ¹H spectrum of pure C₈-PI(4,5)P₂ (red).

Fig. 7. Structure of the myristoylated MA (green)– C₈-PI(4,5)P₂ (blue) complex calculated by HADDOCK.⁴⁴ Phosphates are shown in orange and red.

Fig. 8.
The myrMA and C₈-PI(4,5)P₂ complex showing the interaction between myrMA lysines (green) and C₈-PI(4,5)P₂ phosphates (red).

Fig. 9. Structure of the MA (a) and myrMA (b) as van der Waals surface representations (gray) with lysine residues highlighted in blue to show the binding cavity created by the first, second and third helices of myrMA bound to a molecule of C₈-PI(4,5)P₂ (cyan).