Partitioning of HIV-1 Gag and Gag-Related Proteins to Membranes†

L. S. Ehrlich,‡ S. Fong,‡ S. Scarlata,*,§ G. Zybarth,‡ and C. Carter‡

Departments of Molecular Genetics & Microbiology and Physiology & Biophysics, State University of New York at Stony Brook, Stony Brook, New York 11794

Received September 29, 1995; Revised Manuscript Received January 12, 1996⊗

ABSTRACT: The binding of HIV-1 Gag and Gag-related proteins to model membranes was examined using three experimental systems: (i) large unilamellar phospholipid vesicles (LUVs) and recombinant Gag purified from *Escherichia coli*; (ii) LUVs added to a mammalian cell extract in which Gag proteins were expressed by a coupled transcription/translation system; and (iii) inside-out plasma membrane vesicles purified from human red blood cells (RBC) and recombinant, purified Gag from *E. coli*. Several novel aspects of HIV-1 Gag membrane interactions were observed: (i) Gag proteins bound with high affinity to both model membranes with a negatively charged surface and to RBC membranes. (ii) Binding of the Gag precursor and mature Gag proteins exhibited different sensitivities to ionic strength indicating that the precursor directed membrane binding through interactions that were qualitatively and quantitatively distinct from those of any of its individual domains. Studies using energy transfer between tryptophan residues in the proteins and anthroyloxy-containing probes inserted in the LUVs indicated that the orientation of the precursor and of the mature proteins on the membrane surface were distinct; (iii) Gag oligomers appear to have facilitated high-affinity binding under high salt conditions, suggesting that protein−protein interactions led to formation of stronger electrostatic or new hydrophobic membrane binding determinants. Since binding studies with model membranes permit quantitative analysis, these experimental approaches may permit identification of interactions that drive Gag assembly on the membrane.

The human immunodeficiency virus type 1 (HIV-1), a member of the Retrovirus family, is an RNA virus which is encapsulated by a lipid membrane derived from the host cell (Dickson et al., 1985). Experimental evidence suggests that the viral precursor, Gag, associates with the plasma membrane of the host either preceding or concurrent with the viral RNA [see Gelderblom (1991) and Wills and Craven, (1991) for reviews]. These protein−nucleic acid complexes, along with other viral proteins, form particles that bud off into the extracellular space, taking with them lipids and proteins from the host cell (Arthur et al., 1992). The immature particles are processed to yield infectious particles as budding proceeds. Part of this processing involves the cleavage of the Gag precursor into several proteins including matrix (MA), capsid (CA), nucleocapsid (NC), and p6 (see Figure 1, upper panel). MA is associated with the membrane, CA forms the structural core of the virion, and NC is associated with viral RNA. The p6 domain plays an essential role during the final stages of budding, but its function and location in the particle are unknown.

While many of the general aspects of viral assembly are understood, there is relatively little known about the molecular interactions that drive Gag binding to the plasma membrane of host cells. This information is vital for the development of inhibitors of this process. For example, although binding signals in Gag have been recently discovered, it is not known whether Gag has the intrinsic ability to associate with membranes without the aid of cellular proteins. Gag is myristylated on its N-terminus, and it has been demonstrated that this signal, along with clusters of positively-charged residues in MA, contribute to the transport and binding of Gag to the host cell membrane (Bryant & Ratner, 1990; Gottlinger et al., 1989; Zhou et al., 1994). Although Gag myristylation is required for efficient plasma membrane binding of HIV-1 and several other retroviruses, the Gag proteins of some members of the Retroviridae family lack this modification (Henderson et al., 1987). Moreover, both myristylated and unmyristylated HIV-1 Gag proteins can associate with membrane-enriched fractions (Platt & Haffar, 1994; Spearman et al., 1994). Consistent with these observations, it has been suggested that the contribution of the myristate group to binding may be relatively insignificant, and that basic residues play a more important role [see Zhou et al. (1994)]. Also, although a highly basic sequence in the N-terminal region of the MA domain has been identified as an important assembly subdomain in HIV Gag (Bennett et al., 1993), this motif is not completely conserved in other retroviruses, and point mutation of some of the basic residues does not detectably affect membrane binding (Freed et al., 1995). These observations raise the possibility that additional, as yet undiscovered, binding signals exist in retroviral precursors. A complete understanding of the binding process will require a quantitative analysis of the several contributing viral components.

Several laboratories have utilized Gag precursor proteins synthesized in an *in vitro* rabbit reticulocyte translation system (RRL) and membranes in disrupted cellular lysates to demonstrate Gag−membrane binding *in vitro* (Platt & Haffar, 1994; Spearman et al., 1994, Zhou et al., 1994). This

† This work was supported by National Institutes of Health Grants GM48294 (C.C.) and GM53132 (S.S.).

* Corresponding author. Tel: (516) 444-3071. FAX: (516) 444-3432. E-mail: scarlata@pofvax.pnb.sunysb.edu.

‡ Department of Moledular Genetics & Microbiology.

§ Department of Physiology & Biophysics.

⊗ Abstract published in *Advance ACS Abstracts,* March 15, 1996.

0006-2960/96/0435-3933$12.00/0

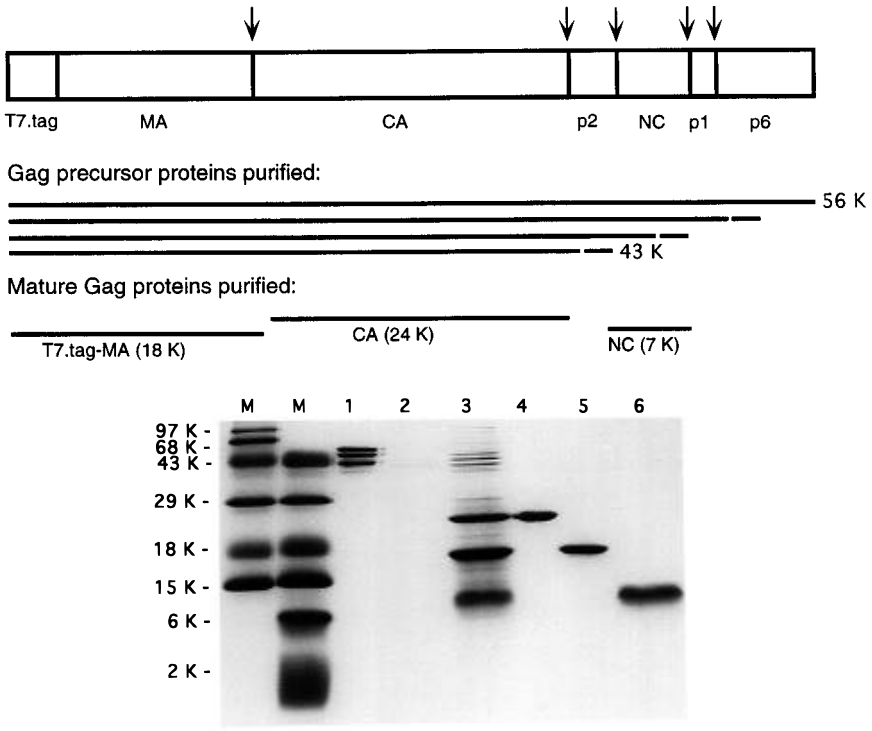

FIGURE 1: Upper panel: Domain organization of the HIV-1 Gag precursor protein synthesized from the expression construct, gpVI-ATG. Positions of cleavage sites recognized by HIV proteinase are indicated by arrows. Shown below this box diagram is a line representation of Gag proteins obtained after purification which include the full-length polyprotein (56 kDa) and three truncated species (dashes indicate approximate C-termini). Incubation of the Gag preparation with HIV-PR results in cleavage at positions indicated by arrows. Three mature Gag proteins (MA, CA, and NC) were obtained in quantities amenable to further purification. Lower panel: Coomasie-stained SDS−polyacrylamide gel with recombinant HIV-1 Gag preparation (lane 1); HIV-1 PR preparation used in proteolytic processing reaction (lane 2); digest after overnight incubation of Gag with PR (lane 3) and preparations of mature CA (lane 4); MA (lane 5) and NC (lane 6) after their separation from the digest. Each lane was loaded with 10 μg of protein except for lane 2, which was loaded with the equivalent amount of PR present in the sample loaded in lane 3.

approach has the advantage of producing N-myristylated Gag proteins by co-translational modification (Wilcox et al., 1987) and of not requiring purification of the protein prior to the membrane binding assay. Gag binding in this system has been shown to be enhanced by myristylation and contributed to by specific regions in the MA (Zhou et al., 1994) and NC domains (Platt & Haffar, 1994). Membrane binding of Gag proteins expressed in RRL has been demonstrated to require Arg-Ala-Pro-Arg-Lys-Lys-Gly, a basic sequence in the NC domain (Platt & Haffar, 1994). This sequence also has been shown to bind RNA (De Rocquigny et al., 1992) and is important for PR maturation (G. Zybarth and C. Carter, unpublished data). A major disadvantage of this experimental system is that the contribution to binding of the several components in the RRL and cell membrane preparations cannot be easily assessed. Most importantly, this system does not permit quantitative analysis of protein−membrane and protein−protein interactions. In the studies described in this report, membrane binding was examined using several experimental systems. Our binding studies used RRL-translated proteins and purified recombinant Gag proteins with cellular or model membranes. The HIV-1 Gag precursor polyprotein was observed to direct membrane binding through interactions that are qualitatively and quantitatively distinct from those of MA, CA, or NC domains. The oligomerization state of the precursor appeared to influence the regions of the protein involved in interaction with the membrane surface. In addition, the lipid composition of the target membrane was a critical determinant of binding affinity.

MATERIALS AND METHODS

Protein Analysis. Protein concentration was determined using the Bradford dye binding assay (Bio-Rad). Electrophoresis of denatured protein samples was done using the Laemmli discontinuous buffer system in 12.5% acrylamide gels (SDS−polyacrylamide gel, SDS−PAG). Protein bands were visualized either by staining with Coomassie Blue dye or by immunoblotting.

Expression and Purification of HIV-1 Gag Precursor Proteins. The HIV-1 sequence [pBH10 nt 333−2130 (Ratner et al., 1985)] encoding the *gag* gene and that part of the *pol* gene encoding proteinase (PR) was cloned into pET 3 vector (Studier et al., 1990) for T7 RNA polymerase promoter-driven expression in *Escherichia coli* strain BL21 (DE3) using two fragments. One fragment was derived from pHIV-FSII (Krausslich et al., 1988) and contained the sequence between the *Cla*I site at nt 375 and the *Eco*RI restriction site. The *Eco*RI site in FSII follows two stop codons placed immediately downstream of the PR coding region which terminates at nt 2130. The second fragment was derived by PCR and encoded the N-terminal region of the *gag* gene and a 5′ *Bam*HI restriction site. The expression construct, gpVI-ATG, also encodes an inactivating mutation in the catalytic site of PR (D25A) introduced by site-directed mutagenesis (Kunkel, 1985). Large-scale cultures of bacterial cells carrying gpVI-ATG were grown and induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) in a 14 L fermenter (Micro fern MMF-14) as previously described (Ehrlich et al., 1990). A 10 g amount of cell paste was resuspended in 90 mL of buffer A (50 mM Tris, pH 7,

with 1 mM EDTA) and lysed by French press. The lysate was centrifuged at low speed for 15 min. The supernate was mixed with crystalline NaCl (to 1 M), shaken at 5 °C for 60 min, and centrifuged at 10 000*g* for 30 min. The supernate fraction was mixed with crystalline ammonium sulfate (to 10% w/v), incubated with stirring at 5 °C for 60 min, and then centrifuged again. Precipitated proteins were redissolved in 200 mL of buffer A containing 0.2 M NaCl and loaded on a DEAE-cellulose (DE-52; Whatman) column that had been pre-equilibrated with this buffer. The column was washed stepwise with 250 mL of buffer A, followed by 250 mL of buffer A + 0.2 M NaCl, and finally with 250 mL of buffer A + 1 M NaCl. Proteins in the eluate collected from the latter wash were precipitated with ammonium sulfate (20% w/v), pelleted by centrifugation at 10 000*g* for 60 min, and redissolved in 10 mL of buffer A + 1 M NaCl. Typical yields were ~20 mg of Gag-related protein/10 gm of cell paste. This final solution contained Gag precursor proteins purified to 90%−95% homogeneity.

Proteolytic Processing of Recombinant Gag Precursor by HIV-1 PR and Purification of the Products. To prepare the mature Gag proteins from the recombinant precursor, the purified Gag proteins were incubated with partially purified PR (Ehlich et al., 1990) for at least 1 h at 37 °C. The extent of proteolytic cleavage was monitored by SDS−PAGE of an aliquot of the digest. When necessary, incubation time was extended or more PR was added. The digest was subsequently diluted 10-fold with buffer A and loaded onto a pre-equilibrated sulfoxyethyl cellulose column (SE-53; Whatman) that had been pre-equilibrated with buffer A containing 0.1M NaCl. The flow-through fraction was collected, and the column was washed stepwise with buffer A containing increasing concentrations of NaCl from 0.1 to 1.0 M. The protein-containing fractions were identified by SDS−PAGE, and the identity of mature MA, CA, and NC proteins was confirmed by immunoblot analysis (MA and CA) and by N-terminal amino acid sequencing. The CA protein did not bind to the SE-53 column. MA eluted between 0.4 and 0.6 M NaCl. NC eluted between 0.6 and 0.8 M NaCl. All proteins except MA, which contained an N-terminal tag of 11 amino acids (Studier et al., 1990), possessed the apparent molecular weight and N-terminal amino acid sequence of the authentic product (Henderson et al., 1992). Yields of the p6 protein were invariably too low to permit its large-scale isolation due to nonspecific C-terminal truncation of ~65% of the Gag protein (see Figure 1, lower panel). This product was, however, detected by immunoblotting (not shown). For some experiments, an MA−CA cleavage intermediate was isolated by partial proteolysis with PR followed by ion-exchange chromatography of the digest. Immunoblot analysis indicated that this protein contained the T7 tag, MA, and CA sequences.

Preparation of Lipid Vesicles and Protein Labeling. Purified proteins used in fluorescent spectroscopy analyses were dialyzed at 5 °C against 40 mM HEPES buffer, pH 7.0, containing 0.5 M NaCl. Proteins were covalently labeled with dansyl chloride (DNS) by adding the probe at 4−10-fold molar excess and incubating overnight at 5 °C. Unreacted probe was removed by dialysis against several changes of buffer. Large unilamellar vesicles (LUVs) were prepared by extrusion, using the method of Hope et al. (1985). Lipid was rehydrated in 40 mM HEPES, pH 7.0, containing 0.5 M NaCl prior to extrusion. Labeled membranes were prepared by mixing 2 mol % DNS-PE (Molecular Probes, Inc.) to a choloform solution of the lipids prior to drying and subsequent hydration with buffer. To prepare sucrose-loaded LUVs, lipid was rehydrated in buffer containing 0.19 M sucrose prior to extrusion (Rebecchi et al., 1992). Fluorescent probes were incorporated into LUVs by adding 1 mol % of Laurodan (Molecular Probes, Inc.) or 2-, 6-, or 12-anthroyloxylstearic acid (AS) from concentrated stocks in ethanol and briefly sonicating at low power in a bath sonicator.

Analysis of Protein−Lipid Binding by Fluorescence. Fluorescence measurements were made using an ISS-K2 fluorimeter (I. S. S., Inc., Champaign, IL) with samples contained in microcuvettes with a path length of 3 mm. Samples were immersed in 40 mM HEPES buffer, pH 7.0, with 0.5 M NaCl. Background corrections were made using unlabeled samples. Control studies for membrane binding experiments were done using IgG (Bio-Rad, Inc.), which does not associate to membranes. All reported intensities were obtained by integrating the area under the emission curve. Emission energies are described in terms of the center of the spectral mass (CM) which takes into account changes in the skewness of the peak and is calculated from the emission energy (EE; units of energy given in kilokaisers of kk, where kk = 1000 cm^{-1}) and intensity (INT) at each wavelength (i):

$$\text{center of spectral mass (CM)} = \sum_i(\text{EE})(\text{INT})/\sum_i(\text{INT})$$

For the intrinsic fluorescence studies samples were excited at 280 nm and scanned from 300 to 420 nm. For the DNS-labeled protein studies, samples were excited 340 nm and scanned from 380 to 580 nm. In these experiments aliquots of LUVs solution were added to 100 μL of protein solution (typically 0.1 mg/mL). For the studies involving membranes labeled with Laurodan, samples were excited at 340 nm and scanned from 380 to 580 nm. In these experiments aliquots of protein (typically 0.1 mg/mL) were added to a LUVs solution (100 μM). In the Trp-AS energy transfer studies, mixtures were excited at 280 and then at 381 nm and emission scanned from 400 to 600 nm.

Analysis of Protein−Lipid Binding by Sedimentation. Protein solutions (0.2 mg/mL) were mixed with sucrose-loaded LUVs (100 μM) or buffer. The LUVs−protein mixture was spun at 44 000 rpm for 30 min at 25 °C in a tabletop ultracentrifuge using a TLA rotor to pellet LUVs and bound proteins. The supernatant fraction was carefully removed and transferred to another tube, and the pelleted fraction was resuspended in fresh buffer. Aliquots from each fraction were analyzed by SDS−PAGE. Proteins were visualized by staining with Coomassie Blue.

Preparation of Inside-Out Erythrocyte Plasma Membranes. Red blood cells were isolated from outdated whole blood from the University at Stony Brook hospital blood bank. Cells were homogenized either with $MgSO_4$, which causes them to vesiculate into the extracellular space and produce right-side out vesicles or in the absence of $MgSO_4$, which causes them to vesiculate into the cell interior and form inside-out membranes [see Steck (1971)]. These two types of membranes were then purified by ultracentrifugation in a sucrose density gradient as described by Steck et al. (1971). The orientation of the membranes (i.e., inside-out or right-side out) was verified using fluorescent-labeled

concanavalin A (F-ConA), which binds tightly to carbohydrates such as glucose-6-*N*-acetylglucosamine (GlcNAG). The two sets of membranes were incubated at room temperature with F-ConA for 20 min and washed three times with buffer. Strong fluorescence was observed from the right-side out samples, but only background signals were obtained for the inside-out samples. The specificity of F-ConA for carbohydrate labeling was confirmed by monitoring the reduction in fluorescent labeling of the cells upon addition of GlcNAG to the samples. Also, addition of the detergent saponin to the inside-out vesicles allowed concanavalin to diffuse into the interior of the vesicles, and labeling was observed.

Expression of Gag by Coupled Transcription/Translation in Vitro. Plasmid gpVI-ATG and constructs derived from this parent encoding mutated Gag proteins were grown in *E. coli* C600 plasmid DNA was prepared using the Promega Magic Mini Prep system and used for translation *in vitro* (Carter & Zybarth, 1994). To ensure uniform translational efficiency of all constructs, equal amounts of supercoiled DNA were used to direct transcription and translation in rabbit reticulocyte lysates (TNT T7 RRL, Promega) in the presence of [^{35}S]Met (specific activity, >1000 Ci/mmol; ICN). Incubation conditions were as recommended by the supplier, at 30 °C for 1 h. Translation products were analyzed by SDS−PAGE as described above.

RESULTS

Expression and Analysis of Recombinant Gag Precursor and Mature Products. To obtain HIV-1 Gag protein for membrane binding studies, we constructed plasmid gpVI-ATG. This construct provides primarily Gag precursor polyprotein (Pr55) due to an inactivating mutation in the catalytic Asp residue of the viral protease (PR). A truncated Gag-Pol precursor protein (Gag-PR) also is expressed at ~5% of the level of Gag due to ribosomal frameshifting (Jacks et al., 1988). Expression of this construct in *E. coli* BL21(DE3) and purification using a non-denaturing protocol generated a protein preparation that contained the full-length Gag precursor polyprotein and three truncated forms (Figure 1, lower panel). The relative amount of the shortest form varied with each preparation. All protein bands were recognized by antibodies directed at the T7 leader sequence and at MA and CA sequences (data not shown). The slowest migrating band also was recognized by monoclonal antibody to the C-terminal portion of the p6 domain in Gag (Veronese et al., 1987), indicating that this band was full-length Gag polyprotein. The shorter proteins apparently resulted from C-terminal truncation, as has been noted previously (Berkowitz et al., 1993; Jowett et al., 1992). Mature Gag proteins MA(p17), CA(p24), and NC(p7) were derived from the precursor protein with purified HIV-1 PR as described in Materials and Methods (Figure 1, lower panel).

The recombinant Gag protein exhibited several of the structural properties expected of the authentic protein. As noted above, the protein was cleaved into MA, CA, NC, and p6 by viral PR, indicating that these sites were accessible, as is the case for the authentic precursor. When analyzed by circular dichroism spectroscopy, the MA and CA products derived from the recombinant precursor exhibited α-helical features consistent with the NMR structure reported for MA (Massiah et al., 1994; Matthews et al., 1994) and the crystal

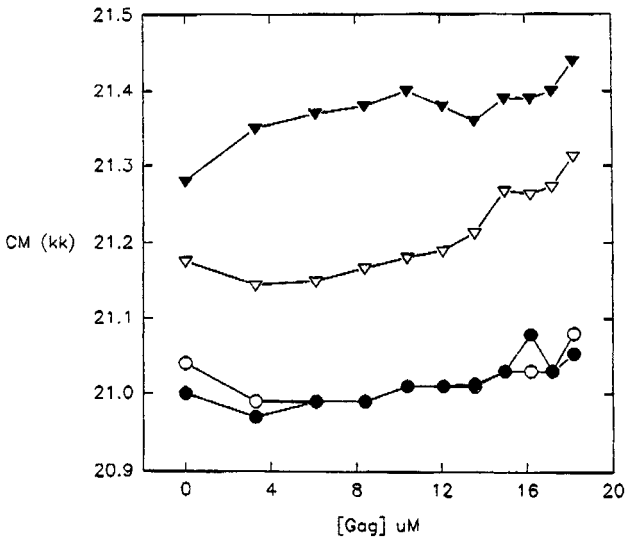

FIGURE 2: Phospholipid requirements for Gag association to membranes at 0.5 M NaCl. Increase in the center of spectral mass (CM) in kk (1 kk = 1000 cm^{-1}) of Laurodan in various membranes as Gag binds. Data points are an average of three to four determinations and are within the instrument error of ±0.025. LUVs composition: (▼) POPS; (○) POPC; (▽) POPC:POPS = 1:2; (●) POPC:POPS = 2:1.

structure of CA (M. Rossmann and C. Carter, unpublished data), indicating that the recombinant precursor protein was correctly folded. The interdomain junctions in the precursor were also found to be exposed when trypsin was used as a probe of accessible regions of the protein (L. S. Ehrlich et al., unpublished data). The functional competence of the recombinant precursor was indicated by efficient binding to RNA and by self-assembly into dimers and pentamers or hexamers, as indicated by electron scattering and scanning transmission electron microscopy (L. S. Ehrlich et al., unpublished data). Negative staining and electron microcopy revealed that the recombinant Gag protein could assemble *in vitro* into spherical particles that resembled immature capsids, although this event was detected at very low efficiency.

Phospholipid Requirements for Membrane Association of the Gag Precursor. Large unilamellar vesicles (LUVs; 0.1 μm diameter) were used as models to permit quantitative assessment of Gag−membrane interactions as these provide a uniform, flat surface for the proteins to adhere. We first measured the partitioning of Gag to electrically neutral phospholipid vesicles to estimate the contribution of hydrophobic interactions to binding. We then examined the effects of incorporating acidic lipids into the LUVs. Membrane association was determined by monitoring perturbation of Laurodan probes incorporated into the membrane. When inserted, the fluorescent head group of Laurodan resides on the membrane surface [see Parasassi et al. (1991)]. As protein molecules partition to the LUV surface, they replace the aqueous environment around Laurodan with a protein environment. Since Laurodan is very sensitive to changes in the charge and polarity of its environment [e.g., Weber and Farris (1979) and Parasassi et al. (1991)], the decrease in local polarity caused by the proteins results in a shift of its fluorescence emission band to higher energies as seen by shifts in the center of spectral mass (CM, see methods).

As shown in Figure 2, the addition of Gag at 0.5 M NaCl elicited a significant increase in CM as compared to error

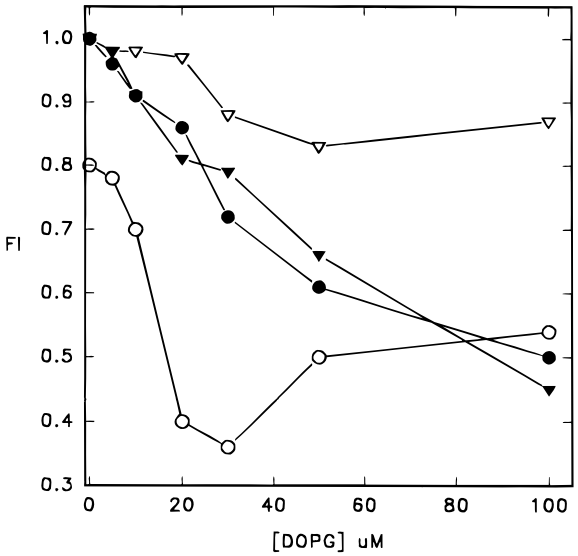

FIGURE 3: Effect of DOPG LUVs on fluorescence intensity of dansyl-labeled Gag, MA, CA, and NC proteins ($n = 2$) at 0.5 M NaCl. (○) Gag precursor; (●) MA; (▼) CA; and (▽) NC. Maximal error is ±4%.

when the LUVs were composed solely of the negatively charged phospholipid, 1,2-palmitoyloleoylphosphatidylserine (POPS). This effect was not seen when Gag was added to LUVs in which the predominant phospholipid was the electrically neutral phospholipid, phosphatidylcholine (POPC). Essentially identical results were obtained with LUVs consisting of esters of both POPC and POPS in a ratio of 2:1. Intermediate results were obtained upon addition of Gag to LUVs consisting of POPC and POPS in a ratio of 1:2. These results indicate preferential partitioning of Gag to negatively charged membrane surfaces.

Reversibility of Gag−membrane association was assessed by following the increase in energy transfer from Gag tryptophan residues to DNS-PE. The addition of 0.6 μM Gag to 50 μM POPS LUVS doped with 2% DNS-PE resulted in significant transfer. The addition of a 10-fold excess of unlabeled POPS vesicles failed to reverse the transfer after 60 min of incubation at room temperature, indicating that the partitioning of Gag to POPS vesicles is an irreversible event.

Membrane Binding of Precursor and Mature Domains Assayed by Quenching of Dansyl Fluorescence and Intrinsic Fluorescence. To investigate the contribution of the Gag domains to membrane partitioning of the precursor, the MA, CA, and NC proteins derived from proteolytically processed Gag were purified independently (Figure 1, lower panel) and labeled with dansyl chloride. (Yields of the p6 protein were insufficient for analysis.) The labeled proteins then were titrated with LUVs of the negatively charged lipid dioleoylphosphatidylglycerol (DOPG). Quenching of the fluorescence emission from Gag was detected with increasing amounts of LUVs indicating interaction of the labeled protein with the membrane (Figure 3). The underlying reason for the quenching (both for these samples and those described below) is not clear but can be attributed to either conformational changes of the protein upon binding or to static quenching of the dansyl by the negatively charged groups on the lipid surface. The emission from MA and CA also was quenched, indicating that both proteins were capable of membrane interaction. In contrast, only a very minor change

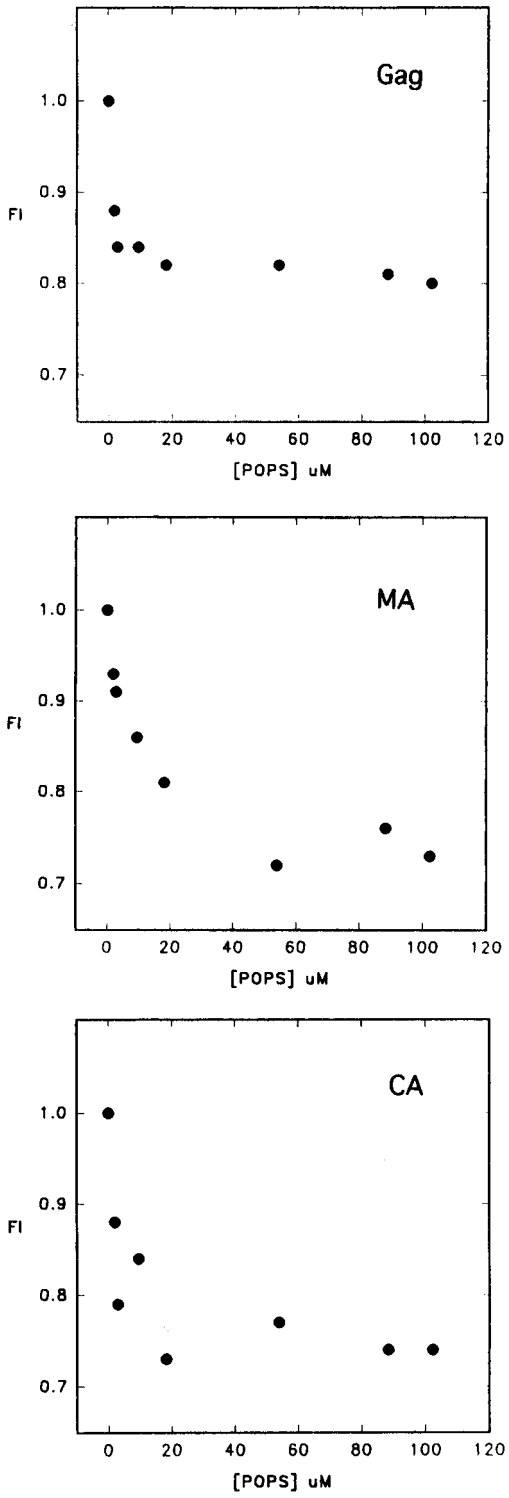

FIGURE 4: Effect of POPS LUVs on intrinsic fluorescence intensity of the Gag precursor ($n = 9$) and mature MA and CA proteins ($n = 6$) at 0.5 M NaCl. Instrumental error is within the size of the symbols. The maximal value of sample to sample error is ±3%.

in the fluorescence intensity of the dansyl-labeled NC protein was detected, indicating that mature NC does not associate to membranes significantly under these conditions.

In conjunction with the above studies using dansyl-labeled proteins, membrane partitioning was also assayed by the change in intrinsic fluorescence of the proteins when lipid is added. As shown in Figure 4, the fluorescence intensity of Gag, MA, and CA proteins was significantly quenched by addition of POPS LUVs with comparable affinities as

Table 1: Partition Coefficients of MA, CA, and Gag[a]

protein	K_{IF} (μM)	K_{Laur} (μM)
GagB	0.88 ± 0.2	0.5 ± 0.2
MA	2.85 ± 0.8	1.1 ± 0.4
CA	2.46 ± 1.0	1.4 ± 0.6

[a] Partition coefficients and corresponding standard deviations of Gag, MA, and CA to POPS LUVs determined by either the changes in intrinsic fluorescence (IF) or the changes in Laurodan (Laur) emission spectrum. Data were an average of three to nine determinations each using independent lipid preparations and three independent protein preparations.

the labeled proteins. These data were analyzed as adsorption isotherms and fitted as a hyperbolic function using Sigmaplot (Jandel Scientific). The partition coefficients are reported in Table 1. (Energy transfer studies using DNS-PE indicated that MA and CA also bound to POPS irreversibly.) The fluorescence intensity of NC was not affected in buffers containing 0.5M NaCl, but was quenched by addition of LUVs in buffer of lower ionic strength (0.1 M NaCl; data not shown). The results indicated that both Gag and the Gag-derived mature products MA and CA, exhibited high-affinity partitioning. We also found that the affinities of the proteins to small, unilamellar vesicles was slightly higher, but within error, as those listed in Table 1, indicating that vesicle size is not important. Moreover, as the partitioning was assayed in high salt, the results suggest that these high-affinity events involved hydrophobic and/or strong electrostatic interactions.

As can be seen from the values in Table 1, the Gag protein exhibited the greatest affinity. Values for MA and CA were within error of each other, suggesting that under the high salt conditions MA and CA partition with similar affinity. The association of the 42 kDa Gag protein in which the C-terminal region was cleaved (see Figure 1) was of slightly lower affinity than the Gag proteins from which it was derived, suggesting that regions in the NC domain may also contribute to membrane association. Also, in control experiments no change was detected in the fluorescence of IgG, which is not expected to bind membranes (data not shown). The results demonstrate that unmyristylated Gag proteins can partition to membranes with micromolar affinity.

Membrane Partitioning of Precursor and Mature Domains as Determined by Perturbation of Laurodan Probes. In the experiments described above, the lipid was added incrementally to the protein. Thus, the surface of the added LUVs was most likely completely covered with protein in the initial stages of the assay when the lipid concentration was low and the protein concentration was relatively high. If larger oligomers partition to membrane with higher affinity than monomers or small oligomers, the interactions fostered at high initial protein concentrations might result in measurements that included contributions from protein−protein interactions. To minimize the contribution of such events, POPS LUVs were labeled with Laurodan and perturbation of this probe was determined in the presence of limiting amounts of protein. As shown in Figure 5, addition of increasing amounts of protein resulted in an increase in the emission energy of Laurodan, indicating that all 3 proteins associated to the membrane under these conditions of limiting initial protein concentrations. In Table 1 we have also tabulated the partition coefficients derived from the titrations using Laurodan-labeled membranes. For all samples the titration data could be analyzed either by the change in the

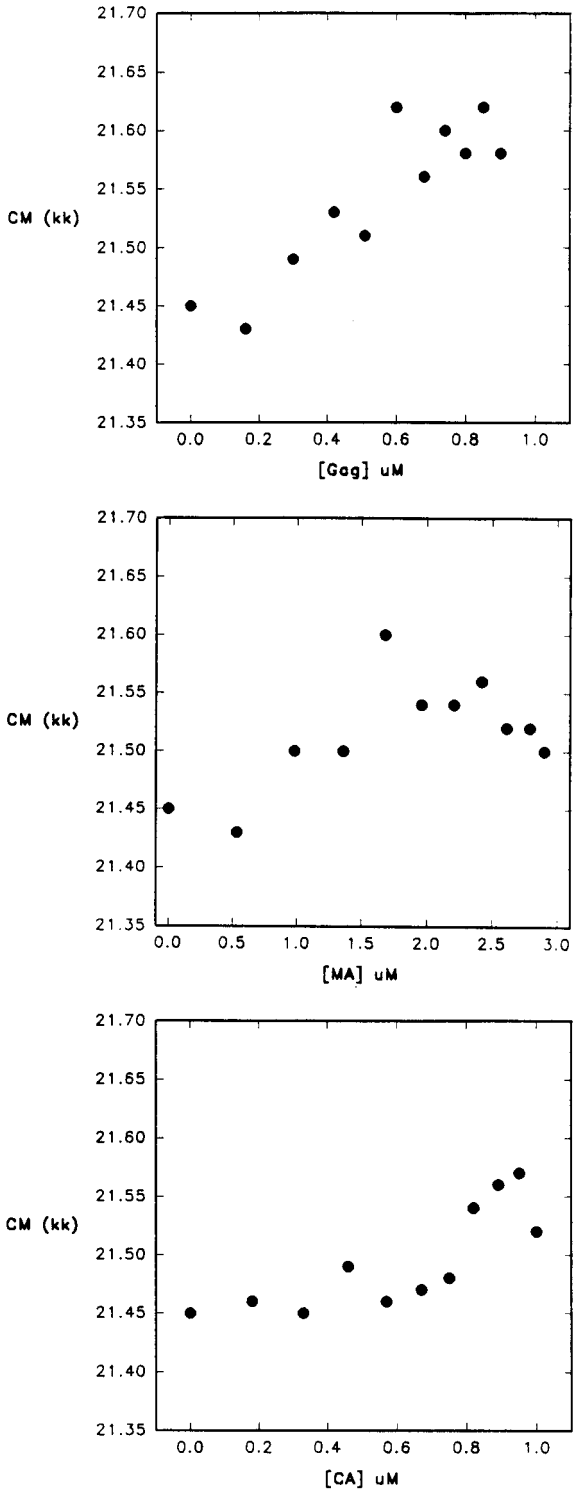

FIGURE 5: Perturbance of the emission energy of Laurodan probes by Gag and mature MA and CA proteins ($n = 3$). LUVs of POPS containing laurodan probes were prepared as described in Methods and Materials. Maximal error is ± 0.025 cm^{-1}.

shift in emission energy or change in emission intensity; both give comparable results. For all three proteins, the partition coefficients determined by intrinsic fluorescence were higher than those determined using Laurodan. These results suggests that protein−protein interactions may indeed have contributed to the association events at the membrane surface.

Membrane Partitioning of Gag Proteins as Determined by Sedimentation. Membrane partitioning was directly assayed using a sedimentation method developed by Rebecchi and co-workers (1992) in which LUVs loaded with

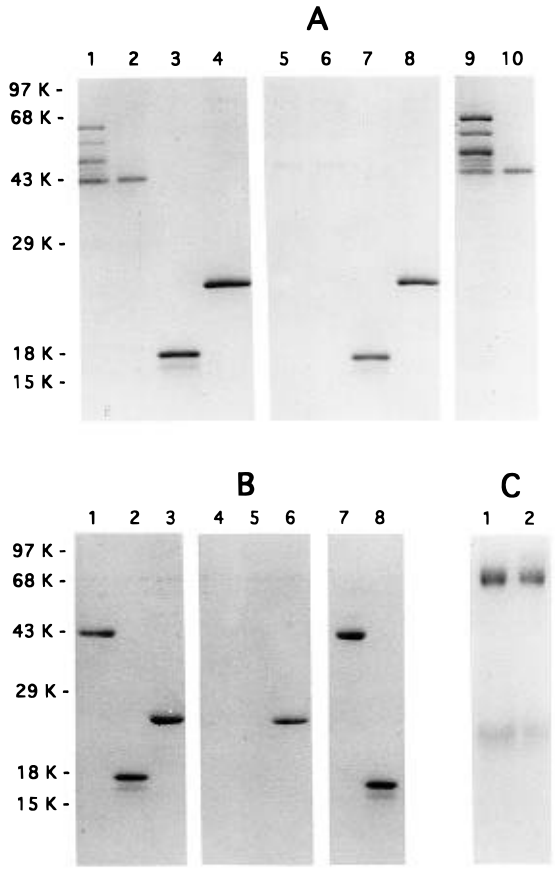

FIGURE 6: Co-sedimentation of Gag-related proteins with lipid vesicles. Panel A: sedimentation at high salt conditions (0.5 M NaCl). Supernatant fractions from protein−buffer mixtures (lanes 1−4: 1, Gag; 2, MA−CA; 3, MA; and 4, CA). Supernatant fractions from protein−LUVs mixtures (lanes 5−8: 5, Gag; 6, MA−CA; 7, MA; and 8, CA). Pellet fractions from protein−LUVs mixtures (lane 9, Gag; lane 10, MA−CA). Panel B: sedimentation at low salt conditions (0.1 M NaCl). Supernatant fractions from protein−buffer mixtures (lanes 1−3: 1, MA−CA; 2, MA; and 3, CA). Supernatant fractions from protein−LUVs mixtures (lanes 4−6: 4, MA−CA; 5, MA; and 6, CA). Pellet fractions from protein−LUVs mixtures (lanes 7, MA−CA; lane 8, MA). Panel C: sedimentation of IgG at high salt conditions. Supernatant fractions from a protein−buffer mixture (lane 1) and a protein−LUVs mixture (lane 2). Sedimentation at obtained at low salt conditions gave identical results.

sucrose (0.19 M) were used as the membrane substrate. The high density of the entrapped sucrose permits isolation of the vesicles by sedimentation. Proteins attached to the membrane will sediment with the vesicles and be effectively removed from the supernatant. This assay is similar to assays previously described by others for analysis of membrane binding of *in vitro* translated proteins (see below; Platt & Haffar, 1994; Zhou et al., 1994). To assess the relative contributions of electrostatic and hydrophobic interactions, the partitioning of Gag, the 42 kDa MA−CA processing intermediate, mature MA and CA, and IgG was tested in both high (0.5 M NaCl) and low (0.1 M NaCl) ionic strength buffers (Figure 6). In the absence of added LUVs, all of the proteins remained in the supernatant irrespective of the ionic strength of the buffer (panel A, lanes 1−4; panel B, lanes 1−3; panel C lane 1). As expected, the IgG control protein remained in the supernatant in the absence or presence of LUVs (panel C, lanes 1 and 2). Full-length Gag protein and the truncated forms partition to LUVs under both high and low ionic strength conditions and were cleared from the supernatant and recovered quantitatively in the pellet fractions (panel A, lanes 5, 6, 9, 10; panel B, lanes 4 and 7). These results indicate that the NC and p6 domains do not play measurable roles in stabilizing Gag−membrane interactions in this assay system. The MA protein partitioned to LUVs under low ionic strength conditions (panel B, lanes 5 and 8) as expected (Zhou et al., 1994) but failed to bind to LUVs and was recovered in the supernatant fraction in high ionic strength buffer (panel A, lane 6). No association was detected for the CA protein using high or low ionic strength conditions (panel A, lane 8; panel B, lane 6, respectively). These observations suggest that the partitioning of MA and CA in the fluorescence assays at high salt are of relatively low affinity compared to Gag binding. Using intrinsic fluorescence we found that the partition coefficients of the proteins to sucrose-loaded vesicles were within error of the values reported in Table 1, indicating that any possible difference in the surface of sucrose-loaded vesicles could not account for this discrepancy. Alternately, it is possible that the fluorescence studies are detecting the membrane association of small populations of MA and CA that are not detected in the gels. This infers that the mature MA and CA proteins have minor conformations that allow for membrane partitioning consistent with suggestions of post-assembly functions (Bukrinsky et al., 1993; Gallay et al., 1995; Steinkasserer et al., 1995).

Orientation of Gag, MA and CA Proteins on the Membrane Surface. The observed efficient partitioning of Gag and truncated Gag forms but not of MA or CA proteins suggests that the MA and CA domains in the precursors participate in membrane interactions that are different in the mature products. To examine the positioning of the proteins relative to the bilayer, energy transfer was measured between Trp donors in the proteins and anthroyloxy moieties incorporated into the LUVs at various distances from the membrane surface (Haigh et al., 1977). Energy transfer from donors in the membrane-bound protein to acceptor moieties is influenced by both donor−acceptor distance and orientation. Stearic acids with anthroyloxy (AO) moieties covalently attached at specific positions along the 18 carbon acyl chain (at C2, C6, and C12) were incorporated into LUVs. These modified stearic acids (*n*-AS) insert spontaneously into the lipid bilayer with the carboxyl terminus anchored in the lipid head group region, placing the AO moiety at distances from the surface that depend on the position of the moiety along the acyl chain (Haigh, 1979). For the Trp−AO pair, energy transfer occurs with ~50% efficiency at 20 Å (Kleinfeld, 1995). The distance from the membrane surface to the position of an AO group at carbon 12 in stearic acid is less than 20 Å

To measure energy transfer, we determined the increase in acceptor emission intensity when Trp residues in the donor were excited at 280 nm relative to the intensity observed when only the AO acceptor was excited at 381 nm (INT_{280}/INT_{381}). As shown in Figure 7, efficient energy transfer was observed between Trp donors in Gag and both the 2-AS and 6-AS but not the 12-AS acceptors. Less efficient transfer was detected between MA and probes embedded at different depths. In the case of CA, the most efficient transfer was found using the 2-AS probe. These observations indicate that Trp residues in MA and CA proteins are oriented differently before and after proteolytic processing of the precursor. Taken together with the results of the sedimenta-

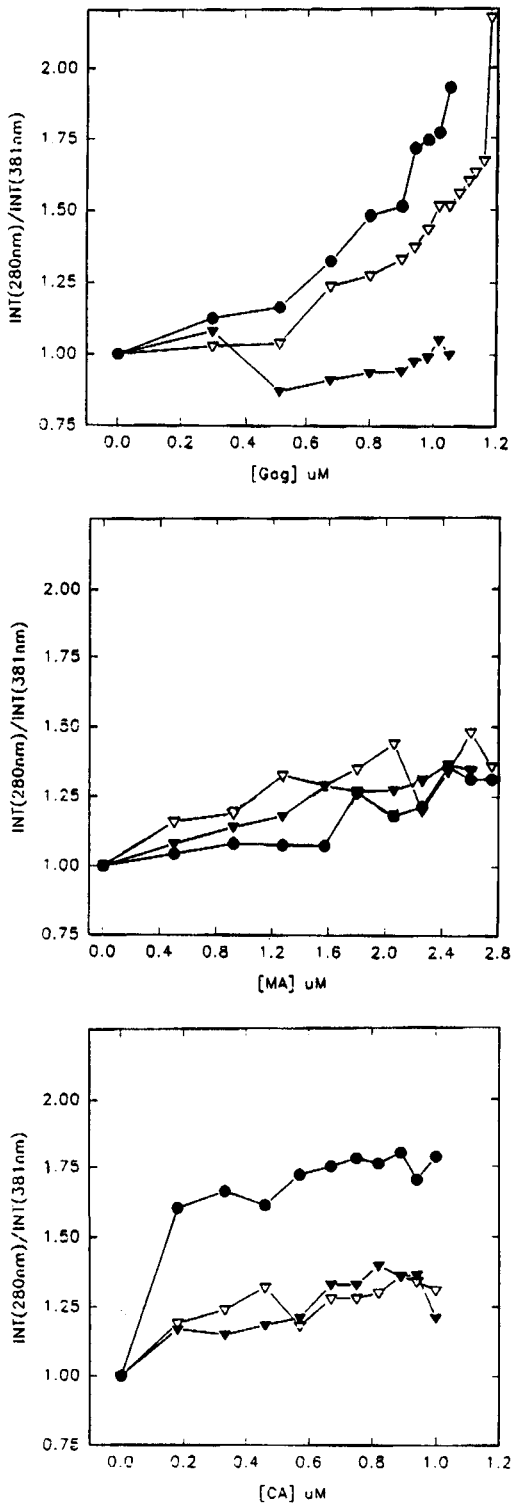

FIGURE 7: Energy transfer from tryptophan donors in Gag, MA, and CA proteins to anthroyloxy (AO) acceptors in the lipid bilayer ($n = 2$). Determined from the increase in AO fluorescence when donor is excited (at 280 nm) relative to only acceptor (at 381 nm). LUVs of POPS containing modified stearic acid with a covalently linked AO group (*n*-AS, where *n* is the position of AO on the 18-carbon acyl chain) was prepared as described in Methods and Materials. (●) 2-AS; (▽) 6-AS; and (▼)12-AS.

tion assay described above, these findings provide strong evidence that determinants of membrane partitioning in the Gag precursor are not identical to the determinants in the mature Gag products.

Membrane Binding of Gag Protein Expressed in RRL. Several laboratories have described membrane binding of

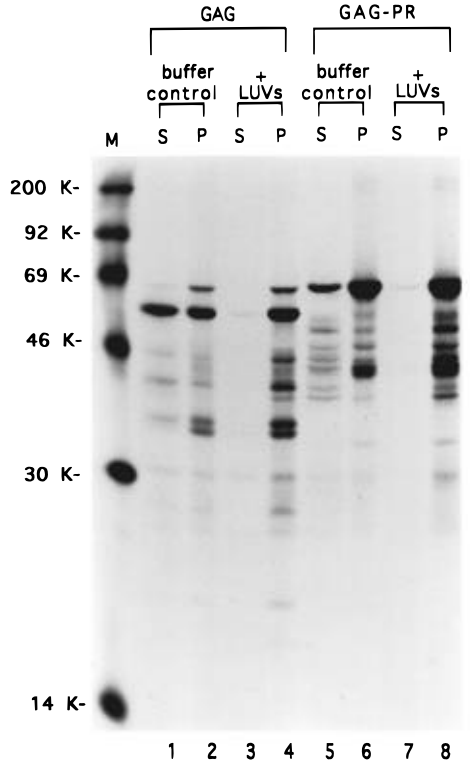

FIGURE 8: Cosedimentation of RRL-synthesized Gag and Gag−PR proteins with lipid vesicles. Shown are the supernatant (S) and pellet (P) fractions from mixtures of translation lysates with buffer alone (buffer control) and sucrose-loaded vesicles (+LUVs). Lanes 1−4, sedimentation of translation lysate containing containing synthesized Gag; lanes 5−8, sedimentation of translation lysate containing synthesized Gag-PR.

Gag proteins expressed in RRL to cellular membrane-enriched fractions *in vitro*. This experimental approach offers the advantage that Gag is synthesized under conditions that more closely mimic the natural setting, and the protein is not subjected to the manipulations that accompany purification. To examine the partitioning of these proteins to LUVs, the DNA construct encoding the Gag precursor protein (used above) was used to direct protein synthesis in RRL in the presence of [^{35}S]Met.

Membrane binding of RRL-synthesized Gag and of the truncated Gag-Pol precursor, Gag-PR, was examined using sucrose-loaded POPS LUV in low ionic strength buffer (0.1 M NaCl) and the sedimentation assay described above (Figure 8). In the absence of LUVs, about half of the Pr55gag, and most of the frame-shifted truncated Gag-Pol precursor (P69), was detected in the pellet fraction (lanes 1, 2 and 5, 6). This has been noted previously (Platt & Haffar, 1994). In the presence of LUVs, the remaining Pr55gag and P69 were effectively removed from the supernatant fraction to the pellet fraction (lanes 3, 4 and 7, 8). These observations are consistent with the results of others (Platt & Haffar, 1994; Spearman et al., 1994; Zhou et al., 1994) and indicate that the proteins synthesized in RRL bind to model membranes efficiently. No significant differences in membrane binding of Gag and Gag-PR were detected, indicating that the p6* and PR domains at the C-terminus of Gag-PR did not significantly affect protein−membrane interactions as measured in this system.

Partitioning of Gag, MA and CA Proteins to Inside-Out Human Erythrocyte Plasma Membranes. To assess the relative importance of lipids *versus* other natural membrane

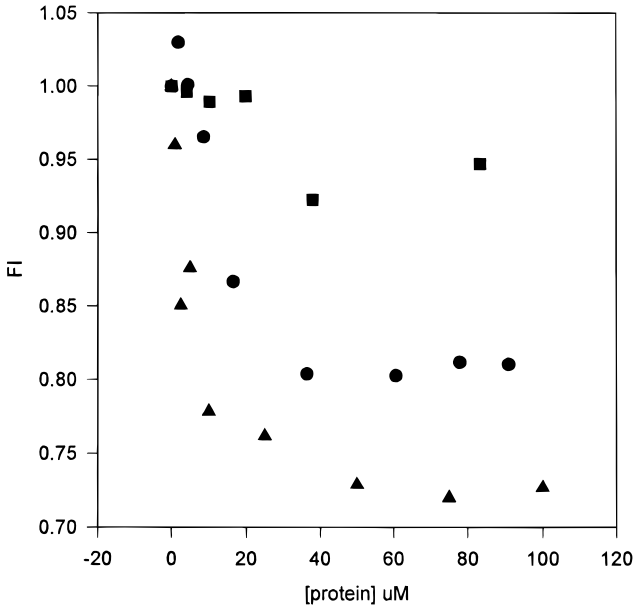

FIGURE 9: Decrease in the fluorescence intensity (FI) of Laurodan incorporated into inside-out RBC vesicles upon the binding of recombinant Gag to inside-out RBC vesicles where the total lipid concentration was 100 μM ($n = 2$); (●) Gag precursor; (▲) MA protein; and (□) CA protein. Maximal sample error is ±3%.

components such as steroids and proteins, vesicles containing the plasma membrane of human RBC on their outer surface were prepared and used for partitioning assays. The membranes were labeled with Laurodan and changes in Laurodan fluorescence were measured in the presence of increasing amounts of Gag, MA, or CA. To preserve the integrity of the membranes, an ionic strength of 0.1 M was used. In Figure 9, we show the change in emission intensity when Gag, MA, and CA are added to RBC membranes at a 0.1 mM lipid concentration and note that identical results are obtained when viewing the change in the emission energy. Both Gag and MA partitioned efficiently to the surface of the RBC vesicles while CA showed reduced binding as compared to LUVs. Thus, the acidic phospholipid component of the membrane appears to be the most important determinant of binding for the Gag and MA proteins while the partitioning of CA may be influenced by other membrane components.

DISCUSSION

Assembly of the Gag precursor of HIV-1 takes place on the cytoplasmic side of the host cell plasma membrane and requires no other viral proteins. The fact that this step in replication occurs in several heterologous systems (Chazal et al., 1995; Gheysen et al., 1989; Jowett et al., 1992; Shioda & Shiuta, 1990; Smith et al., 1993) suggests that membrane binding involves ubiquitous membrane components or highly conserved cellular proteins. Several laboratories have described the binding of Gag proteins to membrane fragments derived from disrupted cells. In this report, we present evidence for several novel features of the Gag−membrane interaction that have not been previously described in detail.

Our studies reveal that the acidic phospholipid content of the target membrane is an important determinant of association and that HIV-1 Gag proteins can partition directly to phospholipids without the aid of other cellular factors. Both the neutral phospholipid, POPC, and the negatively charged phospholipid, POPS, which are common components of eukaryotic cell membranes, are found in HIV-1 particles (Aloia et al., 1993). Gag was found to exhibit a preference for the acidic phospholipids. While MA and Gag have clusters of basic residues that could account for this preference, CA does not. The partitioning that we observed using purified recombinant Gag protein and these model membranes was highly efficient, with a calculated partition coefficient that was comparable or better than those of various peripheral proteins (Parola, 1993). Lipid association was demonstrated using both purified recombinant Gag proteins and Gag proteins which were expressed in RRL and which underwent no purification treatments prior to assay of association. Comparable efficiency also was demonstrated using a natural membrane derived from RBC. These results indicated that Gag-related proteins have the intrinsic ability to associate to membranes and raise the possibility that phospholipid might serve a nonspecific receptor for membrane association of the Gag precursor proteins. It is important to note that the lipid compositions of the viral envelopes of HIV, VSV, and RSV are distinctly different than that of their hosts (Aloia et al., 1993; Pessin & Glaser, 1980) suggesting that Gag, like the M protein from VSV (Weiner et al., 1985), could alter the organization of the lipid surface.

A second finding in our studies is that modification by myristylation or phosphorylation was not required for lipid association to LUVs or to the plasma membrane vesicles derived from RBC. Although myristylation is a critical determinant of Gag targeting in infected cells (Gottlinger et al., 1989; Bryant & Ratner, 1990), our finding is not surprising since it has been estimated that the contribution of the myristate group to membrane binding *per se* is small compared to the measured affinities (i.e., 10^{-4} versus 10^{-6} M; Peitzch & McLaughlin, 1993). Consistent with this, the calculated partition coefficient of Gag for LUVs was ~10-fold higher than that reported for the myristylated alanine-rich C kinase substrate (MARCKS), a major cellular substrate of protein kinase C (Kim et al., 1994). Moreover, we observed membrane affinity of the same order of magnitude in *in vitro* studies with equine infectious anemia virus (D. Ebberts-Reed and C. Carter, unpublished data), which is a related lentivirus whose Gag precursor is not myristylated in its natural form (Henderson et al., 1987). These results clearly indicate that stable association does not require this modification.

The Gag precursor directed membrane partitioning through interactions that appeared to be both qualitatively and quantitatively different from its constituent domains. Gag and the MA−CA processing intermediate were both capable of partitioning to membranes in high salt. The protein−membrane interaction was of high affinity, it was detected in both the fluorescence and the sedimentation assays. However, the amount of association of the mature MA protein was far less extensive in high salt compared to low salt since the interaction was detected by fluorescence but not by sedimentation. Similarly, interactions of the CA protein were detected only by fluorescence, and the mature NC protein did not exhibit association in either assay under these conditions. Our studies indicate that under high ionic strength conditions, the mature MA and CA domains possess comparable partition coefficients (Table 1). In low ionic strength buffers, MA associates more efficiently than CA

seen in the RRL studies. The three-dimensional structure of MA indicates as a surface location and platform-like structure for the highly basic N-terminal region (Massiah et al., 1994; Matthews et al., 1994) which plays as a major role in electrostatic interactions with the membrane surface. In contrast to MA, CA does not contain highly basic regions but does contain an amphipathic domain that appears to contribute to association (D. Ebberts-Reed and C. Carter, unpublished observations). The high affinity partitioning exhibited by the Gag precursor in high salt relative to the mature products suggests as a distinct structure for the MA domain in the precursor and/or that sequences in the CA domain participate directly in membrane association.

Our studies also suggest that the oligomerization state of Gag, either before or after binding, is an important determinant of membrane association. Partitioning of Gag was stable in high salt, while partitioning of the precursor protein synthesized in RRL was not detected in high salt. It is quite possible that the higher concentration of recombinant Gag opposed to RRL Gag (i.e., micromolar opposed to nanomolar) promoted protein−protein interactions in the aqueous phase prior to association which facilitated membrane association under the high salt conditions. Comparison of the partition coefficients obtained from intrinsic fluorescence to intrinsic Trp fluorescence to those obtained from Laurodan-labeled membranes measurements (Table 1), suggest that Gag-Gag interactions occur on the membrane surface. Determination of the self-association of Gag is presently underway. Furthermore, the observation that energy transfer between the Trp residues in Gag and probes continued to rise at protein concentrations where association should be complete supported the idea that Gag-Gag interactions were promoted concomitantly with binding.

This study demonstrates that the HIV-1 structural proteins can partition to the surface of model membranes. Our observations suggest that sites in MA and CA domains can participate in association. Although our data indicate that the NC domain does not participate directly in membrane association, we note that this region appears to play a role in Gag−membrane association in the RRL system (Platt & Haffar, 1994). Admittedly, the composition of cellular plasma membranes, to which Gag proteins are bound during virus assembly, are much more complex than that of model or enriched membrane fractions used in this and other studies (Zhou et al., 1994; Platt & Haffar, 1994). Our observation that Gag has intrinsic membrane affinity makes it conceivable that nonspecific binding can be mediated by interaction of the polyprotein portion of Gag with acidic phospholipids. Specific targeting and functional retention, however, requires participation of the *N*-myristate moiety. We postulate that as the membrane concentration of Gag increases, interactions between CA and the membrane become replaced by interactions between neighboring Gag molecules. We speculate that the introduction of RNA and its association to NC helps to give Gag the membrane orientation most favorable for budding (i.e., where only MA−membrane contacts are intact). The system described in this report provides an experimental method in which these Gag−membrane interactions can be quantitated. Elucidation of critical interactions of the assembling viral complex may facilitate identification of events that are feasible to target for antivrial drug design.

ACKNOWLEDGMENT

The authors thank Heather McBath and Indra Jayatilaka for their technical assistance and an anonymous reviewer for a helpful comment.

REFERENCES

Aloia, R. C., Tian, H., & Jensen, F. C. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5181−5185.
Arthur, L. O., Bess, J. W., Jr., Sowder, R. C., II, Benveniste, R. E., Mann, D. L., Chermann, J.-C., & Henderson, L. E. (1992) *Science 258*, 1935−1938.
Bennett, R. P., Nelle, T. D., & Wills, J. W. (1993) *J. Virol. 67*, 6487−6498.
Berkowitz, R. D., Luban, J., & Goff, S. P. (1993) *J. Virol. 67*, 7190−7200.
Bryant, M., & Ratner, L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 523−527.
Bukrinsky, M. I., Haggerty, S., Dempsey, M. P., Sharova, N., Adzhubei, L., Spitz, L., Lewis, P., Goldfarb, D., Emerman, M., & Stevenson, M. (1993) *Nature 365*, 666−669.
Chazal, N., Gay, B., Carriere, C., Tournier, J., & Boulanger, B. (1995) *J. Virol. 69*, 365−375.
De Rocquigny, H., Gabus, C., Vincent, A., Fournie-Zaluski, M.-C., Roques, B., & Darlix, J.-L. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6472−6476.
Dickson, C., Eisenman, R., & Fan, H., Hunter, E., & Teich, N. (1985) in *RNA Tumor Viruses* (Weiss, R., Teich, N., Varmus, H., & Coffin, J., Eds.) pp 513−648, Cold Spring Harbor Laboratory Press, Plainview, NY.
Ehrlich, L. S., Krausslich, H-G., Wimmer, E., & Carter, C. A. (1990) *AIDS Res. Hum. Retroviruses 6*, 1169−1175.
Freed, E., Englund, G., & Martin, M. (1995) *J. Virol. 69*, 3949−3954.
Galley, P., Swingler, S., Aiken, C., & Trono, D. (1995) *Cell 80*, 379−388.
Gheysen, D., Jacobs, E., de Foresta, F., Thiriart, C., Francotte, M., Thines, D. & De Wilde, M. (1989) *Cell 59*, 103−112.
Gelderblom, H. R. (1991) *AIDS 5*, 617−638.
Gottlinger, H. G., Sodroski, J. G., & Haseltine, W. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5781−5785.
Haigh, E., Thulborn, K., & Sawyer, W. (1979) *Biochemistry 18*, 3525−3532.
Henderson, L. E., Bowers, M. A., Sowder, R. C., II, Serabyn, S. A., Johnson, D. G., Bess, J. W., Jr., Arthur, L. O., Bryant, D. K., & Fenselau, C. (1992) *J. Virol. 66*, 1856−1865.
Henderson, L. E., Sowder, R. C., Smythers, G. W., & Oroszlan, S. (1987) *J. Virol.* 61, 1116−1124.
Hope, M., Bally, M., Webb, G., & Cullis, P. (1985) *Biochem. Biophys. Acta 812*, 55−65.
Jacks, T., Power, M. D., Masiarz, F. R., Luciw, P. A., Barr, P. J., & Varmus, H. E. (1988) *Nature 331*, 280−283.
Jowett, J. B. M., Hockley, D. J., Nermut, M. V., & Jones, I. M. (1992) *J. Gen. Virol. 73*, 3079−3086.
Kim, J., Shishido, T., Jiang, X., Aderem, A., & McLaughlin, S. (1994) *J. Biol. Chem. 269*, 28214−28219.
Kleinfeld, A. M. (1985) *Biochemistry 24*, 1874−1879.
Krausslich, H.-G., Schneider, H., Zybarth, G., Carter, C. A., & Wimmer, E. (1988) *J. Virol. 62*, 4393−4397.
Kunkel, T. (1985) *Proc. Natl. Acad. U.S.A. 82*, 488−492.
Massiah, M. A., Starich, M. R., Paschall, C., Summers, M. F., Christensen, A. M., & Sundquist, W. I. (1994) *J. Mol. Biol. 244*, 198−223.
Matthews, S., Barlow, P., Boyd, J., Barton, G., Russell, R., Mills, H., Cunningham, M., Meyers, N., Burns, N., Clark, N., Kingsman, S., Kingsman, A., & Campbell, I. (1994) *Nature 370*, 666−668.
Parasassi, T., Stasio, G., Ravagnan, G., Rusch, R., & Gratton, E. (1991) *Biophys. J. 60*, 179−189.
Parola, A. H. (1993) in *Biomembranes: Physical Aspects* (Shinitzky, M., Ed.) pp 159−277, VCH Publishers, New York.
Peitzsch, R. M., & McLaughlin, S. (1993) *Biochemistry 32*, 10436−10443.
Pessin, J. E., & Glaser, M. (1980) *J. Biol. Chem. 255*, 9044−9050.

Platt, E. J., & Haffar, O. K. (1994) *Proc. Natl. Acad. U.S.A. 91*, 4594−4598.
Ratner, L., Haseltine, W., Patarca, R., Livak, K. j., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Petteway, S. R., Jr., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Ghrayeb, J., Chang, N. T., Gallo, R. C., & Wong-Staal, F. (1985) *Nature 313*, 277−284.
Rebecchi, M., Peterson, A., & McLaughlin, S. (1992) *Biochemistry 31*, 12742−12747.
Royer, M., Cerutti, M., Gay, B., Hong, S.-S., Devauchelle, G., & Boulanger, P. (1991) *Virology 184*, 417−422.
Shioda, T., & Shibuta, H. (1990) *Virology 175*, 139−148.
Smith, A. J., Cho, M.-I., Hammarskjold, M.-L., & Rekosh, D. (1990) *J. Virol. 64*, 2743−2750.
Spearman, P., Wang, J.-J., Heyden, N. V., & Ratner, L. (1994) *J. Virol. 68*, 3232−3242.
Steck, T. L., Fairbanks, G., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2617− 2624.
Steinkasserer, Harrison, R., Billich, A., Hammerschmid, F., Werner, G., Wolff, B., Peichl, P., Palfi, G., Schnitzel, W., Mlynar, E., & Rosenwirth, B. (1995) *J. Virol. 69*, 814−824.
Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorf, J. W. (1990) *Methods Enzymol. 185*, 60−89.
Weber, G., & Farris, F. (1979) *Biochemistry 18*, 3075−3084.
Wiener, J. R., Pal, R., Barenholz, Y., & Wagner, R. R. (1985) *Biochemistry 24*, 7651−7658.
Wilcox, C., Hu, J-S., & Olson, E. N. (1987) *Science 238*, 1275−1278.
Wills, J. W., & Craven, R. C. (1991) *AIDS 5*, 639−654.
Zhou, W., Parent, L. J., Wills, J. W., & Resh, M. D. (1994) *J. Virol. 68*, 2556− 2569.

BI952337X