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Conditions for Copackaging Rous Sarcoma Virus and Murine Leukemia Virus Gag Proteins during Retroviral Budding

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Rous sarcoma virus (RSV) and murine leukemia virus (MLV) are examples of distantly related retroviruses that normally do not encounter one another in nature. Their Gag proteins direct particle assembly at the plasma membrane but possess very little sequence similarity. As expected, coexpression of these two Gag proteins did not result in particles that contain both. However, when the N-terminal membrane-binding domain of each molecule was replaced with that of the Src oncoprotein, which is also targeted to the cytoplasmic face of the plasma membrane, efficient copackaging was observed in genetic complementation and coimmunoprecipitation assays. We hypothesize that the RSV and MLV Gag proteins normally use distinct locations on the plasma membrane for particle assembly but otherwise have assembly domains that are sufficiently similar in function (but not sequence) to allow heterologous interactions when these proteins are redirected to a common membrane location.

Many eukaryotic proteins are synthesized on cytosolic ribosomes and subsequently targeted to the cytoplasmic faces of very different membranes. Two examples include NADH cytochrome b_5 reductase, which is involved in lipid biosynthesis on the cytoplasmic face of the endoplasmic reticulum, and the Src protein, which is involved in signal transduction events on the inner face of the plasma membrane. Membrane targeting is thought to involve the N-terminal membrane-binding domains of such proteins (3, 6, 14, 15, 18–20, 22, 25, 26, 31, 35), but the sorting mechanism by which specific membranes are recognized is not understood. Furthermore, little is known about the number of distinct compartments that exist on the cytoplasmic faces of each of the various membranes of the eukaryotic cell.

The Gag proteins of retroviruses also exhibit specific membrane targeting following their synthesis on free ribosomes in the cytosolic compartments of the cell (36). Most infectious retroviruses, such as human immunodeficiency virus (HIV), murine leukemia virus (MLV), and Rous sarcoma virus (RSV) (an avian retrovirus), express Gag proteins that mediate budding from the cytoplasmic face of the plasma membrane; however, certain endogenous viruses bud into the lumen of the endoplasmic reticulum to produce what are known as intracisternal type A particles. Specific membrane targeting (and budding) does not require any virus-encoded product other than the Gag protein. That is, the glycoproteins (*env* gene products), the reverse transcriptase and integrase activities (*pol* gene products), and the viral RNA genome are all dispensable for budding from the appropriate membrane. Although the Env proteins of some retroviruses appear to direct budding to the basolateral membrane in polarized cells (reviewed in reference 36), even without Env the Gag proteins of these viruses are still directed to the plasma membrane.

Individual N-terminal membrane-binding domains from Gag proteins are capable of directing heterologous proteins to the appropriate membrane (37, 44, 45), but the mechanism(s) by which this happens is not known. It seems likely that Gag proteins would take advantage of cellular mechanisms normally utilized to sort host proteins to different membranes (e.g., those for NADH cytochrome b_5 reductase and Src), but, as mentioned above, these events are poorly understood. Likewise, it is unclear whether Gag proteins that are targeted to the same membrane (e.g., those of HIV, MLV, and RSV) assemble into particles at molecularly distinct locations on that membrane.

In the experiments described below, we have examined the possibility that Gag proteins from two distantly related retroviruses—RSV and MLV—might interact during particle assembly and budding. Although these viral proteins direct assembly events that are morphogenetically and kinetically similar, they possess little sequence similarity. Only 72 of the 538 residues of MLV Gag (13%) can be scored as identical to RSV Gag after optimizing the alignment of the sequences (data not shown). Many of these residues are widely scattered, and most of the similarity resides in the C-terminal halves of the molecules. That part of Gag contains the major homology region of the capsid sequence and the zinc finger motifs (Cys-X_2-Cys-X_4-His-X_4-Cys) of the nucleocapsid (NC) sequence (36). However, even in this most conserved portion of Gag, only 24% of the residues are identical in the optimized alignment. No sequence similarity is found in the N-terminal membrane-binding domains of the RSV and MLV Gag proteins. Indeed, it is well established that MLV Gag is dependent on the addition of myristate whereas RSV Gag is not myristylated at all (7, 21, 30). For all of these reasons, it seemed unlikely that the RSV and MLV Gag proteins would be capable of interacting during the assembly process. On the other hand, a variety of experiments have shown that the assembly domains of the RSV Gag protein (named M, L, and I) can be replaced by those from other distantly related Gag proteins, such as that of HIV (1, 9, 23, 24).

Briefly, assembly domains provide the functions of Gag required for budding (see Fig. 1A) (reviewed in references 5, 9, and 36). The M (membrane-binding) domain is located at the N terminus and provides the information needed for binding/targeting to the plasma membrane. M mutants have very tight blocks to budding. The I (interaction) domains are located in

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the NC sequence and provide the major region of interaction, during which ~1,500 Gag molecules come together to form a particle. I mutants release low-density particles, if any are produced at all. The L (late) domain is needed just prior to release, as the particle separates from cell. L can be located at different positions in different Gag proteins and can function in a positionally independent manner. L mutants accumulate tethered particles on the membrane, and the few that are released have normal density.

The ability to exchange assembly domains among distantly related Gag proteins suggests that their functions may be conserved while their amino acid sequences are not. Thus, it was difficult to know what to expect from our attempts to copackage the RSV and MLV Gag proteins. To our surprise, we found that these two Gag proteins could be copackaged during budding, but only when the same membrane-binding domain (that of the Src protein) was placed at their N termini. This provides unprecedented evidence that distinct membrane-binding sites may exist for different Gag proteins.

MATERIALS AND METHODS

Parental *gag* genes. The wild-type RSV *gag* gene is from pATV-8, an infectious clone of the RSV Prague C genome (34). The wild-type MLV *gag* and *pol* genes are from pRR88, an infectious clone of the Moloney murine leukemia virus (MMLV) genome in plasmid pGCcos3neo (12) (kindly provided by Alan Rein, Frederick Cancer Research and Development Center). All of the expression plasmids used in this study have been described previously and are briefly explained below. They were propagated in *Escherichia coli* DH-1 by using medium supplemented with 25 μg of ampicillin per ml.

RSV Gag mutants. Several derivatives of the RSV Gag protein were used (Fig. 1A). The wild-type protein was expressed by pSV.Myr0 (41, 42). A myristylated form of this protein containing the first 10 residues of the Src oncoprotein was expressed by pSV.Myr1 (41, 42) and is here given the name R.M1. A protease-minus form of this chimera, here named R.M1.Pr−, was expressed by pSV.Myr1.D37S (4). An unmyristylated form of R.M1, the result of changing the second amino acid from Gly to Glu (2), was produced by pSV.Myr1(−) and is here referred to as R.M(−). The product of pSV.Bg-Bs (38, 39) is a C-terminally truncated form of R.M1 lacking the I domains, and the product of pSV.T-10C (23, 40) has an internal deletion that removes the L domain.

MLV Gag mutants. The wild-type MLV Gag protein (Fig. 1B) was expressed by two different plasmids. One of these, pSV.MLV, also produces a nearly full-length (lacking the last 168 of the 1,737 residues) protease-plus form of Gag-Pol, whereas the other, pSV.MLV.Pr−, produces a protease-minus form (2). We also expressed chimeras of MLV Gag in which the first 39 residues are replaced with the first 10 residues of Src. The Src chimera produced by pSV.M.M1 also expresses the MLV protease, whereas that produced by pSV.M.M1.Pr− does not (2). A myristate-minus (G2E) form of the Src-MLV chimera was expressed by pSV.M.M(−) (2).

Chimeric RSV-MLV *gag* genes. pSV.BgM (Fig. 1C) contains a chimeric *gag* gene in which the first half of the *myr1* allele of RSV *gag* is linked to the second half of the wild-type MLV *gag* gene (within their capsid-coding sequences) and has been described previously (2). Plasmids that express a myristate-minus form of this chimera, pSV.BgM.M(−), or an internally deleted chimera, pSV.T10M.Pr−, have also been described previously (2).

Expression and analysis of Gag proteins. COS-1 cells were transfected by the DEAE-dextran-chloroquine method, as described previously (41). The plasmid DNAs were digested with *Xba*I and ligated at a concentration of 25 μg/ml before transfection. This removes the bacterial plasmid sequence and joins the 3′ end of the *gag* or *pol* genes with the simian virus 40 late polyadenylation signal (41).

Cells contained in 35-mm plates were labeled at 48 h after transfection for 2.5 h in a manner similar to that described previously (41). Because the MLV Gag protein contains very few methionine residues, labeling was performed with 150 μCi of L-[4,5-^{3}H(N)]leucine. The cells and growth medium from each labeled culture were mixed with standard radioimmunoprecipitation assay lysis buffer (final concentration, 25 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% deoxycholate) containing protease inhibitors, and the Gag proteins were immunoprecipitated for 1 h on ice with polyclonal rabbit serum against whole RSV (38) or polyclonal goat serum against MLV CA (kindly provided by Alan Rein). Immunoprecipitated proteins were separated by electrophoresis in SDS–10% polyacrylamide gels which were then fixed and dried. The radiolabeled proteins were detected by fluorography with Fluoro-Hance (Research Products International, Inc.) and Kodak X-Omat AR5 film at −80°C. Overnight exposures were typically required.

RESULTS

The overall purpose of our experiments was to determine whether the Gag proteins of RSV and MLV could be copackaged into viral particles when coexpressed in simian (COS-1) cells. The properties of the individual Gag derivatives used in our experiments are described below and are followed by the results of the two types of coexpression experiments used to investigate copackaging.

Expression of RSV Gag derivatives. To study the process of particle assembly mediated by the RSV Gag protein, we have been using a simian virus 40-based vector that expresses Gag in the absence of all other viral proteins (41). The wild-type protein, Pr76 (Fig. 1A), is not myristylated and therefore has been referred to as Myr0 when expressed alone in mammalian cells. Myr0 expression in COS-1 cells results in the rapid release of membrane-enclosed, virus-like particles into the culture medium (Fig. 2, lanes 1). These are similar to authentic RSV particles with regard to their rate of release from the cell, their morphology in electron micrographs, and their density in isopycnic sucrose gradients (16, 38, 41). Moreover, during or immediately after particle release, the viral protease cleaves the Myr0 protein into products that are indistinguishable from the mature products of RSV, with the most prominent protein being CA. Proteolytic processing is not a prerequisite for budding, and many of the experiments described below make use of protease (PR)-minus derivatives.

Several years ago (41, 42), we constructed a myristylated derivative of the RSV Gag protein, originally named Myr1 but here referred to as R.M1, in which the first 10 residues are replaced with those from the Src oncoprotein (Fig. 1A). This segment of Src contains a signal for the addition of myristic acid and, together with the adjacent amino acid sequence, provides an excellent plasma membrane-binding domain (3, 6, 18, 26, 35). The R.M1 protein is released efficiently from the cells in virus-like particles and processed by the viral protease (Fig. 2, lanes 2). Elimination of the myristic acid addition site by replacing glycine at residue 2 with glutamate [creating R.M(−) (Fig. 1A)] blocked particle assembly and reduced the level of cell-associated processing by the viral PR (Fig. 2, lanes 3). This demonstrates that R.M1 is dependent on the myristylated Src sequence and demonstrates the importance of the extreme N terminus of RSV Gag for particle assembly and budding (i.e., the M domain has been inactivated by substitution of the Src sequence [21]). The reduction in cell-associated Gag processing observed for the myristate-minus derivative is indicative of the requirement of membrane binding for PR activity and provides an indirect assay for the targeting of Gag proteins to the plasma membrane (21, 42).

Expression of MLV Gag derivatives. To investigate MLV assembly in COS-1 cells, we utilized pSV.MLV, which expresses the *gag* and *pol* genes of MMLV (Fig. 1B). The products of this vector are the MLV Gag protein (Pr65) and a larger fusion protein (Gag-Pol) that arises from in-frame suppression of a UAG codon at a frequency of 5% (29). Digestion with *Xba*I before transfection (see Materials and Methods) leaves the PR and reverse transcriptase (RT) coding sequences intact, but only the first 239 codons of integrase (IN) remain. Upon transfection, MLV Gag proteins were synthesized and released into the medium in particles that predominantly contained Gag cleavage products (e.g., CA), but noticeable levels of uncleaved Gag molecules were consistently observed (Fig. 2, lanes 4).

A Src-MLV chimera, M.M1, was constructed by replacing the first 39 amino acids of Gag with the first 10 residues of the Src protein (Fig. 1B). This chimera was detectably smaller than

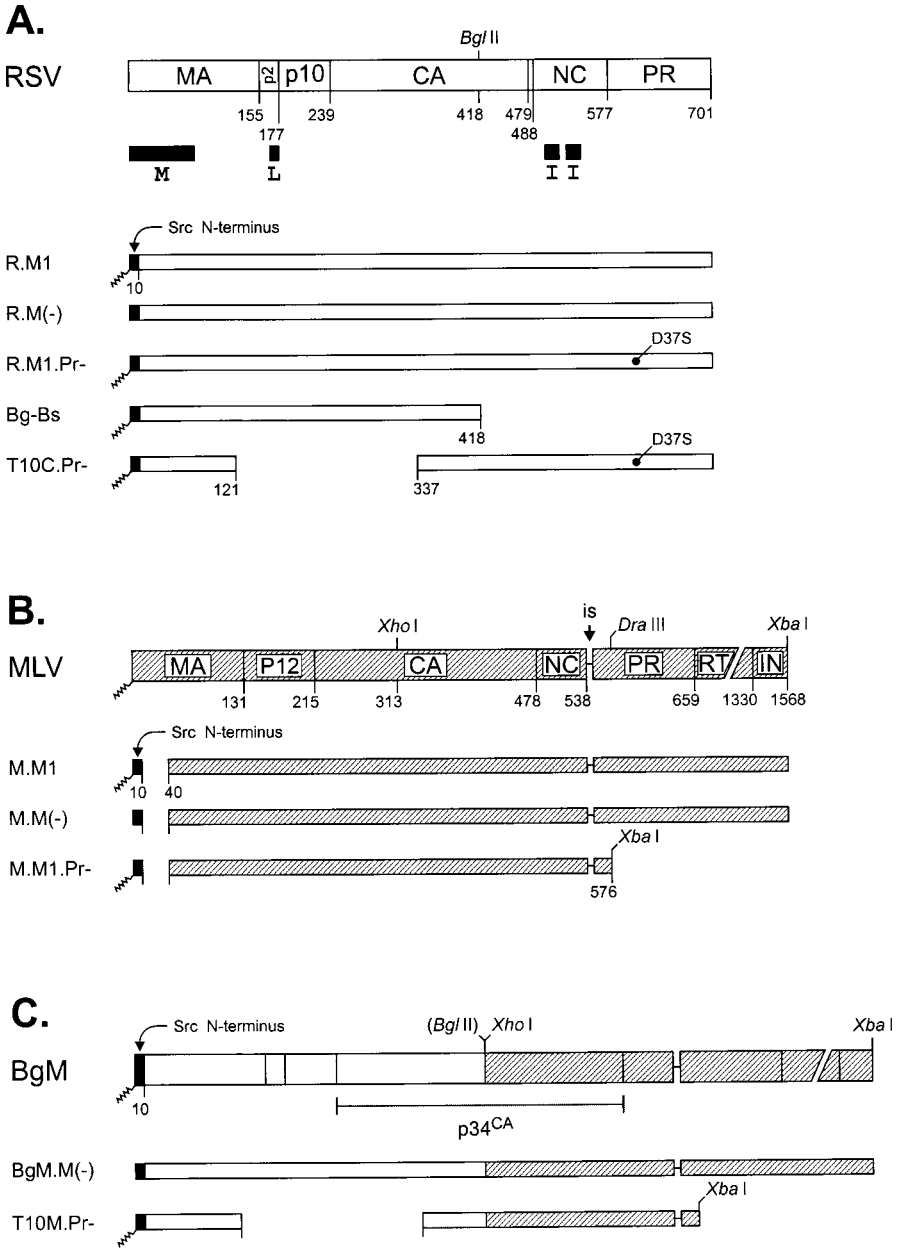

FIG. 1. Derivatives of the RSV and MLV Gag proteins. RSV sequences are represented by open boxes, and those of MLV are represented by hatched boxes. The restriction sites used to create the chimeras are noted above each sequence. Myristylation is indicated by a squiggly line, and M(−) indicates that the glycine encoded by the second codon is replaced, eliminating the site of myristic acid addition. Numbers below the Gag proteins refer to amino acids counted from the N terminus of each Gag protein and mark positions cleaved by the viral protease. (A) Derivatives of the RSV Gag protein. The wild-type molecule is illustrated at the top, where the positions of domains essential for budding are indicated with black bars (M, L, and I). Gag derivatives in which the first 10 residues are replaced with those of the Src oncoprotein are also depicted. The location of the inactivating amino acid substitution in the RSV protease, D37S, is noted. (B) Derivatives of the MLV Gag protein. The wild-type protein is illustrated at the top, and versions in which the first 39 amino acids are replaced with the first 10 residues of the Src protein are also shown. The site of in-frame suppression ("is") of the stop codon between the *gag* and *pol* genes is indicated. (C) RSV-MLV Gag chimeras. The RSV *Bgl*II site was destroyed, while the MLV *Xho*I site was restored during construction of the chimeric gene.

the full-length MLV molecule but was released and processed in a manner identical to the authentic MLV protein (Fig. 2, lanes 5). Because deletions within the first 40 residues of wild-type MLV Gag have been shown to disrupt the budding process (13), it is clear that the heterologous membrane-binding domain from Src can substitute for this M domain in a manner similar to that of the RSV Gag protein.

The particles made by the wild type and the Src chimera of MLV Gag appeared to be normal in every way that we measured. Both contained normal levels of RT activity relative to the authentic virus (data not shown). Moreover, sucrose density gradients showed that the derivatives are released into the medium only within particles of a density similar (MLV, 1.18 g/ml) or identical (M.M1, 1.16 g/ml) to authentic retrovirions (data not shown). The significance of the minor shift in density observed with the wild-type MLV construct is unknown, but

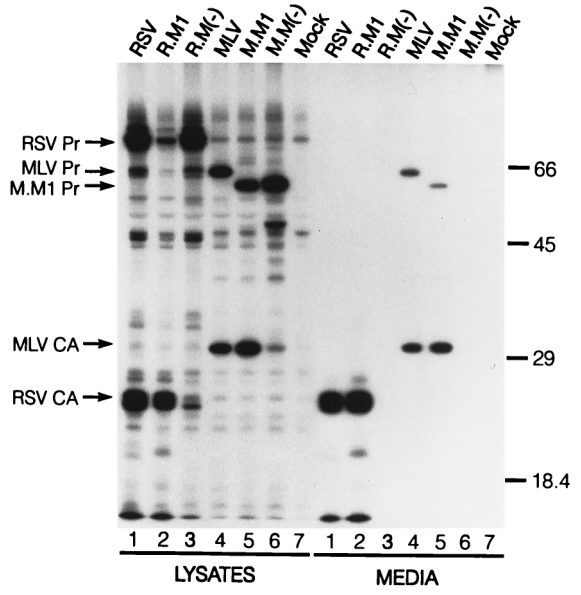

FIG. 2. Expression of RSV and MLV Gag derivatives. COS-1 cells in 35-mm dishes were transfected with the indicated DNAs and labeled 48 h later with [^{3}H]leucine for 2.5 h. The RSV and MLV proteins from the cell lysates or media samples were immunoprecipitated with anti-RSV and anti-MLV serum, respectively. The proteins were electrophoresed on an SDS–10% polyacrylamide gel and visualized by fluorography. Positions of the Gag precursors (Pr) for RSV, MLV, and M.M1, along with the capsid (CA) cleavage products, are indicated on the left. The positions of molecular mass standards are indicated (in kilodaltons) on the right.

similar shifts have been reported for particles made by expressing the RSV Gag protein in mammalian cells (42).

When the site of myristic acid addition was destroyed by changing Gly to Glu at the second residue of the Src-MLV chimera, the resulting protein [M.M(−) (Fig. 1B)] was not released into the medium (Fig. 2, lanes 6). Therefore, the Src chimera of MLV, like its RSV counterpart, is dependent on the foreign membrane-binding domain for particle assembly at the plasma membrane. In addition, intracellular processing was reduced for the myristate-minus chimera, indicating that membrane binding is required for PR activity in MLV. Indeed, this mutant behaves identically to the unmyristylated mutant of MMLV Gag originally described by Rein et al. (30), which was unable to bind to membranes or assemble virions and demonstrated no proteolytic activity.

RSV-MLV Gag chimeras. We also made use of an RSV-MLV chimera named BgM in which the I domains of RSV are replaced with that of MLV (Fig. 1C). We have previously shown that this chimera produces particles at the wild-type rate and density (2). In contrast, the same N-terminal sequence from RSV without the MLV sequence (mutant Bg-Bs [Fig. 1A]) is released poorly and the particles have a very low density (1, 39). The MLV protease is capable of cleaving BgM to release the chimeric capsid sequence, p34^{CA}, and this product is not observed when MLV PR is mutated (2). Moreover, both the uncleaved Gag protein and p34^{CA} were reactive to RSV and MLV antisera (not shown). When the site of myristylation was destroyed [BgM(−) Fig. 1C], the chimera was unable to produce particles and the level of cell-associated proteolytic processing was reduced (data not shown), as was the case for the myristate-minus Src derivatives of the RSV and MLV Gag proteins.

The derivative of BgM used here is named T10M.Pr− (Fig. 1C). It lacks RSV residues 122 to 336, which contain the L domain needed for efficient release of particles (Fig. 1A). Although this chimera has the membrane-binding domain from Src, it exhibits a severe block to budding. The magnitude of this defect is similar to that of RSV mutant T10C.Pr− (23, 40), which contains the very same deletion (Fig. 3A; compare lanes 3 and 4). A derivative of T10M that expresses the functional MLV protease was also unable to produce particles (not shown), as would be expected for a mutant lacking L domain

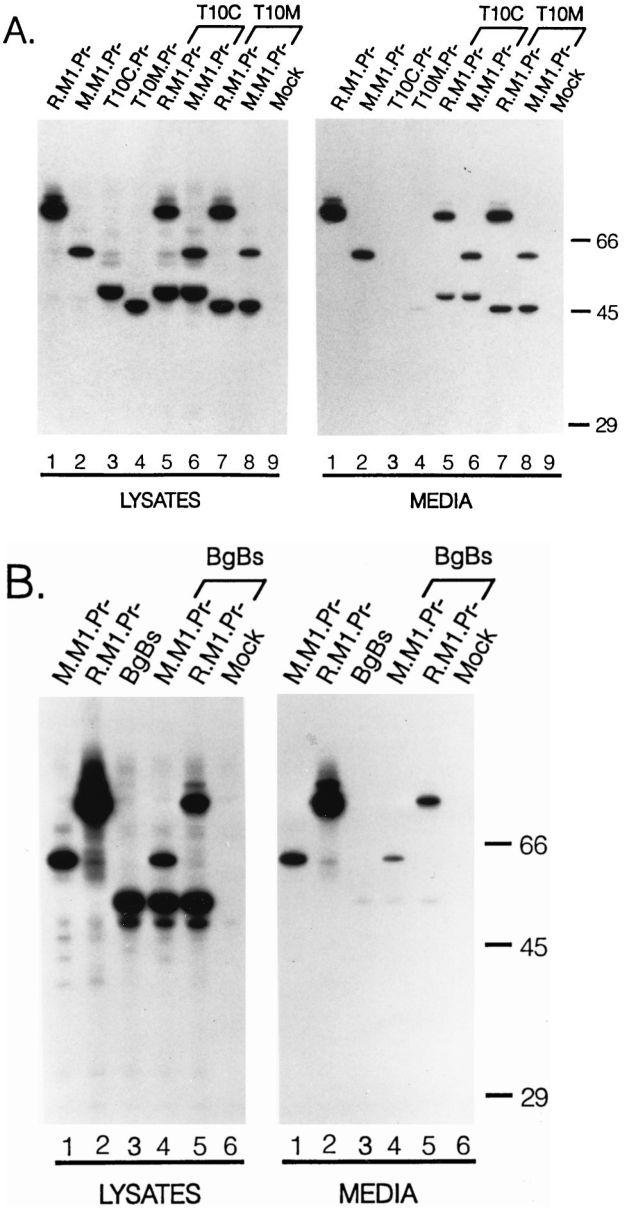

FIG. 3. Complementation analysis with RSV and MLV Gag derivatives. COS-1 cells transfected with the indicated DNAs or combinations of DNAs were metabolically labeled with [^{3}H]leucine for 2.5 h. Immunoprecipitations were performed with a mixture of RSV and MLV antisera. The proteins were separated on an SDS–10% polyacrylamide gel and visualized by fluorography. The positions of molecular mass markers are indicated (in kilodaltons) on the right. (A) Analysis of T10C derivatives. These mutants lack the L domain function of RSV and are blocked at a late step in budding, after membrane binding, but retain the I domain sequences involved in interactions among Gag proteins. (B) Analysis of mutant Bg-Bs. This RSV deletion mutant lacks the region of Gag-Gag interaction.

activity. Because BgM is dependent on the L domain of RSV, we conclude that the C-terminal sequence from MLV Gag must not possess L domain activity.

Genetic evidence for copackaging of heterologous Gag proteins. We have previously shown that L domain mutants of RSV are associated with membranes and can be readily rescued into particles when coexpressed with assembly-competent RSV Gag proteins (40). An example of this is shown in Fig. 3A, where it is clear that T10C.Pr− is not released when expressed alone but is easily detected in the medium when coexpressed with R.M1.Pr− (compare lanes 3 and 5). The high efficiency of this complementation experiment allowed us to investigate the ability of heterologous Gag proteins to rescue L domain mutants. To our surprise, RSV mutant T10C.Pr− was readily released into the medium when the Src-MLV chimera (M.M1.Pr−) was coexpressed (Fig. 3A, lanes 6). This is remarkable because the only sequence in common in these two molecules is the small piece of the Src protein found at their N termini (Fig. 1). Identical results were obtained with the RSV-MLV chimera T10M.Pr−, which is defective for budding on its own but was rescued by the Src chimeras of both RSV and MLV (Fig. 3A; compare lanes 4 with lanes 7 and 8).

The ability to rescue RSV deletion mutant T10C.Pr− with M.M1.Pr− was dependent upon the Src N terminus. That is, when the wild-type membrane-binding domain of RSV was present on T10C.Pr−, the authentic MLV Gag protein was unable to rescue the T10C derivative (data not shown). Although it seemed unlikely that the very small segment from Src (10 residues) would be able to provide interactions between Gag proteins while simultaneously interacting with the membrane, we addressed this possibility experimentally. Mutant Bg-Bs (Fig. 1A), which contains the Src sequence but no I domains, was coexpressed with assembly-competent Src chimeras of RSV and MLV. Neither R.M1.Pr− nor M.M1.Pr− were able to enhance the release of Bg-Bs (Fig. 3B, lanes 4 and 5), even though this I domain mutant has been shown to be membrane associated (42). These experiments suggest that the I domains located in the C-terminal regions, and not the Src sequences at the N termini, are responsible for the interactions among heterologous Gag molecules in the complementation experiments.

Direct interactions between RSV and MLV Gag sequences. Although the genetic evidence for copackaging of RSV and MLV Gag molecules is strong, it does not alone rule out the possibility that budding-competent molecules might indirectly exert an effect on ones that are defective. That is, mutant T10C (and presumably T10M) is blocked at a very late step in budding, just prior to the virus-cell separation step, and it seemed possible that any budding activity by assembly-competent Gag proteins in the vicinity of these arrested particles might be enough to trigger their release into the medium without copackaging. Therefore, it was necessary to determine whether the Src chimeras of RSV and MLV Gag were actually present in the very same particles.

To test for direct interactions among coexpressed RSV and MLV Gag proteins, a series of coimmunoprecipitation experiments was used (Fig. 4A). Cells expressing the Src-Gag chimeras—R.M1.Pr−, M.M1.Pr−, or both—were radiolabeled, and the particles released into the medium were collected by ultracentrifugation. As an important control, R.M1.Pr− particles and M.M1.Pr− particles that had been made separately were mixed together. The pelleted particles were disrupted with lysis buffer, and the released proteins were immunoprecipitated with either anti-MLV or anti-RSV serum. R.M1.Pr− and M.M1.Pr− produced particles when expressed alone, as expected (Fig. 4B, lanes 1 and 4). When these separately made

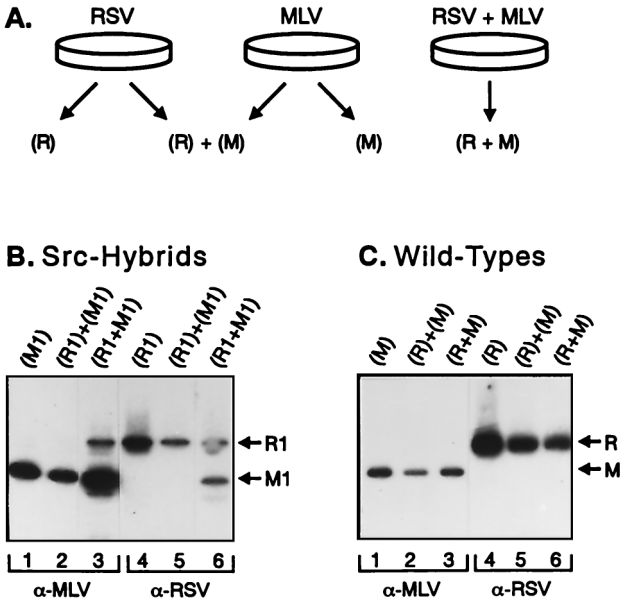

FIG. 4. Coimmunoprecipitation analysis of RSV and MLV Gag proteins. (A) Diagram of the transfection and mixing protocol. COS-1 cells expressing derivatives of the RSV Gag protein, the MLV Gag protein, or both were metabolically labeled for 6 h with [^{3}H]leucine at 48 h after transfection. (B) Analysis of Src-Gag chimeras. The labeled particles were collected by centrifugation through a cushion of 20% sucrose, resuspended in buffer, and divided into two samples of equal volume. In the case of the singly transfected cells, one sample was immunoprecipitated with anti-MLV or anti-RSV serum. The remaining two samples were mixed, split into equal aliquots, and then immunoprecipitated with anti-MLV or anti-RSV serum. In the case of the particles obtained from cotransfected cells, the two aliquots were immunoprecipitated with either anti-MLV or anti-RSV serum. The proteins were electrophoresed on an SDS–10% polyacrylamide gel and visualized by fluorography. The identity of each protein in the gel is indicated on the right. (C) Analysis of wild-type Gag proteins. The experiment was set up exactly as described above except that wild-type constructs were used in place of the Src chimeras.

particles were mixed prior to disruption and immunoprecipitation, only the M.M1.Pr− protein was detected by the anti-MLV serum and only the R.M1.Pr− protein was detected with the anti-RSV serum, indicating that they could not interact once the barrier of the viral membrane was removed (Fig. 4B, lanes 2 and 5). In contrast, when the Src chimeras of RSV and MLV were coexpressed, both Gag proteins were immunoprecipitated with either antiserum, indicating that direct interactions had taken place between them (Fig. 4B, lanes 3 and 6). The amount of MLV protein collected with anti-MLV serum was much greater than that obtained with anti-RSV serum, but the amount of RSV protein was about the same with either serum. This is the expected result given that the level of expression of RSV Gag was less than MLV Gag in this particular experiment. Thus, it appears that all of the RSV protein may have been copackaged, leaving the excess MLV protein to produce particles alone. While it is not possible to make more quantitative conclusions, these data clearly demonstrate that the Src chimeras of RSV and MLV can interact during assembly and become packaged together within the same particle.

As predicted by the genetic complementation experiments, copackaging required the Src membrane-binding domain. This was shown by repeating the coimmunoprecipitation experiment with the wild-type Gag proteins from RSV and MLV (Fig. 4C). In this case, the only Gag proteins detected with the antisera were the immunoreactive species, regardless of whether the RSV and MLV proteins were made separately (Fig. 4C, lanes 2 and 5) or coexpressed (Fig. 4C, lanes 3 and 6).

Therefore, it appears that a critical condition for copackaging these two Gag proteins is providing them with the same membrane-binding domain.

DISCUSSION

Evidence for particles that contain Gag proteins from different retroviruses has never been reported; indeed, our experiments show that the wild-type Gag proteins of RSV and MLV cannot be copackaged even when expressed at high levels in the same cells. From this observation, we hypothesized that the mutually exclusive properties of these two Gag proteins might result from (i) the use of different transport pathways to, or different sites of assembly on, the plasma membrane; (ii) an inability of the molecules to interact even though present at the same membrane location; or (iii) both. Our studies of the Src chimeras of RSV and MLV in budding assays support the first possibility and show that the barrier to copackaging can be removed by placing a common membrane targeting/binding signal on the N termini of these two Gag molecules. The evidence presented here comes from complementation and coimmunoprecipitation assays. A third line of evidence consistent with copackaging has been obtained by coexpressing the protease-active form of BgM with protease-minus forms of the two Src chimeras. In this case, particles that contain unique cleavage products as a result of *trans*-processing among the copackaged Gag molecules (2) are released into the medium.

We interpret our results to mean that the wild-type RSV and MLV Gag proteins are normally directed to different (as yet undefined) locations on the plasma membrane by their unique N-terminal M domains (Fig. 5A). When each of these domains are replaced with the membrane-binding domain of the Src protein, the chimeras arrive at the same membrane location, allowing them to interact and assemble particles containing both Gag molecules (Fig. 5B). The mechanisms of specific and localized membrane targeting are completely unknown, not only for Gag proteins but also for the many cellular proteins that are made in the cytosolic compartments of the cell and then transported to the cytoplasmic faces of the endoplasmic reticulum, Golgi, or plasma membranes (see the introduction). Thus, it appears to us that an unrecognized set of protein-sorting mechanisms remains to be discovered.

The idea that retroviruses utilize unique sites on the plasma membrane as they exit the cell, much as they utilize distinct sites as they enter, is further supported by the emerging properties of L domains. In particular, it appears that L domains participate in specific interactions with host proteins, recruiting them to the site of budding to mediate the membrane fusion event needed for the virus to separate from the cell (reviewed in reference 9). The L domain of RSV (P-P-P-P-Y) has been shown to interact in vitro with proteins containing WW domains (11, 40, 43), and the L domain of equine infectious anemia virus (Y-X-X-L) has been shown to interact both in vitro and in vivo with the plasma membrane-localized, clathrin-associated adapter protein complex (AP-2), a component of the endocytosis machinery (27, 28). Clearly, it is important to learn more about the local molecular environment in and around the sites of budding.

We have not tested any other Gag proteins for the ability to interact in coexpression experiments, so there is little that we can say at this point about which combinations will work. Those wishing to pursue these sorts of experiments need to keep four things in mind. First, RSV and MLV are both oncoviruses. It has become clear from numerous studies that the Gag proteins of oncoviruses and lentiviruses have many important differences, including different arrangements of their assembly domains (M-L-I versus M-I-L, respectively [23]) and the locations of their particle size determinants (10, 16). Second, RSV and MLV have similar, rapid kinetics of budding (half-life, ≈30 min). It is difficult to predict whether Gag molecules from retroviruses with lower rates of budding would be able to be copackaged with RSV. Similarly, Gag proteins from type B and D retroviruses, which assemble in the cytoplasm prior to transport to the membrane, might not be compatible with Gag molecules that assemble only on the membrane. On the other hand, a mutant of Mason-Pfizer monkey virus that results in the assembly of this type D virus only on the membrane has been identified, and it might well be capable of copackaging (32). Third, the MLV Gag protein is very different from that of RSV in that it cannot be rescued into particles when its membrane-binding domain is absent (33). This and other recently described data suggest that MLV Gag proteins do not initiate interactions among themselves until they arrive at, and are concentrated on, the plasma membrane (2). This property might be essential for obtaining definitive results in copackaging experiments. For example, if two Gag molecules that are normally targeted to different sites on the plasma membrane are capable of interacting in the cytoplasm to make complexes that can go to either site, then it would be impos-

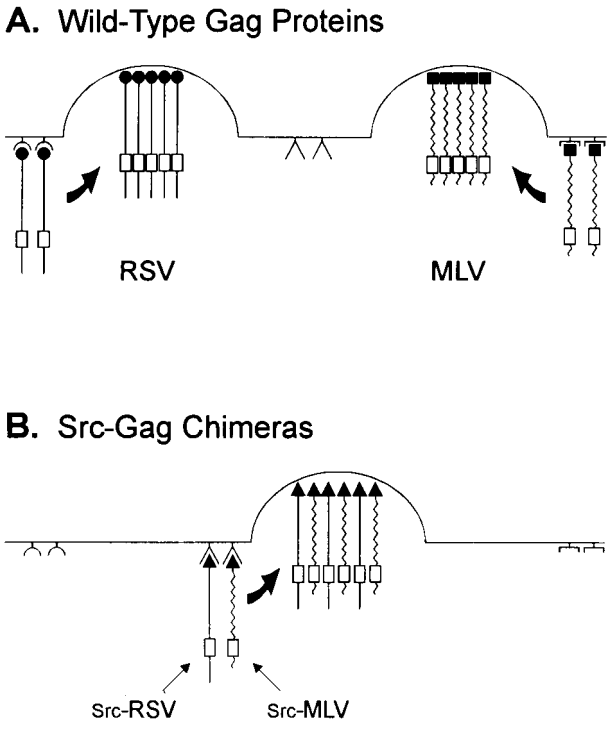

FIG. 5. Distinct sites utilized by Gag proteins during particle assembly on the plasma membrane. (A) The wild-type Gag proteins of RSV and MLV have unrelated membrane-binding domains (depicted by solid circles and solid squares, respectively) and do not encounter one another when expressed in the same cell. Molecular interactions between Gag molecules of a single type occur through the I domain located within the NC sequence (open rectangles) and lead to the emergence of particles of a single type. (B) When the membrane-binding domains of each type of Gag molecule are replaced with that of the Src protein (solid triangles), particles containing both species are released from the cell. This model suggests that the membrane-binding domains of RSV Gag, MLV Gag, and the Src protein interact with distinct regions on the inner surface of the plasma membrane. Therefore, Gag proteins must be targeted to the same membrane location for copackaging to occur. The molecular nature of the proposed receptors is unknown.

sible to discern the existence of the two unique sites of budding. MLV Gag would also be useful in those cases where cytoplasmic interactions among heterologous molecules produce complexes that are incapable of being transported to any membrane. Fourth, the yeast two-hybrid system has been used to look for direct interactions among Gag proteins from different retroviruses, and positive results have been obtained only with the most closely related molecules (8, 17). RSV and MLV Gag have not been tested. Whether negative results from this artifical assay are predictive of what can happen during budding remains to be seen.

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