Biochemical Characterization of Rous Sarcoma Virus MA Protein Interaction with Membranes

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The MA domain of retroviral Gag proteins mediates association with the host cell membrane during assembly. The biochemical nature of this interaction is not well understood. We have used an in vitro flotation assay to directly measure Rous sarcoma virus (RSV) MA-membrane interaction in the absence of host cell factors. The association of purified MA and MA-containing proteins with liposomes of defined composition was electrostatic in nature and depended upon the presence of a biologically relevant concentration of negatively charged lipids. A mutant MA protein known to be unable to promote Gag membrane association and budding in vivo failed to bind to liposomes. These results were supported by computational modeling. The intrinsic affinity of RSV MA for negatively charged membranes appears insufficient to promote efficient plasma membrane binding during assembly. However, an artificially dimerized form of MA bound to liposomes by at least an order of magnitude more tightly than monomeric MA. This result suggests that the clustering of MA domains, via Gag-Gag interactions during virus assembly, drives membrane association in vivo.

The assembly and budding of retroviruses are driven by protein-nucleic acid, protein-protein, and protein-lipid interactions. These inter- and intramolecular interactions are mediated by the viral structural polyprotein Gag. Membrane binding of Gag is an essential step in assembly and budding, and the N-terminal MA domain of Gag mediates this interaction. For most retroviruses, Gag multimerization traditionally has been assumed to take place at the plasma membrane (94) but more recently has been suggested to commence at internal membranes (53, 58, 83). It is unclear to what extent Gag-Gag interactions also occur prior to membrane binding. While the membrane binding signals of many retroviral Gag proteins have been identified, little is known in biochemical terms about the intrinsic affinity of Gag for membranes in the absence of cellular factors.

For most retroviruses, Gag-membrane binding signals consist of two elements, an N-terminal myristate fatty acid modification and a patch of basic residues (8, 24, 36). However, the Gag proteins of a few retroviruses, notably the alpharetroviruses (the prototype of which is Rous sarcoma virus [RSV]) and the lentivirus equine infectious anemia virus (EIAV), lack myristate modification. Bipartite myristate-plus-basic membrane binding signals are common among cellular membrane binding proteins, for example, the oncprotein Src (9). The myristate moiety attached to the N terminus contributes hydrophobic interactions to membrane binding, and in the case of human immunodeficiency virus type 1 (HIV-1), is a well-established requirement for proper assembly and budding (8, 32, 57, 74, 89, 97). However, studies of the binding of myristylated peptides to liposomes imply that the fatty acid modification alone does not provide sufficient energy to support stable protein-membrane interactions (62). It is unclear to what extent the myristylation of HIV-1 Gag is sufficient for membrane association and assembly. On the one hand, in vivo studies monitoring Gag localization, membrane binding, and particle release identified specific regions within MA, in addition to the myristate moiety, that are critical for plasma membrane association (60, 89, 96, 97). The first 31 amino acids of HIV-1 MA form a highly basic patch on the surface of the protein (37) which has been inferred to interact with acidic lipids in the inner leaflet of the plasma membrane. Both in vitro and in vivo studies corroborate that this region can mediate protein-lipid interactions (60, 89, 97). On the other hand, large portions of the basic region of HIV-1 MA can be deleted with little effect upon membrane binding in vivo and virus-like-particle (VLP) release (14, 20, 88). Indeed, HIV-1 VLPs can be released from transfected cells if the entire MA domain is replaced with a myristylation signal (32, 74). Thus, while basic residues in HIV-1 MA probably do contribute to membrane binding, the main functions of the downstream sequences within MA might be to target Gag to the correct cellular membrane where assembly occurs, perhaps by regulating exposure of the myristate moiety (20, 26, 60, 61, 88, 90, 98). Discrepancies in the inferred importance of downstream regions of MA for the membrane binding of Gag in vivo may be attributable at least in part to differences in protein expression. The considerable divergence among the results on HIV-1 Gag membrane binding in vivo highlights the difficulty in drawing conclusions from experiments that cannot measure the intrinsic membrane affinity of a protein in the absence of cellular factors.

Few in vitro studies of retroviral Gag protein-membrane associations have been published. In one type of protocol, radioactive MA or Gag proteins synthesized in reticulocyte extracts were mixed with natural membranes or liposomes and binding was monitored by sedimentation (18, 26, 68, 97, 98).
With this method, the N-terminal region of HIV-1 MA was shown to contain an electrostatic membrane binding signal (97). While reticulocyte expression systems are easy to manipulate and offer the advantage of proper secondary modifications, such as myristylation, the vast excess of crude cell components makes results from such experiments difficult to interpret in biochemical terms. Similar problems are inherent in the use of natural membranes as targets for binding. Furthermore, sedimentation assays cannot unambiguously distinguish membrane-bound proteins from protein aggregates. In a second type of protocol, purified MA proteins derived by Escherichia coli expression were used in fluorescence-quenching studies to monitor membrane associations (18, 70, 81). Both EIAV MA (which is not naturally myristylated) and unmyristylated HIV-1 MA were reported to interact tightly with acidic liposomes, apparently reinforcing the importance of electrostatic interactions for Gag-membrane binding (18, 70). However, the report by the same authors that HIV-1 CA, which presumably does not interact with membranes in vivo, also associates with acidic membranes with a high affinity (18) calls into question the biological relevance of the results from the fluorescence-based assays.

To date, information about RSV Gag-membrane binding has come from studies of virions and living cells. Early work showed that in virions, MA (then called p19) could be specifically cross-linked to phosphatidylethanolamine, thus identifying this protein as the retroviral matrix protein, later named MA (64). An extension of this study revealed that the site of cross-linking was in the first 35 amino acids of MA (63). More recently, the functional membrane binding region of RSV Gag was mapped to the first 87 amino acids of MA (50, 91), which therefore was labeled the membrane binding domain (MBD). Even small deletions in the MBD block assembly and budding (50). The MBD has an overall charge of +4 at a neutral pH, and although the charges are not clustered in the primary amino acid sequence, the solution structure reveals that several basic residues form a patch on the surface of the protein (38). Since MA is not myristylated, this patch has been hypothesized to be responsible for mediating membrane binding during assembly through interactions with negatively charged lipids in the inner leaflet of the plasma membrane. In support of this hypothesis, mutations that alter the overall charge of the MBD affect virus particle production (11). While changing any single basic amino acid residue did not compromise budding, changing any two basic amino acid residues to neutral ones blocked the release of VLPs from transfected cells expressing RSV Gag tagged with green fluorescent protein (GFP).

In the present study, we describe an in vitro flotation assay for directly measuring RSV MA-membrane interactions. The biological relevance of this analysis is supported by the requirement for acidic lipids and by the binding properties of a mutant MA protein. These experimental results are complemented by computational modeling of the membrane association of MA. Both types of analysis are in quantitative agreement and imply that the MA-lipid interaction is electrostatic and relatively weak. Artificially induced dimerization of MA increased its affinity for acidic liposomes 10- to 100-fold. We interpret this result to mean that clustering of MA domains via Gag-Gag interactions is necessary for efficient membrane association during virus assembly.
The frozen cell pellets were resuspended in lysis buffer (20 mM Tris [pH 8], 500 mM NaCl, 10% glycerol, 0.5% [wt/vol] TX-100, 0.5% [wt/vol] DOC, 1 mM phenylmethylsulfonyl fluoride) at 25 ml/mg for unlabeled and 100 ml/mg for radiolabeled pepts. The cells were broken by sonication and the lysates were centrifuged at the equivalent of 440,000 x g for 45 min. The His-tagged proteins were purified by nickel affinity chromatography (QIAGEN), and purified proteins were stored in elution buffer plus 10 mM dithiothreitol at 4°C for no more than 1 month. Protein concentrations were determined by spectrophotometry using the Advanced Protein Assay reagent (Cytoskeleton), and counts per minute were determined for radiolabeled pepts by scintillation counting.

**Liposome preparation.** Purified phosphatidylcholine (PC) from egg, phosphatidylethanolamine from E. coli, phosphatidylserine from brain, phosphatidylinositol,4,5-phosphate (PIP2) from brain, and cholesterol from wood greese were purchased from Avanti Polar Lipids and stored in chloroform at -80°C. Liposomes were prepared by mixing the desired lipids in siliconized glass tubes and evaporating the chloroform under a stream of nitrogen. The dried lipids were resuspended by vortexing in lipid buffer (20 mM Tris [pH 8], 5 mM NaCl, 5 mM EDTA) to a final concentration of 50 µg/µl. The resulting multimamellar vesicle suspension was then passed at least 30 times through a 100-nm nitrocellulose filter in an Avanti extruder apparatus to yield liposomes of uniform size. Liposomes were prepared fresh before each experiment.

**Liposome binding assay.** Unless otherwise stated, the protein concentration was estimated by the Advanced Protein Assay reagent (Cytoskeleton), and counts per minute were determined for radiolabeled peptides by scintillation counting. We found that at much lower protein concentrations, the results of the binding assays were less reproducible. Protein concentrations, lipid concentrations, salt concentrations, and lipid compositions varied as described in the text. Each binding reaction mixture was incubated with purified Gag(1-157) containing 4% (wt/wt) sucrose, both in the same buffer. The gradients were centrifuged for 45 min at 100,000 rpm, and unless otherwise noted, 30-ml fractions were collected from the bottom. The fractions were subjected to electrophoresis in 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and the gels were dried and scanned using the ImageQuant program (Molecular Dynamics).

Proteins in the top three fractions were counted as liposome-bound. These fractions contained approximately 50% of the recoverable lipid from each gradient. To determine the amount of recoverable lipid, we floated increasing concentrations of liposomes labeled with [3H]cholesterol through gradients in the absence of protein. After fractionation, the centrifuge tubes were rinsed with SDS to solubilize any remaining lipid, which comprised approximately 5% of the radioactivity in the input, and this was termed unrecoverable lipid. Of the recoverable lipid, 80% appeared in the first three fractions and 20% appeared in the pooled fractions from the rest of the gradient. Since the centrifugation conditions used should be sufficient to float all of the liposomes, we interpreted the counts not found in the top fractions as resulting from the technical difficulty of fractionating small (240 µl) gradients. Thus, our measurements may underestimate the amount of protein actually bound to liposomes.

To interpret the binding experiments, we applied a simple mass action equation which assumes that the protein binds reversibly to the lipid. Previous studies have shown that although the phenomenon being studied is actually a partitioning event, not a binding event, one can still apply a simple mass action equation to describe the protein-lipid interaction if the concentration of protein is kept much lower than that needed to saturate the surface of the membrane (5). Since MA does not penetrate the lipid bilayer, the accessible lipid is limited to the outer leaflet of the liposome and therefore is equal to one-half of the total lipid added to the binding reaction, as described previously (62). One estimate for what constitutes a saturating protein concentration is based on the assumption that the inner membrane surface of the RSV virion is covered by mature MA. The weight ratio of MA to lipid in virions is approximately 1:1 (wt/wt) (92), and thus we assumed that for the in vitro binding experiments this ratio is saturating. However, based upon surface area calculations, this is likely an underestimate of the total amount of MA required to saturate an equal amount of lipid. From the nuclear magnetic resonance structure of the RSV MBD, one molecule of MA would be expected to cover approximately 13 nm² of membrane surface, which corresponds to about 20 lipid molecules. Based on these approximations, in our binding assays lipids were always in excess of proteins.

**Computational modeling of RSV matrix constructs.** The crystal structure of the RSV MBD (Protein Data Bank [PDB] identifier, 1a6s) (38) is composed of residues 1 to 87 of the RSV Gag polyprotein. Three protein secondary structure prediction programs, PSSPRED (39), 3D-PSSM (30), and SSPRO-2 (69), predicted that residues 88 to 100 of RSV Gag form an a-helix, which may be structurally comparable to the fifth helix observed in the MA proteins from EIAV and HIV-1 (PDB identifiers, Ihek [25] and Ihiv [27], respectively); these programs predicted that residues 101 to 157 of RSV Gag are predominately unstructured. An alignment of the known and predicted secondary structure elements of RSV Gag(1-100) with the secondary structure elements of retroviral MAs with known structures was used as a guide to create a three-dimensional model for the sequence of Gag(88-100) based on the C-terminal regions of other MAs. A composite homology model for RSV Gag(1-100) was constructed with the program Nest (67), using the structure of the RSV MBD (PDB no. 1a6s) as a template for residues 1 to 87 and the fifth a-helix of EIAV MA as a template for residues 88 to 100. The composite model for RSV Gag(1-100) scored well according to the structure evaluation program Verify3D (35). All three constructs, i.e., RSV MBD, Gag(1-100), and RSV MA, are predicted to have the same electrostatic component for binding to PS-containing membranes.

The protein threading and fold recognition programs FUGUE (84), 3D-PSSM, and 123D+ (1) did not detect any suitable templates for Gag(101-157) among structures in the PDB. The ab initio-like program HMMSSTR (10) was used to obtain a model of Gag(101-157). The final model for RSV MA, i.e., Gag(1-157), was constructed in Nest as a composite model of the Gag(1-100) and the HMMSSTR model of Gag(101-157). This larger composite model also scored well according to Verify3D. The crystal structures of MA dimers from EIAV (PDB no. 1hek) and Moloney murine leukemia virus (PDB no. 1m8b) (75) were used as templates to produce various oligomeric models of the RSV MBD. For example, a dimeric model of the RSV MBD was obtained by a structural superimposition of 1a6s on both subunits of the EIAV MA dimer by use of the program Combinatorial Extension (85). The program CHARMM (7) was used to produce mutant forms of the RSV MBD.

**Electrostatic calculations.** Our calculations are based on solutions to the Poisson-Boltzmann equation, which is an equation from classical electrostatics (29). This methodology has been widely applied to characterize the electrostatic properties of protein/membrane, protein/protein, and protein/nucleic acid systems (3, 29, 46, 48). Electrostatic potentials and free energies were obtained from a modified version of the DelPhi program (22) that solves the nonlinear Poisson-Boltzmann (PB) equation for protein-membrane systems. Briefly, the DelPhi program employs the finite difference PB (FDBP) method to produce finite difference solutions to the PB equation for a system for which the solvent is described in terms of a bulk dielectric constant and concentrations of mobile ions, whereas solutes (in this case, RSV MBD, MA, and PC/PS phospholipid bilayers) are described in terms of coordinates of the individual atoms as well as their atomic radii and partial charges (6, 44). The FDBP method is described in detail in a number of papers and yields results that are in excellent agreement with experimental studies of the binding of peptides and proteins to membranes (6, 47, 48). The electrostatic free energy of interaction between the protein and the membrane, ΔGia, is determined as the difference between the electrostatic free energy for the protein/membrane system and the electrostatic free energies for the protein and membrane taken separately. For the calculations reported here, the ΔGia values are precise to within 0.2 kcal/mol. The GRASP program (52) was used to visualize the electrostatic equipotentials, as calculated with the nonlinear PB equation, of the RSV MBD/membrane system.

**RESULTS.**

We have developed an in vitro liposome-binding assay to study the molecular requirements for the RSV MA-membrane interaction. Briefly, unlabeled and 35S-radiolabeled preparations of recombinant MA domain-containing proteins were purified in parallel from E. coli by nickel affinity chromatography. The proteins were incubated with 100 mM PS-containing liposomes under conditions in which the surface of the bilayer was not saturated with protein (see Materials and Methods). The binding reaction mixtures were adjusted to 53% sucrose, transferred to a centrifuge tube, overlaid with a step sucrose gradient, and then centrifuged to separate the liposomes and any bound protein (which float) from unbound protein (which
remain at the bottom of the gradient). Fractions were collected from the top and subjected to SDS-polyacrylamide gel electrophoresis and quantitation with a phosphorimager. This assay, which is similar to assays used to monitor membrane binding of other viral matrix proteins (78, 79) and binding of retroviral envelope proteins to exposed fusion peptides (15, 17), allowed several parameters that affect protein-membrane interactions to be determined. Complementary calculations based on the FDPB method (6, 22, 29, 46) were performed with atomic models of RSV MA and phospholipid membranes to provide a molecular basis for the experimentally observed membrane binding.

Dependence of binding on ionic strength. The N-terminal half of the MA domain of RSV Gag is required for membrane binding of Gag in vivo (50, 91). This region, i.e., the MBD, has a patch of positive residues on its surface (38) which has been shown to be critical for membrane binding and budding (11) (Fig. 2A). While these results support the hypothesis that the RSV MA-lipid interaction is electrostatic, in vivo studies cannot eliminate the possibility that the basic residues in MA play some other role in assembly and budding.

To address the nature of the MA-lipid interaction, we incubated 50 μg of unlabeled MA-p10 (Fig. 1) with 200 μg 2:1 PC:PS liposomes under conditions of increasing ionic strength. PC is a zwitterionic, neutral phospholipid and PS is a negatively charged, acidic phospholipid. At 75 mM NaCl, almost half of the protein floated to the top three fractions, which contained approximately 80% of the recoverable liposomes (see Materials and Methods) and which we interpret to represent liposome-bound protein (Fig. 3). As the concentration of NaCl was increased, the amount of bound protein decreased, and at 500 mM NaCl, binding was undetectable. These results demonstrate that the RSV MA-membrane interaction is primarily electrostatic in nature.

In agreement with these experiments, our FDPB calculations predicted that binding to 2:1 PC/PS membranes would decrease dramatically as the ionic strength increases. Specifically, the electrostatic interaction of MA with the membrane is predicted to decrease 80-fold as the concentration of NaCl is increased from 100 mM to 500 mM. In addition, our calculations indicated that the membrane interaction of MA should be observable in experimental assays when the ionic strength is 100 mM (KD, ~10^-2 M; ΔG_{eq}, ~−4.1 kcal/mol) but undetectable when the ionic strength is 500 mM (KD, ~10^-2 M; ΔG_{eq}, ~−1.5 kcal/mol), as we observed experimentally. This agree-
ment between experiment and theory strongly suggests that there is a large electrostatic component to the membrane interaction of RSV MA.

**Dependence of binding on lipid concentration.** Experimental and computational studies of the binding of basic peptides (such as pentalysine) to 2:1 PC:PS liposomes have led to the conclusion that each basic residue contributes approximately 1.5 kcal/mol in free energy toward the protein-lipid interaction, corresponding to a dissociation constant ($K_D$) of approximately $10^{-4}$ M per basic residue (6). For example, the $K_D$ of pentalysine is approximately $10^{-4}$ M. However, the peptide-lipid interaction is not defined solely by the number of charged residues but also by their spacing. Inserting neutral or acidic amino acids between the basic residues of pentalysine reduces the affinity by about 1 order of magnitude (42). Thus, an accurate prediction of a protein’s electrostatic affinity for a membrane requires either knowledge of the protein structure to generate computational models or biochemical measurements (or both) (5).

To determine the apparent $K_D$ for the RSV MA-lipid interaction, we incubated constant, limiting ($\mu$g) amounts of cold MA with trace amounts of radiolabeled protein (for quantitation) in the presence of increasing concentrations of 2:1 PC:PS liposomes. Three representative flotation gradients are shown in Fig. 4A. The fraction of protein that floated in each experiment was plotted against the concentration of accessible lipid to generate binding curves (Fig. 4B). The $K_D$ for the protein-membrane interaction can be determined from each binding curve as the concentration of lipid which supports half-maximal protein binding. This value was found to be approximately $10^{-3}$ M for mature MA (Fig. 4B, diamonds), based on the assumption that one-half of the lipid, i.e., the outside of the liposomes, is accessible to the protein.

To confirm that the interaction being analyzed in these experiments is biologically relevant, we included MA from the K18N/K23N-Gag mutant, which had been shown in vivo to be incapable of membrane binding or budding (11), as a negative control. This mutant Gag protein has two mutations of lysine residues in MA to asparagine. MA(K18N/K23N) was severely impaired in its ability to bind liposomes (Fig. 4A and B, triangles). The slight binding at the highest lipid concentrations presumably was due to the remaining positive charges in the MBD, which could interact with acidic lipids. The binding was too weak to permit a calculation of the $K_D$, but wild-type MA bound at least an order of magnitude more tightly than the mutant. The correlation between these results and the in vivo experiments lends confidence in the in vitro binding assay as a biologically relevant tool to study membrane interactions.

Computational studies corroborated the experimentally derived $K_D$. The calculations provided approximate $K_D$ values based on the minimum electrostatic free energy of interaction of MA with the membrane (6). For 2:1 PC/PS and 100 mM NaCl, the predicted $K_D$ for wild-type MA was $10^{-3}$ M, in agreement with the experimentally derived value. Figure 2 illustrates the molecular basis of this interaction: the calculations accounted not only for the atomic detail of the molecules and hence the complex distribution of charge on the protein surface (Fig. 2A) but also for how the protein and membrane affect each other’s potentials, as depicted in Fig. 2B. In addition, our calculations predicted that the double mutation K18N/K23N would decrease the association 20-fold, suggesting that the decrease in membrane binding is due to a change in the electrostatic character of the protein upon the introduction of the mutations.

We next investigated whether MA proteins that are C-terminally extended to include downstream domains of Gag (Fig. 1) behave similarly to mature MA. This inquiry was motivated...
by a report that HIV-1 Gag appears to have a higher affinity for membranes than does mature HIV-1 MA (18, 88, 98). As shown by way of example, MA-NTD (Fig. 4, squares) bound to liposomes with an affinity similar to that of MA. All other extended forms of RSV MA that were tested also bound similarly (data not shown). Proteins extending through the entire CA could not be tested because MA-NTD is the longest MA-domain-containing protein that we have been able to purify in a soluble form from E. coli. The results from these experiments imply that at least for this in vitro system, downstream sequences through the N-terminal domain of CA do not modulate the affinity of MA for liposomes.

Role of cooperativity in lipid binding. The nature and extent of MA-MA self-association are not understood in detail. HIV-1 MA crystallizes as a trimer (27), and a lattice of trimers has been used to model the disposition of MA inside the virion (19). The existence of trimers of unmyristylated HIV-1 MA has been reported for some conditions (41), but this observation is controversial. The more biologically relevant myristylated form of HIV-1 MA exists in a monomer-trimer equilibrium in solution, with the myristate moiety playing a major role by promoting trimerization (90). However, it is unknown if RSV MA forms oligomers in solution.

If MA can self-associate to form multimers, either in solution before membrane binding or on the membrane itself, then the multimers should have a higher intrinsic affinity for liposomes than the monomers, resulting in an apparent cooperativity for binding. We addressed the hypothesis that RSV MA could bind cooperatively to liposomes by carrying out flotation experiments at different protein concentrations. Liposomes (500 µg of 2:1 PC:PS) were incubated with trace amounts of 35S-labeled protein and increasing amounts of unlabeled protein. The ratio of bound protein to total protein for each gradient are plotted on the y axis. MA data are averages of two independent experiments. MA-NTD data are averages of three independent experiments. Error bars represent standard deviations from the means.

FIG. 5. Effect of protein concentration on MA-lipid interaction. Liposomes (500 µg of 2:1 PC:PS) were incubated with approximately 3 µg of 35S-labeled protein and increasing amounts of unlabeled protein. The ratio of bound protein to total protein for each gradient are plotted on the y axis. MA data are averages of two independent experiments. MA-NTD data are averages of three independent experiments. Error bars represent standard deviations from the means.

CA with the last 25 amino acids of p10 (25-NTD) suggested that this region of Gag may be capable of dimerization (49). It seemed possible that longer MA proteins including this portion of Gag might bind to liposomes cooperatively, even though MA itself did not. Therefore, we carried out the same experiment to test the concentration dependence of MA-NTD. The results were similar to those for MA (Fig. 5, squares), suggesting that under these conditions dimerization did not occur.

Effect of forced dimerization on binding. The weak affinity of RSV MA for acidic liposomes apparently would not provide enough energy to support efficient Gag-membrane binding during assembly in vivo. However, during assembly, Gag-Gag interactions bring several MA domains together, which should result in a stronger avidity for lipid bilayers than that of the individual domains alone. Experimental evidence that Gag multimerization in vivo enhances membrane binding comes from studies of both HIV-1 and RSV, which showed that deletions of the NC domain which abrogate Gag-Gag interactions also block membrane binding (31, 68, 80). However, it is important that two of these studies (68, 80) used sedimentation analysis to quantitate membrane-bound proteins and that other interpretations of these data are difficult to exclude. For example, cellular proteins might interact with NC, indirectly affecting membrane binding.

We wanted to determine whether dimers of RSV MA have a higher affinity for acidic liposomes than MA monomers. To create dimers, we fused the HIV-1 C-terminal domain of CA (CTD), together with the immediately upstream linker sequence (comprising CA residues 145 to 231), to the C-terminal end of RSV MA (Fig. 1). The HIV-1 CTD is known to self-associate with a moderate affinity ($K_D \sim 10^{-5} \text{M}$) (16, 23, 76). This self-association can be abrogated by changing two hydrophobic amino acids at the dimer interface (W184A and M185A) and can be enhanced by an order of magnitude ($K_D \sim 10^{-7} \text{M}$) by mutating a glutamine residue (Q192A) (16). We incorporated both sets of mutations into fusion proteins, creating MACA_{CTD(W184A/M185A)} and MACA_{CTD(Q192A)} (Fig. 1). These proteins were expressed, purified, and examined by velocity sedimentation on sucrose gradients. The analysis confirmed that in the concentration range of 10 µM, the two proteins behaved as monomers and dimers, respectively (data not shown).

To determine the binding properties of the MACA_{CTD} chimeras, we incubated trace amounts of each radiolabeled fusion protein with unlabeled proteins at concentrations sufficient to promote HIV-1 CA_{CTD} dimerization and measured liposome binding with increasing concentrations of 2:1 PC:PS liposomes. MACA_{CTD(W184A/M185A)} (Fig. 6, diamonds) bound with an affinity indistinguishable from that of MA (Fig. 6, squares; these data were reproduced from Fig. 3 for comparison), ruling out nonspecific effects of the HIV-1 polypeptide sequence itself. In contrast, MACA_{CTD(Q192A)} (Fig. 6, triangles) bound more than an order of magnitude more tightly to acidic liposomes. The wild-type MACA_{CTD}, i.e., the protein without either the W184A and M185A mutations or the Q192A mutation, behaved like MACA_{CTD(Q192A)} at the concentrations tested (data not shown). Taken together, these data are entirely consistent with the hypothesis that Gag-Gag multimerization during RSV assembly drives membrane binding. Mul-
timerization might induce a conformational change in MA, exposing a high-affinity binding site, or alternatively, the more MA domains which are tethered together, the tighter the overall membrane binding affinity of the complex becomes. This concept of membrane binding domain multimerization would explain how a very weak membrane binding motif, such as that measured for RSV MA, could support assembly at the plasma membrane in vivo.

We created several models for dimers of RSV MBD based on retroviral MA dimer structures that have been solved recently (25, 75). In one example, the MBD monomer structure was superimposed on each of the subunits of the EIAV MA dimer structure (Fig. 7). Calculations predicted that this model dimer would interact with 2:1 PC/PS in 100 mM NaCl 100-fold more strongly than the monomer. Similar results were obtained for dimer models based on the dimer structures observed for Moloney murine leukemia virus MA. Most likely, the MA domains in the MACACTD(Q192A) constructs are not as tightly associated as depicted here, since they are likely to be tethered at some distance. This would reduce the cooperativity in binding, as observed for other constructs with two membrane binding motifs, e.g., the N-terminal portion of Src, the MARCKS protein, and the C-terminal portion of K-Ras (40). However, the model may approximate how RSV MA domains cooperate to produce enhanced membrane association and how they interact at the membrane surface during assembly and in the mature virion, in which the concentrations of retroviral proteins are very high.

Dependence of membrane interaction on concentration of PS. Studies of basic peptides have shown that membrane binding does not occur with uncharged (PC) liposomes and depends directly upon the mol% of PS present in the liposomes (5, 6, 42, 45, 47). To determine the role of negative charge density on the membrane binding of MA, we prepared PC liposomes with increasing mol% PS and carried out parallel flotation experiments using 500 μg of lipid. The fraction of total protein that floated to the buoyant density of the liposomes was plotted against the mol% PS. As before, the mutant MA(K18N/K23N) (Fig. 8, squares) did not float at this lipid concentration. In contrast, the binding of wild-type MA (Fig. 8, diamonds) increased with increasing mol% PS. Binding to 100% PS liposomes was similar to that at 45% PS (data not shown).

FIG. 6. Effect of protein dimerization on apparent binding constant. Proteins were incubated with increasing amounts of 2:1 PC:PS liposomes. The ratio of bound protein to total protein for each gradient are plotted on the y axis. The MA data were reproduced from Fig. 3 for comparison. The apparent binding constant for CTD dimers is approximately 10^{-7} M. CTD dimer data are averages of four independent experiments. CTD monomer data are averages of two independent experiments. Error bars represent standard deviations from the means.

FIG. 7. Enhanced positively charged membrane binding surface produced by dimerized MBD. The figure shows a hypothetical dimer model for RSV MA based on the dimer structure observed for EIAV MA (25). See Fig. 1 for a comparison. The equipotential contours (blue = +1 kT/e and red = −1 kT/e) were calculated and visualized in GRASP (52) for 100 mM NaCl.
MA, reaching a maximum at approximately 30% PS (Fig. 8, y plotted on the PS. The ratio of bound protein to total protein for each gradient are of protein was incubated with 500 μg/H9262 tions from the means. two independent experiments. Error bars represent standard devia-

FIG. 8. Effect of PS on MA-lipid interaction. Approximately 30 μg of protein was incubated with 500 μg liposomes of increasing mol% PS. The ratio of bound protein to total protein for each gradient are plotted on the y axis. MA data are averages of four independent experiments. MA(K18N/K23N) and CTD dimer data are averages of two independent experiments. Error bars represent standard deviations from the means.

shown). No protein-lipid interactions were observed with 100% PC liposomes, confirming that the interaction is electro-

FIG. 9. Prediction of dependence of MA-membrane association on mole percent PS. The electrostatic free energy of interaction between RSV MA (diamonds) and PS-containing membranes decreases sharply as the mole percent PS increases to 33, but it levels off after this point. The K18N/K23N RSV MA mutant (squares) is predicted to bind weakly to membranes regardless of the mole percent PS. brane association of wild-type MA (diamonds) was predicted to increase dramatically (120-fold) when the mole percent PS in the membrane was increased from 11 to 33. In contrast, the membrane association of the K18N/K23N mutant MA protein (squares) was predicted to be about 30-fold weaker. Fur-

We also examined the effect of lipid composition on MA binding. The in vitro assembly of the HIV-1 Gag protein into correctly shaped virus particles requires either inositol phosphates or phosphatidylinositol phosphates (PIPs), and this re-

DISCUSSION

In this report, we present the first example of an in vitro liposome binding assay to directly measure Gag-membrane interactions in the absence of cellular factors. Using this assay, complemented by computational modeling, we have deter-

The calculated electrostatic free energy of membrane inter-

The MACACTD(Q192A)-lipid interaction was similar to that of PS, which is approximately equal to the concentration of PS present in the inner leaflet of the RSV envelope (66). Thus, under the experimental conditions used, the RSV MA-lipid interaction occurs at biologically relevant levels of PS, consistent with results from studies of basic peptides (5, 6, 42, 45, 47). The MACACTD(Q192A)-lipid interaction was similar to that of MA, reaching a maximum at approximately 30% PS (Fig. 8, triangles). However, MACACTD(Q192A) required half as much acidic lipid to support half-maximal protein binding, consistent with its higher affinity for membranes.

The calculated electrostatic free energy of membrane inter-

The possibility that the assembly of RSV Gag is affected by these compounds has not been tested rigor-

The lipid envelopes of RSV particles have been known since the late 1970s to be enriched in cholesterol and sphingomyelin compared with the plasma membrane (66, 71–73). These findings were later confirmed for other retroviruses (2, 87). To determine whether MA has a specific affinity for these lipids, we monitored binding to PC-PS liposomes containing PI(4,5)P2 (PIP2) and monitored protein binding. MA bound with a similar affinity to both PC-PS and PC-PIP2-PS liposomes (data not shown). We interpret this result to suggest that unlike HIV-1 Gag, RSV MA does not interact specifically with PIPs. However, these results do not rule out the possibility that a PIP interaction occurs elsewhere in RSV Gag, perhaps in conjunction with the MA domain, nor do the results address the possibility that more highly phosphorylated PIPs could bind MA, as found for HIV Gag (12).

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mined the molecular requirements for membrane binding of the purified RSV MA protein. The MA-lipid interaction is electrostatic and depends on the presence of a biologically relevant concentration of the negatively charged lipid PS. The intrinsic affinity of MA for negatively charged membranes is weak, and extending MA through the N-terminal domain of CA does not change this property. However, the membrane affinity of monomeric MA can be augmented >10-fold by appending a dimerization domain.

It is well established that the membrane binding of retroviral Gag proteins is mediated by the N-terminal part of Gag, which becomes the mature MA protein after proteolytic maturation late in virus assembly. The method of membrane binding is less clear in mechanistic terms, although for retroviral Gag proteins such as that of HIV-1, both myristylation and an adjoining positively charged surface on MA contribute to binding (8, 32, 89, 97). RSV Gag is unusual in that it is not myristylated, suggesting a simpler binding mechanism. To date, studies with purified Gag proteins and liposomes of defined composition have been limited to HIV-1 (18, 81) and EIAV (70). In those experiments, membrane associations were monitored indirectly by fluorescence-quenching techniques. Both HIV-1 MA and EIAV MA were reported to have much higher affinities for PS-containing liposomes than what we have found for RSV MA. Furthermore, the HIV-1 CA protein, used as a control in those studies, was reported to interact with membranes as strongly as MA, which is difficult to reconcile with the known role of CA in assembly. In our assay, RSV CA failed to float with either neutral or negatively charged liposomes. In preliminary experiments, unmyristylated HIV-1 MA bound liposomes more weakly than RSV MA. Furthermore, only trace amounts (~1%) of HIV-1 CA bound PS-containing liposomes at low salt and high liposome concentrations, while none bound these liposomes at high salt concentrations (0.5 M). If this limited binding of HIV-1 CA is attributable to the native protein, as opposed to a small fraction of the protein that might have been denatured or otherwise abnormal, then the binding constant would be too weak to be measured, at least 5 orders of magnitude less than that reported previously (18).

Several arguments support the conclusion that our results are biologically relevant. First, the MA-liposome interaction is sensitive to the mole percent of negatively charged lipids. Theoretical and experimental studies which measure the binding of short basic peptides to acidic liposomes show sigmoidal curves (42) similar to the one presented here for MA. For both MA and these model peptides, membrane binding reaches a maximum at approximately 2:1 neutral:acidic lipid, a value that approximates the amount of PS found in the inner leaflet of the virion membrane and the plasma membranes of most cells (2, 21, 71, 73).

Second, in the flotation assay a mutant MA protein behaved as predicted from its in vivo properties. Callahan and Wills created a collection of Gag mutants with alterations in the basic residues in the MBD and tested these mutants for the budding of virus-like particles and for subcellular localization as Gag-green fluorescent protein fusions (11). Their conclusion was that any 10 of the 11 positively charged residues are sufficient for plasma membrane localization and budding but that 9 residues are insufficient. We tested one of their mutants containing only nine basic residues and found that it failed to associate significantly with acidic liposomes.

Third, the measured binding constant for MA matches that predicted by molecular modeling. The agreement between theory and experiment for ionic strength dependence, mole percent PS dependence, mutations of basic residues of MA, and oligomerization strongly indicates that the MA/membrane interaction is electrostatic in nature. Taken together, these diverse approaches provide a model for how the unmyristylated MA domain targets RSV Gag to the plasma membrane.

One factor that could contribute to efficient RSV MA-membrane binding is its maturation state. Both in vivo and in vitro, full-length HIV-1 Gag appears to bind more efficiently to membranes than does mature HIV-1 MA (18, 56, 88, 98). In one study, the extension of HIV-1 MA through just the N-terminal portion of CA was found to be sufficient to support membrane association in vivo at a level comparable to that of full-length Gag (56). Although these results are consistent with the idea that the basic residues of CA contribute to binding, the measured binding affinities for all available MA-containing proteins proved to be identical. This result suggests that there are no additional RSV Gag sequences, at least at the N-terminal domain of CA, that contribute directly or indirectly to membrane binding.

The simplest hypothesis to explain the discrepancy between the weak membrane binding of RSV Gag and the efficient plasma membrane binding of RSV Gag during assembly is Gag multimerization. Gag multimerization, both for assembly in vitro and assembly in vivo (4, 13, 31, 93, 95). Deletion or mutation of NC reduces or abrogates efficient membrane binding of Gag in vivo, reinforcing a role for multimerization in membrane binding (31, 80). For HIV-1, the relationship between protein-protein interactions and the membrane binding of Gag was made clearer by the recent discovery that Gag-Gag interactions lead to exposure of the myristate group (90), presumably promoting membrane association. Under monomeric conditions, the myristate group is buried within a hydrophobic pocket in the globular head of the MA domain; Gag-Gag interactions favor extrusion of the myristate from the pocket and thereby play a key role in maintaining the accessibility of the bipartite membrane binding signal. This myristyl-switch model was supported by a recent in vivo membrane binding study which showed that the presence of the HIV-1 MA domain inhibits Gag-membrane binding at low levels of protein expression but that Gag multimerization at high levels of protein expression can overcome this MA-induced block to membrane binding (65).

It has not been possible to directly test the membrane binding of full-length RSV Gag in these liposome binding assays and therefore to test the effects of multimerization, because the intact protein cannot be recovered in a soluble form from E. coli (13). Therefore, to address how protein-protein interactions affect RSV MA-membrane binding, we constructed an artificial MA dimer in which the MA domain was fused to the C-terminal domain (CTD) of HIV-1 CA, which is known to dimerize in solution (23). Fusion of the CTD to the end of MA preserves the natural orientation of the two domains with respect to each other, as they would be found in Gag. Further-
more, although at the protein concentrations used in this study RSV MA appeared to be monomeric, cross-linking evidence suggests that in intact virions, in which protein concentrations are in the millimolar range, MA dimers do exist (63). This lends additional support to the biological relevance of studying MA dimers by using the liposome binding assay. The chimeric MA protein dimers bound at least 1 order of magnitude more tightly to liposomes than did monomeric MA. Presumably, higher-order oligomers would bind still more tightly, effectively locking the protein complex onto the membrane. These results provide biochemical validation of the principle that Gag-Gag multimerization drives membrane binding in vivo (80, 94, 96).

If the membrane binding of RSV Gag is strictly electrostatic and the MA domain harbors no additional membrane binding signals, it seems reasonable that the even more basic NC domain might also participate in membrane binding in vivo. Indeed, we found that Gag proteins lacking the MBD but containing the NC domain [4MBD(13)] do float with acidic liposomes (data not shown). While the role of NC in nucleic acid interactions is well established, an additional function in direct membrane binding has not been considered previously, although the NC domain has been shown to indirectly promote the efficient binding of HIV-1 Gag to the plasma membrane (80). The interaction of NC with the phosphodiester backbone of RNA might preclude its interaction with the acidic lipids on the membrane, or RNA might displace the lipid. These models should be testable by use of the flotation assay.

Sphingomyelin and cholesterol are the hallmarks of lipid rafts, which are ordered microdomains in the plasma membrane that are thought to serve as platforms for a variety of events, including signaling and the budding of some viruses (43, 86). The observation that retroviral envelopes are enriched in raft lipids sparked efforts to link retroviral assembly with lipid rafts, but the results have been conflicting. One method for determining the lipid raft association of a protein involves the extraction of membranes at 4°C with a nonionic detergent such as Triton X-100 (TX-100). This technique solubilizes the bulk, fluid membrane but leaves at least some of the more ordered raft domains intact. Some proteins associated with lipid rafts before extraction remain associated with the rafts after a cold TX-100 treatment and can be separated from the bulk, solubilized material by sucrose gradient flotation. Cold TX-100 experiments performed in our lab have revealed no evidence of an association between RSV Gag and lipid rafts in infected cells (data not shown). Similarly, the virus lipid envelope itself is not detergent resistant (data not shown). Since detergent extraction studies can alter the behavior of both the proteins and lipids being studied (28, 82), we used the in vitro liposome binding assay to confirm that RSV MA does not specifically bind to raft-like liposomes. The origin of the lipid composition of the virion envelope remains to be elucidated.

Since PIPs are highly enriched in some cellular membranes, such as multivesicular bodies (54, 58, 77), the interaction of HIV-1 Gag with PIPs may explain the preferential targeting of Gag to some membranes over others (12, 53, 58, 83). PIPs are well-known docking sites for cellular proteins (33). Alternatively, or in addition, perhaps raft domains enriched in cholesterol are involved in targeting Gag to the sites of assembly and budding, as suggested by several studies (28, 34, 51, 59). It is more difficult to understand how electrostatic interactions alone would be able to target RSV Gag to the membranes at which assembly is initiated. We have found no evidence that RSV MA or MA-NTD has a preference for PIP2, but our experiments have not been exhaustive. We hypothesize that as for HIV-1, the specificity of targeting of RSV Gag and other retroviral Gag proteins requires additional interactions, either with cellular proteins or with specific lipids. This hypothesis remains to be tested.

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