

Suppression of Retroviral MA Deletions by the Amino-Terminal Membrane-Binding Domain of p60^{src}

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The molecular mechanism by which retroviral Gag proteins are directed to the plasma membrane for the formation of particles (budding) is unknown, but it is widely believed that the MA domain, located at the amino terminus, plays a critical role. Consistent with this idea, we found that small deletions in this segment of the Rous sarcoma virus Gag protein completely blocked particle formation. The mutant proteins appear to have suffered only localized structural damage since they could be rescued (i.e., packaged into particles) when coexpressed with Gag proteins that are competent for particle formation. To our surprise, the effects of the MA deletions could be completely suppressed by fusing as few as seven residues of the myristylated amino terminus of the oncoprotein p60^{src} to the beginning of the mutant Gag proteins. Particles produced by the chimeras were of the same density as the wild type. Two myristylated peptides having sequences distinct from that of p60^{src} were entirely unable to suppress MA deletions, indicating that myristate alone is not a sufficient membrane targeting signal. We hypothesize that the amino terminus of p60^{src} suppresses the effects of MA deletions by diverting the Rous sarcoma virus Gag protein from its normal site of assembly to the Src receptor for particle formation.

Retroviruses are enveloped and acquire their lipid bilayer by budding at the plasma membrane of the host cell. The retroviral protein that is responsible for directing the formation of particles in this manner is the Gag protein, and it can do so in the absence of all the other virion components, including the viral glycoproteins (*env* products), the reverse transcriptase and integrase activities (*pol* products), and the viral RNA genome (7-9, 12, 41, 45). Very little is known about the molecular events that take place during the formation of particles, but it can be surmised that the Gag protein contains functional domains for its specific targeting to the plasma membrane and also for stable binding once it arrives. It is widely believed that the matrix (MA) domain (22), located at the amino terminus of Gag, provides these two potentially distinct functions since it resides near the periphery of the virion (11) and can be cross-linked to the lipids and glycoproteins of the viral envelope (10, 25, 27, 28).

The structural features of MA proteins that enable their specific interaction with the plasma membrane remain poorly understood. It is thought that the fatty acid (myristate) attached to the amino-terminal glycine of most Gag proteins plays a role in targeting and binding to the site of assembly (37, 40, 42), but the nature of that role is unknown. Quite clearly, substitution of glycine with other amino acids blocks myristylation and results in a loss of membrane binding and particle formation (3, 15, 30, 33). Nevertheless, it is difficult to imagine how myristate alone could provide a sufficiently specific signal for directing a Gag protein to the plasma membrane, since a variety of myristylated host proteins have been found to reside at other membrane locations or even free in the cytoplasm (40, 42). Indeed, it has been found recently that small, internal deletions in the MA domain of the myristylated Gag protein of Mason-Pfizer monkey virus completely block particle formation, even

though the addition of myristic acid to the amino terminus is not affected (34). Combined with the fact that several retroviruses encode fully functional Gag proteins that are never myristylated (9, 16, 38), it seems clear that MA proteins must have additional features, presumably contained within the three-dimensional arrangement of their amino acid sequences, that are required for Gag-membrane interactions.

The host-encoded functions involved in transporting and binding Gag proteins to the plasma membrane are understood far less than are the functions of MA proteins. Because retroviruses normally do not bud indiscriminately at all cell membranes (8, 35), we imagine that their Gag proteins must be directed to the appropriate site for assembly by means of a receptor. We further suppose that these receptors correspond to cell-encoded proteins, primarily located on the cytoplasmic face of the plasma membrane, because viral functions other than Gag clearly are not needed for particle formation. It should be noted, however, that host proteins are largely excluded from retrovirions and none are known to be present in equimolar amounts with Gag (29). Thus, it would appear that any Gag-receptor interactions that do take place must occur transiently or among just a few molecules in the Gag population; in either case, Gag receptors may prove difficult to identify and characterize.

Further credence to the idea of host-specified Gag receptors, and for the inferred role of the MA domain in membrane interactions, would be provided if the membrane-binding domain of an unrelated (e.g., non-Gag) protein could be used to specifically suppress MA defects. The results described in this report, obtained by using the Gag protein of Rous sarcoma virus (RSV) and the membrane-binding domain of an oncoprotein, p60^{src}, suggest that this can be done.

MATERIALS AND METHODS

DNAs and cells. The wild-type RSV *gag* gene was obtained from pATV-8, a molecular clone containing an infectious,

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sequenced copy of the RSV Prague C genome (39). MGAG, the recombinant M13mp19 bacteriophage containing RSV *gag* and used for oligonucleotide-directed mutagenesis, has been described previously (45). Plasmids pSV.Myr0, pSV.Myr1, pSV.Myr2, and pSV.GagX are simian virus 40 (SV40)-based expression vectors containing the *myr0*, *myr1*, *myr2*, and *gagX* alleles of the *gag* gene, respectively, and have been described previously (45). The ATG- mutant was generously provided by Marty Stoltzfus (University of Iowa). Carboxy-terminal deletion mutants 3h and Bg-Bs have been described previously (44). Standard protocols were used for all of the DNA manipulations (36). Bacteriophage stocks were routinely propagated in *Escherichia coli* MV1190 (36) grown in LB medium. Plasmids were propagated in *E. coli* DH-1 (36), using LB medium with ampicillin (25 µg/ml). The Gag proteins were expressed in SV40-transformed African green monkey kidney (COS-1) cells grown in Dulbecco's modified Eagle's medium (GIBCO Laboratories) supplemented with 3% fetal bovine serum and 7% calf bovine serum (Hyclone, Inc.).

Site-directed, in vitro mutagenesis. Deletion mutant MA1 was constructed by removing the small DNA fragment that lies between the unique *SauI* and *XhoI* sites in pSV.Myr1 (45). The *SauI* site begins at nucleotide (nt) 401, which lies in codon 8 of the p60^{src}-coding sequence present at the 5' end of the *myr1* allele; the *XhoI* site lies at the middle of the MA-coding sequence (nt 630). The noncomplementary ends produced by the two restriction enzymes were treated with the Klenow fragment of DNA polymerase I to make them blunt and to preserve the *gag* reading frame. The large DNA fragment was agarose gel purified, circularized by using T4 DNA ligase, and then used to transform *E. coli* DH-1 to ampicillin resistance. Plasmid clones containing the desired deletion were identified by restriction mapping.

Deletion mutant B1c was created by cutting pSV.Myr0 DNA at the unique *XhoI* site in the MA-coding sequence and then digesting it with the exonuclease Bal31, using previously described methods (46). The endpoints of the resulting deletions were determined by DNA sequencing using the dideoxy method. The B1c deletion was transferred to pSV.Myr1, pSV.Myr2, and pSV.Myr3 by removing the wild-type *SacII-SacII* fragment (nt 543 to 1806) of each and replacing it with the mutated fragment from pSV.Myr0.B1c. Transfer of this DNA fragment, in the proper orientation, was confirmed by restriction endonuclease mapping and DNA sequencing.

Deletion mutants MA3 and MA4 were made by oligonucleotide-directed mutagenesis using single-stranded, uracil-containing template DNA isolated from MGAG (1, 20, 45). The sequence of the mutagenic oligonucleotide for MA3 was 5'-GCGTGTA AACCTATGGGTTGCTTATGTC-3', and that for MA4 was 5'-GGCTATGATACTGGGGTTACATCTGAGC-3'. The success of the mutagenesis was confirmed by DNA sequencing using the dideoxy method. The MA4 allele was moved from the replicative form DNA into pSV.Myr0 and pSV.Myr1 by a restriction fragment exchange of the *SacII-SacII* fragments (nt 543 to 1806). In the case of MA3, the deletion fell upstream of the *SacII-SacII* fragment (and all other convenient restriction sites), and thus it was necessary to create the mutation separately in MGAG and MMYR1. The latter is an M13mp19 derivative identical to MGAG except containing the *myr1* allele of *gag*. The MA3 mutations were moved from the replicative form DNAs into the SV40 expression vector by exchanging the *SacI-BglII* fragments (nt 255 to 1630).

The *myr3* allele was created by oligonucleotide-directed

mutagenesis using a 48-mer with the sequence 5'-TCAAGCATGGGACAAGAATTAAGCCAGCATGAGCGCGCGTGTAAACC-3', which encodes the amino terminus of the Mason-Pfizer monkey virus MA protein. This mutation creates a *Bss*HII site and was confirmed by DNA sequencing. It was moved into the SV40-based expression vector by using *SacI* and *BglII* as previously described for the *myr0*, *myr1*, and *myr2* alleles (45).

To cripple particle formation in the cell fractionation experiments, the 3' end of the Gag-coding sequence was removed from pSV.Myr0 and pSV.Myr2 as previously described for pSV.Myr1 (44). Briefly, the plasmids were digested with *BglII* (nt 1630) and *Bss*HII (nt 2724), and the noncomplementary ends were made blunt by using the Klenow fragment of DNA polymerase I prior to joining them together with T4 DNA ligase. Clones containing the desired deletion were identified by restriction mapping and have been designated Bg-Bs. All were found to express proteins of the expected size and were unable to direct particle formation, as described before for Myr1.Bg-Bs (44).

To guard against inadvertent mutations that might have arisen elsewhere in *gag*, duplicate or triplicate clones from each of the above-described mutagenesis experiments were initially screened to be sure that their *gag* products exhibited the same phenotype. In all, only one such aberrant clone was found and discarded.

Transfection of mammalian cells and metabolic labeling. Prior to transfection, the bacterial plasmid sequence in the SV40-based expression vectors was removed by digestion with *XbaI*, and the DNA was ligated at a concentration of 25 µg/ml. This manipulation joins the 3' end of the *gag* gene to the SV40 late polyadenylation signal and is essential for high levels of Gag expression (45). COS-1 cells in 35-mm plates were transfected by the DEAE-dextran/chloroquine method as described previously (1, 44, 45). The transfected cells were metabolically labeled with L-[³⁵S]methionine (>1,000 Ci/mmol; ICN Biomedicals) 48 h after transfection, also as described previously (1, 44, 45). The duration of the labeling period is indicated in the text.

Cell lysis, immunoprecipitation, and detection of Gag proteins. The labeling medium was removed from the cells, microcentrifuged for 1 min to remove any loose cells, and mixed with detergents to solubilize any particles that were present, as previously described (45). Cell lysates were prepared and the nuclei were discarded, also as previously described (45). Gag-related proteins were immunoprecipitated from the lysate and medium fractions by using an excess of rabbit antiserum raised against purified RSV (1, 44). The antigen-antibody complexes were collected with fixed *Staphylococcus aureus* cells, and then the immunoprecipitated proteins were dissolved in sample buffer and separated in 1.5-mm-thick sodium dodecyl sulfate (SDS)-12% polyacrylamide gels as previously described (1, 44, 45). The positions of labeled proteins in the gels were determined by fluorography, using Fluoro-Hance (Research Products International, Inc.) and X-Omat AR5 film (Eastman Kodak Co., Rochester, N.Y.) at -70°C.

RESULTS

We have chosen the RSV Gag protein, Pr76^{gag}, for our investigations (Fig. 1A). This Gag protein is among those that function without the need for myristate (9, 16, 38), though it can be added without seriously reducing infectivity (9). Although RSV is an avian retrovirus, its unmyristylated Gag protein (designated Myr0; Fig. 1A) can drive particle

A. Chimeric Gag Proteins:

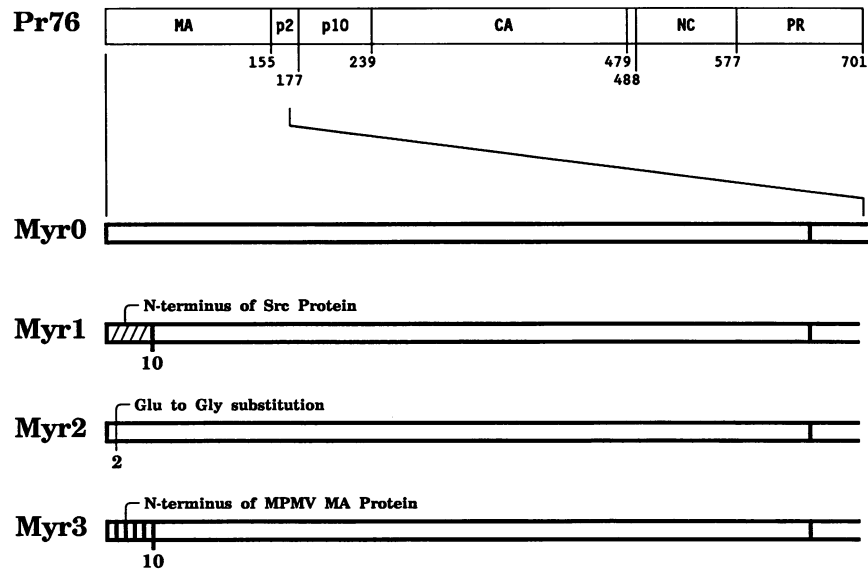


FIG. 1. Schematic diagrams of the RSV Gag derivatives. (A) The upper bar depicts the arrangement of the Gag cleavage products within the precursor, Pr76^{gag}. Myr0 is the name given to this unmyristylated protein when it is expressed in mammalian cells. The Myr1, Myr2, and Myr3 proteins are full-length, myristylated derivatives which differ only at their N termini, as indicated. (B) MA deletions B1c, MA3, and MA4 were introduced into the Gag proteins as described in Materials and Methods. Mutants ATG- and GagX have a truncated N terminus, but the initiation codon used for their synthesis is uncertain, as indicated. Mutant MA1 was constructed by using a restriction site unique in the *src* sequence and thus could be expressed only with the Myr1 N terminus. Numbers below the maps refer to amino acid residues.

formation in mammalian cells from quite different species, including cells of simian (COS-1 and CV-1) and murine (NIH 3T3) origin (9, 44, 45). This suggests that any host-encoded proteins that bind to the RSV Gag protein must be rather conserved in structure. However, our previously reported yields of particles obtained by expressing Myr0 at high levels in mammalian cells have been much lower than that obtained with avian cells (9, 45), potentially indicating a reduced affinity or reduced number of the presumptive receptors in mammalian cells.

The heterologous membrane-binding domain that we have used in our experiments is the myristylated amino terminus of p60^{src}. This protein is the product of the *src* oncogene and normally resides on the cytoplasmic face of the plasma membrane as well as on other membranes in the cell (18, 40,

42). The first few amino acids of the Src protein have been shown to be sufficient for myristylation and have been used to direct heterologous proteins to the plasma membrane (4, 6, 17, 18, 21, 24). Also, it has been shown that these residues, if myristylated at the amino terminus, are sufficient for recognition by a 32-kDa plasma membrane-associated receptor (13, 31, 32). We introduced this sequence by substituting the first 10 codons of the *gag* gene with those that encode the first 10 amino acids of the Src protein (45), (M)-G-S-S-K-S-K-P-K-D, where the parentheses indicate that the initiator methionine is removed prior to myristylation. The resulting chimeric protein has been designated Myr1 (Fig. 1A).

Our original reason for creating Myr1 was to determine whether the addition of myristate would enhance particle

formation when the modified Gag protein was expressed in the environment of the mammalian cell. Curiously, we found that Myr1 produces particles at a higher rate than did Myr0 not only in simian (CV-1) and murine (NIH 3T3) cells but also in avian (turkey embryo fibroblast) cells, which are highly permissive for RSV (9, 45). Though we previously have not had a clear hypothesis to explain the enhanced rate of particle formation exhibited by Myr1 (but see below), we have adopted it for most of our studies since the particles it produces are virtually identical to those of authentic RSV in their rate of release, density, and morphological appearance (44, 45). Moreover, they contain Gag cleavage products that comigrate in SDS-polyacrylamide gels with those from RSV, except for the MA protein which has been modified at its amino terminus. The release of these products from the Myr1 precursor is accomplished by the viral protease, and processing occurs with the expected kinetics (1, 45).

Deletion analysis of the RSV MA domain. During the course of our experiments with Myr1, we constructed a large deletion mutant, MA1 (Fig. 1B), which lacks the first 83 of the 155 amino acids of the MA domain but retains most of the amino-terminal residues from p60^{src} (seven, not counting the initiator methionine). We fully expected this mutant to be unable to produce particles, given the blocked phenotype of another MA deletion mutant, GagX (44, 45). We have previously shown that the deletion in the GagX protein is small and have hypothesized (45) that it arises posttranscriptionally as a result of initiating translation at a downstream AUG, either at codon 29 or at codon 40, both of which lie in the *gag* reading frame (Fig. 1B). Since the deletion contained in mutant MA1 is twice as large as, and appears to overlap, that of GagX, we were quite surprised to find that two independent MA1 clones were capable of particle formation, as measured by the release of large amounts of Gag cleavage products into the growth medium (Fig. 2B, lanes 3 and 4). The only difference between the MA1 clones and their Myr1 parent (Fig. 2B, lanes 1) was the expected absence of the normal-length MA products, p19 and p23. (p19 is the mature MA protein which consists of 155 amino acids [43] and migrates as a doublet as a result of phosphorylation [23]. p23 is a processing intermediate and consists of p19 plus a small peptide of 22 residues, called p2, which flanks MA and is proteolytically released at a very slow rate [1, 26].)

The ability of the MA1 protein to be released into the medium prompted us to test our predicted mechanism for the synthesis of the GagX protein. For this purpose, we constructed a derivative of Myr0, named ATG- (Fig. 1B), in which the *gag* initiation codon has been destroyed by changing A to T, thus forcing all translation to begin at downstream AUGs. We found that the ATG- mutant behaved identically to GagX. That is, neither mutant protein was released into the medium (Fig. 2A; compare lanes 1 and 2), and they were found to be identical in size. Moreover, they were both found to be slightly smaller than the wild-type precursor, but bigger than MA1, when analyzed on lower-percentage SDS-polyacrylamide gels (data not shown). We conclude that the amino acids deleted in GagX/ATG- indeed are a subset of those deleted in MA1.

The dramatic difference in phenotypes of mutants MA1 and GagX/ATG- first suggested to us that the amino terminus of p60^{src} might be able to replace the membrane-binding function associated with the RSV MA protein. However, this interpretation was tentative since the mutants do not differ solely by the presence or absence of the Src sequence but also in the size of the deletion. To be rigorous, we constructed three additional deletion mutants (B1c, MA3,

and MA4; Fig. 1B) at internal positions in MA. Each of these deletions was then introduced into the Myr0 protein and into the Myr1 protein to obtain pairs of mutants which differ only at their amino termini (Fig. 1A). If our hypothesis is correct, then none of the new MA deletions would be expected to have any effect on Myr1 (which has the Src amino terminus) but should abolish particle formation for Myr0 (which does not).

Because of the low level of particle formation noted above for Myr0, we were concerned that we would not be able to distinguish mutations that completely blocked Myr0 production from those that merely reduced its low rate of release a little further. Fortunately (and fortuitously), we discovered that in COS-1 cells, the level of particles produced by Myr0 rivals that of Myr1 (Fig. 2A; compare lanes 3 and 4). Using this cell line, we found that all three of the new MA deletions unquestionably destroyed the ability of Myr0 to produce particles (Fig. 2C to E). We estimate that our immunoprecipitation assay is sensitive enough to detect Gag proteins present at only 1 to 5% of the wild-type level, but none were observed in the medium samples. In marked contrast, the presence of the Src amino terminus completely suppressed the effects of each of these deletions and restored the level of Gag protein released into the medium to that of the full-length Myr1 control. Indeed, the only difference between the deleted and full-length Myr1 molecules was the expected, faster migration of the shortened MA species. In the case of mutant B1c, the MA products were harder to detect, suggesting that a major antigenic determinant was also destroyed.

Other myristylated amino-terminal sequences were unable to suppress the effects of the MA deletions. This was determined by moving the B1c deletion into two other Gag derivatives, Myr2 and Myr3 (Fig. 1A). In Myr2, a site for the addition of myristic acid has been created by changing the second amino acid of Gag from glutamic acid to glycine (9, 45); the first 10 residues are thus predicted to be (M)-G-A-V-I-K-V-I-S-S. In Myr3, the first 10 amino acids were replaced with those of the Mason-Pfizer monkey virus MA protein, (M)-G-Q-E-L-S-Q-H-E-R, a substitution which is sufficient to enable myristylation on the RSV Gag protein (data not shown). Although the full-length forms of Myr2 and Myr3 produced particles at high levels in COS-1 cells (Fig. 2A, lanes 5 and 6), they completely lost that ability when the B1c mutation was introduced (Fig. 2E, lanes 4 and 5). From these results, it is clear that myristate alone is not a sufficient signal for suppressing MA deletions but rather the amino acid sequence is also critical. Because Myr2 and Myr3 are as sensitive to MA deletions as is Myr0, we suggest that all three use the same mechanism (e.g., the Gag receptor) for binding to the plasma membrane during particle formation.

Sucrose density gradient analysis. Does suppression of MA deletions by the amino terminus of Src restore the actual process of particle formation? If this were the case, then the mutant proteins should band in isopycnic sucrose gradients at a position corresponding to the density of a retrovirus. COS-1 cells expressing the Myr1 deletion mutants were labeled for 3.5 h with [³⁵S]methionine, and the proteins present in the growth medium were immediately layered on 10 to 50% sucrose gradients and centrifuged at 70,000 × *g* for 16 h. Unlabeled, authentic RSV was included in each centrifuge tube to serve as an internal marker. Fractions containing the labeled Gag proteins were detected by scintillation counting, and those containing RSV were identified by assaying for reverse transcriptase (14). We found that the full-length (wild-type) Myr0 protein and the Myr1 derivative

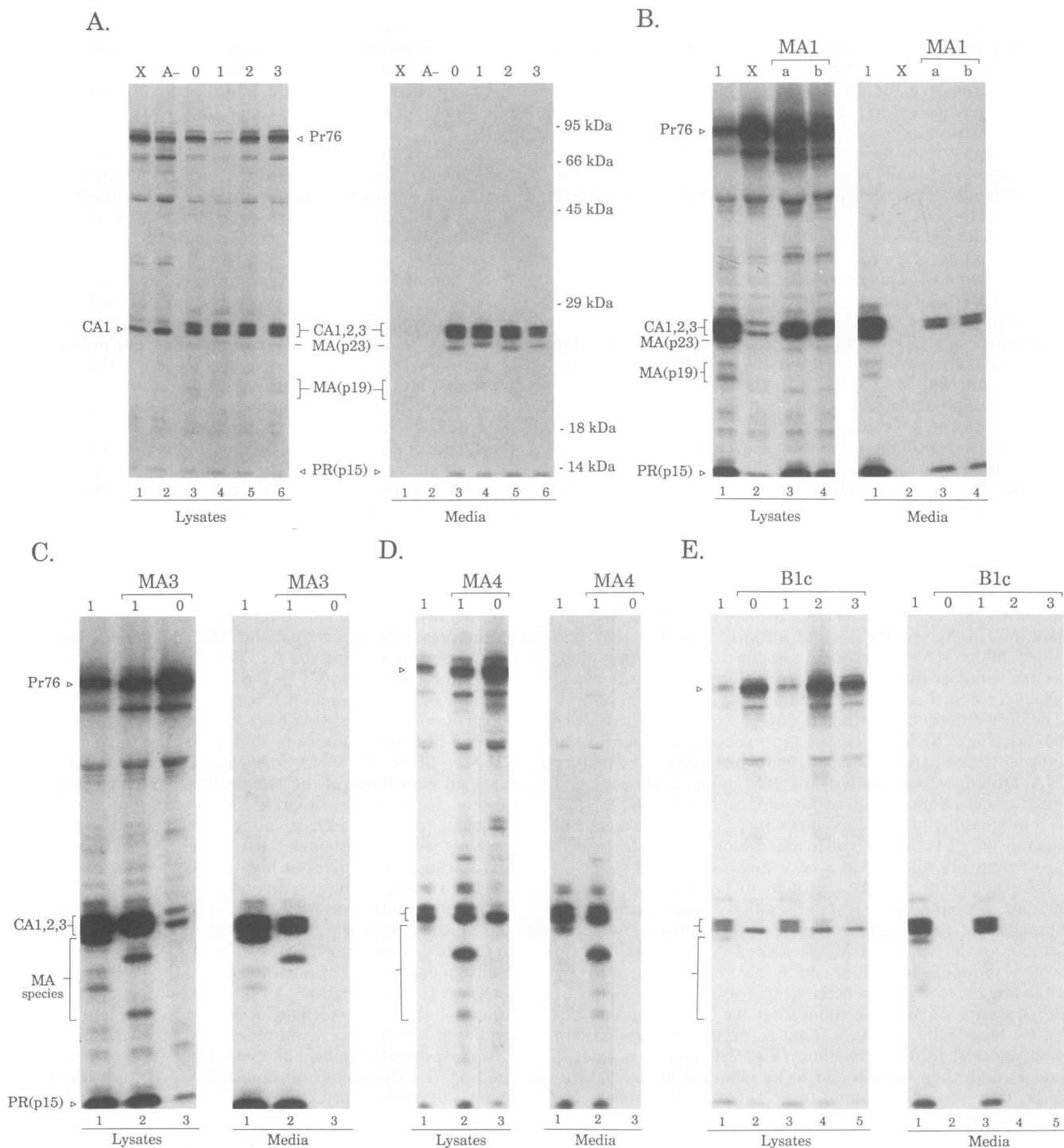


FIG. 2. Immunoprecipitation analysis of the chimeric and mutant Gag proteins. COS-1 cells transfected with the indicated SV40-based expression plasmids were metabolically labeled with [35 S]methionine for 2.5 h, and then the RSV-related proteins present in the cell lysates and in the media were collected by immunoprecipitation using an anti-RSV serum as detailed in the Materials and Methods. The precipitated proteins were electrophoresed in an SDS-12% polyacrylamide gel and visualized by fluorography. (A) Analysis of the full-length Gag proteins. Positions of molecular weight markers are indicated at the right. (B) Analysis of mutant MA1. Lanes a and b represent two independently isolated clones of MA1. (C) Analysis of MA3 mutants. (D) Analysis of MA4 mutants. (E) Analysis of B1c mutants. Lanes: X, GagX; A-, ATG-; 0, Myr0; 1, Myr1; 2, Myr2; 3, Myr3. Lanes corresponding to the deletion mutants are set off with brackets at the top of each panel. The positions of the full-length Gag protein (Pr76) and the cleavage products are indicated. The CA protein is a mixture of three species, CA1, CA2, and CA3, as described previously (1). The heterogeneous MA proteins are marked by larger brackets. PR(p15), protease.

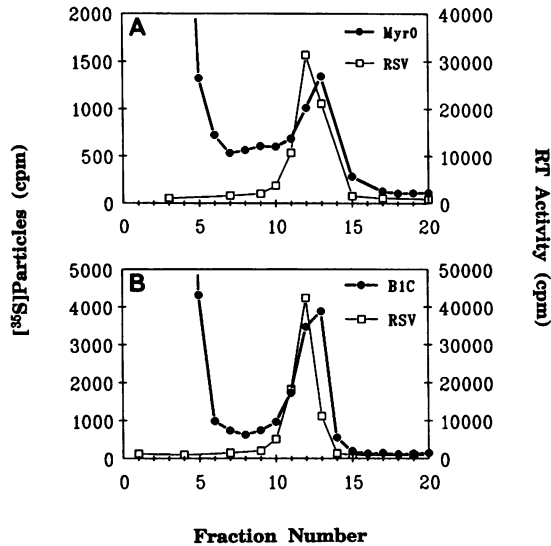


FIG. 3. Analysis of particle density in sucrose gradients. COS-1 cells were transfected with pSV.Myr0 (A) or pSV.Myr1.B1c (B); 48 h later, the cells were labeled for 3.5 h with [³⁵S]methionine. The medium from each plate was then immediately mixed with a sample of unlabeled, authentic RSV and centrifuged at 70,000 × *g* in 10 to 50% sucrose gradients for 16 h. Fractions containing the labeled particles were detected by scintillation counting, and those containing RSV were identified by assaying for reverse transcriptase activity. The large amount of radioactivity at the top of the gradients corresponds to the unincorporated isotope present in the medium. The measured density (grams per milliliter) of each of the peak fractions is noted in the text.

of deletion mutant B1c indeed were contained in particles of a density similar to authentic RSV (Fig. 3A and B, respectively). The reported densities for retroviruses generally fall between 1.15 and 1.18 g/ml (8). In this particular experiment, the measured densities for the RSV controls were 1.1513 (Fig. 3A) and 1.1583 (Fig. 3B), while those for Myr0 and Myr1.B1c were 1.1676 and 1.1811, respectively. Immunoprecipitation analysis of the labeled proteins demonstrated that the lower height of the Myr0 (wild-type) peak was due to a lower level of expression relative to Myr1.B1c and was not due to the presence of low-density particles obscured by the radioactivity near the top of the gradient (data not shown). Virtually identical results were obtained with the full-length Myr1 protein (1.1724 g/ml) and derivatives containing deletions MA1 (1.1588 g/ml) and MA3 (1.1698 g/ml). Mutant MA4 was not tested, but we have no reason to expect that it would behave differently. These results rule out the possibility that the chimeric proteins were released from the cell in a soluble form after somehow entering the secretory pathway or in low-density vesicles formed by blebbing from the plasma membrane.

Complementation analysis. Does the Src sequence work by suppressing global defects in protein folding? If deletions in MA destroy a sequence needed for proper folding of the overall Gag protein—a folding sequence that could be replaced by the amino terminus of Src—then our Myr0 deletion mutants should not be capable of interacting with normally folded Gag proteins during the budding process. To test this, GagX and Myr0.B1c were each coexpressed with a carboxy-terminal deletion mutant named 3h (44). This truncated Gag protein is fully capable of forming particles when expressed alone (Fig. 4, lanes 1) but is detectably smaller

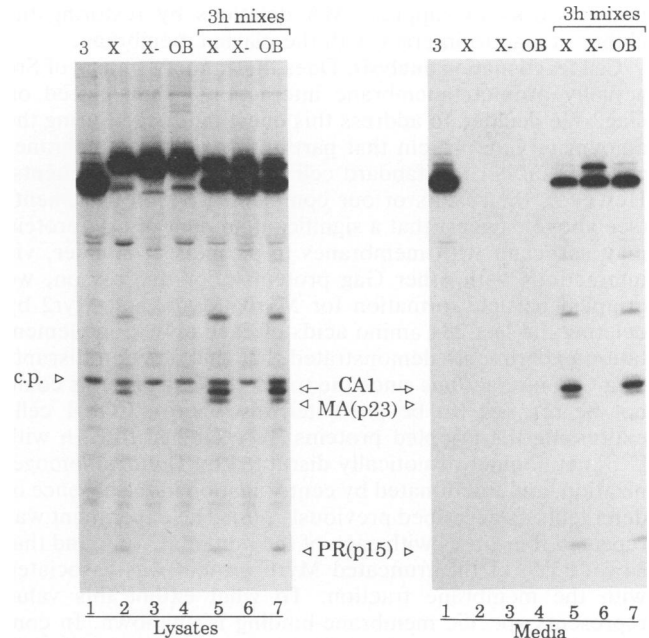


FIG. 4. Packaging of defective Gag molecules by complementation. COS-1 cells were transfected with single DNAs or combinations of DNAs, as indicated. After 48 h, Gag proteins present in the cell lysates and media were immunoprecipitated and separated by electrophoresis as described in the legend to Fig. 2. Mutant Myr1.3h (here abbreviated 3) lacks the viral protease but is capable of particle formation. Mutants GagX(X), GagX.Pro- (X-), and Myr0.B1c (OB) are defective for particle formation but could be packaged if coexpressed with Myr1.3h. c.p., a cellular protein that is present in immunoprecipitates from cell lysates but not the media samples; CA1, the immature form of the CA protein (1).

than the MA mutants because it lacks almost all (117 residues) of the carboxy-terminal protease domain (PR; Fig. 1A). We found that although GagX and Myr0.B1c were unable to produce particles on their own (Fig. 4, lanes 2 and 4, respectively), coexpression with 3h led to the release of readily detectable amounts of all the Gag cleavage products, including the protease (Fig. 4, lanes 5 and 7, respectively). Identical results were obtained with mutant ATG- (not shown). Since 3h does not have the protease, these cleavage products could only have arisen by complementation-packaging of the wild-type protease domain contained on the precursors of the MA mutants. To corroborate these results, we inactivated the protease of GagX by substituting isoleucine for aspartic acid in the active site (44, 45) and repeated the mixing experiment with 3h. As expected, we found no cleavage products in the medium, but the precursor form of GagX.Pro- was quite apparent along with that of 3h (Fig. 4, lanes 6). Control experiments confirmed that GagX.Pro- was unable to form particles alone (Fig. 4, lanes 3). These results clearly demonstrate that our MA-deletion mutants retain the ability to interact with other Gag molecules, and thus, any folding defects they have suffered are expected to be limited to the MA domain. It would be interesting to know where in the cell these Gag-Gag interactions first occur, but our data are not enlightening in that regard. In any case, because the myristylated amino terminus of the Src protein is known to interact with a specific receptor on the cytoplasmic face of the plasma membrane (13, 31, 32), we suggest

that it works to suppress MA deletions by restoring the ability of Gag to interact with the plasma membrane.

Cell fractionation analysis. Does the amino terminus of Src actually promote membrane interactions when placed on Gag? We decided to address this question by measuring the amount of Gag protein that partitions with the membranes and cytosol during standard cell fractionation experiments. However, the results of our complementation experiments (see above) suggest that a significant amount of Gag protein may associate with membranes in an indirect manner, via interactions with other Gag proteins. For this reason, we crippled particle formation for Myr0, Myr1, and Myr2 by deleting the last 284 amino acids of each (44). Complementation experiments demonstrated that this deletion disrupts Gag-Gag interactions since the truncated Gag proteins could not be rescued (to be presented elsewhere). COS-1 cells expressing the crippled proteins were labeled for 2 h with [³⁵S]methionine, osmotically disrupted by Dounce homogenization, and fractionated by centrifugation in the absence of detergents as described previously (46). The experiment was repeated four times with each of the mutants. We found that 34% ($\pm 5\%$) of the truncated Myr0 protein was associated with the membrane fraction. To what extent this value represents specific membrane-binding is unknown. In contrast, the addition of the Src amino terminus produced a quite different result, with 73% ($\pm 6\%$) of the truncated Myr1 protein being found in the membrane pellet. This value is comparable to those reported for p60^{src} (5, 19). The truncated Myr2 protein behaved more like Myr0 than Myr1, with 46% ($\pm 4\%$) pelleting with the membranes. This slightly elevated value might be due to a nonspecific interaction of myristate with membranes; however, as discussed above, myristate alone cannot account for the dramatically different behavior of Myr1. From these results, we conclude that the myristylated amino terminus of p60^{src} indeed promotes membrane association in a sequence-specific manner when placed onto the RSV Gag protein. Further experimentation is in progress to determine whether this association actually occurs on the Src receptor.

DISCUSSION

The experiments described in this report demonstrate that deletions in the MA domain of the RSV Gag protein disrupt the ability of Gag to drive particle formation. At the moment, we do not know where in the pathway Gag transport has been blocked. The ability of the mutant proteins to be rescued by complementation provides convincing evidence that the MA lesions do not perturb the overall structure of the Gag protein and do not block transport to the cellular site where Gag molecules first interact. For RSV and other type C retroviruses, that site has been thought to be on the plasma membrane because of the absence of obvious viruslike structures free within the cytoplasm of infected cells. If Gag-Gag interactions indeed occur only on the membrane, then it would follow that our mutants are transported there but lack the capacity to bind (e.g., via the Gag receptor) in the absence of the complementing molecules. While we favor this model, it is possible that type C Gag proteins form transparent aggregates prior to interacting with membranes.

Is the binding of Gag proteins on the plasma membrane mediated by a receptor? This idea is strongly supported by our finding that a small, membrane-binding domain from a heterologous protein has the capacity to suppress the effects of MA deletions. There are other pieces of evidence, too. For example, electron microscopy has revealed that retro-

viruses generally do not bud from all of the available membrane surfaces within the infected cell but primarily at the plasma membrane. In fact, in polarized cells, they bud only at the basolateral membrane (35). In the case of RSV, it has been reported that the location of particle formation can be redirected into the Golgi apparatus by treating the infected cells with monensin (2). Since monensin blocks the transport of glycoproteins from the Golgi apparatus to the cell surface, and since virus-encoded glycoproteins are not required for particle formation, this observation suggests that a cellular glycoprotein is involved in the binding of Gag molecules to membrane. Finally, a mutation in the MA domain of the Mason-Pfizer monkey virus Gag protein has been described that blocks both the addition of myristic acid and the release of particles from the infected cell but does not prevent this type D retrovirus from forming its characteristic viruslike structures (ICAPS) within the cytoplasm. Electron microscopy has demonstrated that the mutant particles are not distributed randomly in the cell but are clustered deep within the cytoplasm, far from the plasma membrane (32). This finding suggests that Gag proteins are not transported to membranes by simple diffusion. Assuming that Gag receptors exist, it will be interesting to learn whether they are different for different retroviruses and what function(s) they normally provide in the cell.

We do not yet know which portions of the RSV MA domain are required for interaction with the plasma membrane or the hypothetical Gag receptor. However, since this Gag protein appears to be insensitive to myristylation and to changes in sequence within the first 10 amino acids, we hypothesize that the amino terminus of MA, unlike that of Src, is not important. We are currently testing this prediction by deleting residues 3 to 8 from Myr0. The importance of the 5' gag sequence may lie at the RNA level where splicing occurs at a donor site located between codons 6 and 7 during the synthesis of *env* and *src* messengers (39).

Does our chimeric Src-Gag protein (Myr1) actually direct particle formation at the recently identified 32-kDa Src receptor? A definitive answer to this question awaits the results of in vitro membrane-binding experiments of the sort used for p60^{src} (31, 32). Meanwhile, this interpretation of our data certainly provides a sensible explanation for why Myr0 particle production varies from one cell type to another while that for Myr1 is always higher. Cells of different types may simply express different levels of the Myr0 (Gag) receptor relative to the Src receptor. Moreover, since the first 10 residues of Myr0 seem not to be required for interacting with the Gag receptor, it may be that the Myr1 protein displays signals for interacting with both receptors, which could explain why it exhibits higher particle production than does Myr0, even in avian cells.

To further substantiate the results described here, we are currently attempting to suppress our MA deletions by using the membrane-binding domains of proteins that normally reside on the cytoplasmic faces of other membranes (e.g., the rough endoplasmic reticulum). Since one would not expect the composition of cellular proteins to be the same at all sites on all membranes, the success of these experiments would imply that all of the functions required for particle formation are self-contained within Gag except for membrane binding, which also appears to require a cell-encoded receptor.

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REFERENCES

- Bennett, R. P., S. S. Rhee, R. C. Craven, E. Hunter, and J. W. Wills. 1991. Amino acids encoded downstream of *gag* are not required by Rous sarcoma virus protease during Gag-mediated assembly. *J. Virol.* **65**:272–280.
- Bosch, J. V., and R. T. Schwarz. 1984. Processing of gpPr92^{env}, the precursor to the glycoproteins of Rous sarcoma virus: use of inhibitors of oligosaccharide trimming and glycoprotein transport. *Virology* **132**:95–109.
- Bryant, M., and L. Ratner. 1990. Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. *Proc. Natl. Acad. Sci. USA* **87**:523–527.
- Buss, J. E., C. J. Der, and P. A. Solski. 1988. The six amino-terminal amino acids of p60^{src} are sufficient to cause myristylation of p21^{v-ras}. *Mol. Cell. Biol.* **8**:3960–3963.
- Buss, J. E., M. P. Kamps, K. Gould, and B. M. Sefton. 1986. The absence of myristic acid decreases the membrane binding of p60^{src} but does not affect tyrosine protein kinase activity. *J. Virol.* **58**:468–474.
- Buss, J. E., P. A. Solski, J. P. Schaeffer, M. J. MacDonald, and C. J. Der. 1989. Activation of the cellular proto-oncogene product p21Ras by addition of a myristylation signal. *Science* **243**:1600–1603.
- Delchambre, M., D. Gheysen, D. Thines, C. Thiriart, E. Jacobs, E. Verdin, M. Horth, A. Burny, and F. Bex. 1989. The Gag precursor of simian immunodeficiency virus assembles into virus-like particles. *EMBO J.* **8**:2653–2660.
- Dickson, C., R. Eisenman, H. Fan, E. Hunter, and N. Teich. 1984. Protein biosynthesis and assembly, p. 513–648. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), *RNA tumor viruses*, vol. 1. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Erdie, C. R., and J. W. Wills. 1990. Myristylation of Rous sarcoma virus Gag protein does not prevent replication in avian cells. *J. Virol.* **64**:5204–5208.
- Gebhardt, A., J. V. Bosch, A. Ziemiecki, and R. R. Friis. 1984. Rous sarcoma virus p19 and gp35 can be chemically crosslinked to high molecular weight complexes. *J. Mol. Biol.* **174**:297–317.
- Gelderblom, H. R., E. H. S. Hausmann, M. Ozel, G. Pauli, and M. A. Koch. 1987. Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. *Virology* **156**:171–176.
- Gheysen, D., E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. De Wilde. 1989. Assembly and release of HIV-1 precursor Pr55^{gag} virus-like particles from recombinant baculovirus-infected insect cells. *Cell* **59**:103–112.
- Goddard, C., S. T. Arnold, and R. L. Felsted. 1989. High affinity binding of an N-terminal myristoylated p60^{src} peptide. *J. Biol. Chem.* **264**:15173–15176.
- Goff, S., P. Traktman, and D. Baltimore. 1981. Isolation and properties of Moloney murine leukemia virus mutants: use of a rapid assay for release of virion reverse transcriptase. *J. Virol.* **38**:239–248.
- Gottlinger, H. G., J. G. Sodroski, and W. A. Haseltine. 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **86**:5781–5785.
- Henderson, L. E., R. C. Sowder, G. W. Smythers, and S. Oroszlan. 1987. Chemical and immunological characterizations of equine infectious anemia virus *gag*-encoded proteins. *J. Virol.* **61**:1116–1124.
- Kaplan, J. M., G. Mardon, J. M. Bishop, and H. E. Varmus. 1988. The first seven amino acids encoded by the *v-src* oncogene act as a myristylation signal: lysine 7 is a critical determinant. *Mol. Cell. Biol.* **8**:2435–2441.
- Kaplan, J. M., H. E. Varmus, and J. M. Bishop. 1990. The *src* protein contains multiple domains for specific attachment to membranes. *Mol. Cell. Biol.* **10**:1000–1009.
- Krueger, J. G., E. A. Garber, A. R. Goldberg, and H. Hanafusa. 1982. Changes in amino-terminal sequences of pp60^{src} lead to decreased membrane association and decreased in vivo tumorigenicity. *Cell* **28**:889–896.
- Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367–382.
- Lacal, P. M., C. Y. Pennington, and J. C. Lacal. 1988. Transforming activity of *ras* proteins translocated to the plasma membrane by a myristoylation sequence from the *src* gene product. *Oncogene* **2**:533–537.
- Leis, J., D. Baltimore, J. M. Bishop, J. Coffin, E. Fleissner, S. P. Goff, S. Oroszlan, H. Robinson, A. M. Skalka, H. M. Temin, and V. Vogt. 1988. Standardized and simplified nomenclature for proteins common to all retroviruses. *J. Virol.* **62**:1808–1809.
- Leis, J., N. Phillips, X. Fu, P. T. Tuazon, and J. A. Traugh. 1989. Phosphorylation of avian retrovirus matrix protein by Ca²⁺/phospholipid-dependent protein kinase. *Eur. J. Biochem.* **179**:415–422.
- Pellman, D., E. A. Garber, F. R. Cross, and H. Hanafusa. 1985. An N-terminal peptide from p60^{src} can direct myristylation and plasma membrane localization when fused to heterologous proteins. *Nature (London)* **314**:374–377.
- Pepinsky, R. B., D. Cappiello, C. Wilkowski, and V. M. Vogt. 1980. Chemical crosslinking of proteins in avian sarcoma and leukemia viruses. *Virology* **102**:205–210.
- Pepinsky, R. B., R. J. Mattaliano, and V. M. Vogt. 1986. Structure and processing of the p2 region of avian sarcoma and leukemia virus *gag* precursor polyproteins. *J. Virol.* **58**:50–58.
- Pepinsky, R. B., and V. M. Vogt. 1979. Identification of retrovirus matrix proteins by lipid-protein cross-linking. *J. Mol. Biol.* **131**:819–837.
- Pepinsky, R. B., and V. M. Vogt. 1984. Fine-structure analyses of lipid-protein and protein-protein interactions of *gag* protein p19 of the avian sarcoma and leukemia viruses by cyanogen bromide mapping. *J. Virol.* **52**:145–153.
- Putterman, D., R. B. Pepinsky, and V. M. Vogt. 1990. Ubiquitin in avian leukemia virus particles. *Virology* **176**:633–637.
- Rein, A., M. R. McClure, N. R. Rice, R. B. Luftig, and A. M. Schultz. 1986. Myristylation site in Pr65^{gag} is essential for virus particle formation by Moloney murine leukemia virus. *Proc. Natl. Acad. Sci. USA* **83**:7246–7250.
- Resh, M. D. 1989. Specific and saturable binding of pp60^{v-src} to plasma membranes: evidence for a myristyl-*src* receptor. *Cell* **58**:281–286.
- Resh, M. D., and H. Ling. 1990. Identification of a 32K plasma membrane protein that binds to the myristylated amino-terminal sequence of p60^{v-src}. *Nature (London)* **346**:84–86.
- Rhee, S. S., and E. Hunter. 1987. Myristylation is required for intracellular transport but not for assembly of D-type retrovirus capsids. *J. Virol.* **61**:1045–1053.
- Rhee, S. S., and E. Hunter. 1990. Structural role of the matrix protein of type D retroviruses in Gag polyprotein stability and capsid assembly. *J. Virol.* **64**:4383–4389.
- Roth, M. G., R. V. Srinivas, and R. W. Compans. 1983. Basolateral maturation of retroviruses in polarized epithelial cells. *J. Virol.* **45**:1065–1073.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Schultz, A. M., L. E. Henderson, and S. Oroszlan. 1988. Fatty acylation of proteins. *Annu. Rev. Cell Biol.* **4**:611–647.
- Schultz, A. M., and S. Oroszlan. 1983. In vivo modification of retroviral *gag* gene-encoded polyproteins by myristic acid. *J. Virol.* **46**:355–361.
- Schwartz, D. E., R. Tizard, and W. Gilbert. 1983. Nucleotide sequence of Rous sarcoma virus. *Cell* **32**:853–869.
- Sefton, B. M., and J. E. Buss. 1987. The covalent modification of eukaryotic proteins with lipid. *J. Cell Biol.* **104**:1449–1453.
- Smith, A. J., M.-I. Moon, M.-L. Hammarskjold, and D. Rekosh. 1990. Human immunodeficiency virus type 1 Pr55^{gag} and Pr160^{gag-pol} expressed from a simian virus 40 late replacement

- vector are efficiently processed and assembled into viruslike particles. *J. Virol.* **64**:2743–2750.
42. **Towler, D. A., and J. I. Gordon.** 1988. The biology and enzymology of eukaryotic protein acylation. *Annu. Rev. Biochem.* **57**:69–99.
 43. **Vogt, V. M., R. B. Pepinsky, and L. E. Southard.** 1985. Primary structure of p19 species of avian sarcoma and leukemia viruses. *J. Virol.* **56**:31–39.
 44. **Weldon, R. A., Jr., C. R. Erdie, M. G. Oliver, and J. W. Wills.** 1990. Incorporation of chimeric Gag protein into retroviral particles. *J. Virol.* **64**:4169–4179.
 45. **Wills, J. W., R. C. Craven, and J. A. Achacoso.** 1989. Creation and expression of myristylated forms of Rous sarcoma virus Gag protein in mammalian cells. *J. Virol.* **63**:4331–4343.
 46. **Wills, J. W., R. V. Srinivas, and E. Hunter.** 1984. Mutations of the Rous sarcoma virus *env* gene that affect the transport and subcellular location of the glycoprotein product. *J. Cell Biol.* **99**:2011–2023.