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An Assembly Domain of the Rous Sarcoma Virus Gag Protein Required Late in Budding

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The Gag protein of Rous sarcoma virus has the ability to direct particle assembly at the plasma membrane in the absence of all the other virus-encoded components. An extensive deletion analysis has revealed that very large regions of this protein can be deleted without impairing budding and has suggested that the essential functions map to three discrete regions. In the studies reported here, we establish the location of assembly domain 2 (AD2) within the proline-rich p2b sequence of this Gag protein. AD2 mutants lacking the p2b sequence were completely defective for particle release even though their Gag proteins were tightly associated with the membrane fraction and exhibited high levels of protease activity. Mutations that inactivate the viral protease did not restore budding to wild-type levels for these mutants, indicating that the defect is not due simply to a loss of protease regulation. AD2 mutants could be rescued into dense particles in genetic complementation assays, indicating that their defect is not due to a gross alteration of the overall conformation of the protein and that the assembly function is not needed on every Gag molecule in the population. Several mutants with amino acid substitutions in the p2b sequence were found to have an intermediate capacity for budding. Inactivation of the protease of these mutants stabilized the Gag polyprotein within the cells and allowed an increase in particle release; however, the rate of budding remained slow. We favor the idea that AD2 is a dynamic region of movement, perhaps serving as a molecular hinge to allow the particle to emerge from the surface of the cell during budding.

Retroviruses are enveloped. They acquire their lipid bilayers as the internal structural proteins of the virion push on the cytoplasmic face of the plasma membrane, causing buds to emerge from the surface of the infected cell. These subsequently pinch off and go on to infect additional cells. It is now quite clear that only a single viral protein, the Gag polyprotein, is required for the induction of budding. Neither the viral RNA genome nor the products of the *env* and *pol* genes are needed for Gag-mediated particle assembly (reviewed in reference 49).

Very little is known about how Gag proteins work. Because their amino acid sequences exhibit little similarity between retroviruses, it has been difficult to identify functional domains that may be conserved (49). Consequently, investigators have been taking empirical approaches in analyzing a variety of different Gag proteins—dissecting each in hopes of identifying those regions that are needed for budding and those that are not. Efforts in our laboratories have been focused on the assembly domains (AD) of the Gag protein of Rous sarcoma virus (RSV).

The RSV Gag protein, also known as Pr76, contains 701 amino acids (Fig. 1). It is eventually cleaved by the virus-encoded protease (PR) to release the mature products found in the infectious virion: MA, p2, p10, CA, NC, and PR (28). The mechanism of protease regulation and the exact time at which Gag precursors are cleaved are unknown, but processing has been widely thought to be a late event in assembly, beginning just after the bud pinches off. Once initiated, proteolytic processing of the RSV Gag protein brings about a striking, morphological rearrangement of the internal structure of the nascent virion. In particular, immature virions (and virions produced by protease mutants) have electron-luscent centers, whereas mature virions have cores that are electron dense (18, 43, 45, 47). Moreover, immature virions are stable in nonionic detergents, whereas mature virions are not (14, 43). Thus, it would appear that budding requires the formation of a rather rigid structure beneath the plasma membrane, as the assembly domains of the Gag protein work together to bring about the emergence of a particle. Once budding is complete (or perhaps nearly complete), proteolytic processing kicks in to reshape the inside of the virion, revealing those Gag functions that are needed when the virus infects the next cell (49).

How many assembly domains does the RSV Gag protein contain? Our previous studies have suggested three (49). AD1 appears to be the membrane-binding domain and is located at the amino terminus of Gag, within the 155 residues of the MA sequence (Fig. 1A). Small deletions throughout the first half of MA abolish budding activity; however, the effects can be suppressed by replacing AD1 with the well-defined, 8-amino-acid, membrane-binding domain from the Src oncoprotein (51). AD1 can also be replaced with the presumptive membrane-binding domain of the human immunodeficiency virus (HIV) Gag protein (1, 52).

At the other end of the Gag molecule, within the NC sequence, are two copies of AD3 (38, 48). This region appears to provide important interactions between Gag molecules. Mutants lacking both AD3s inefficiently produce particles, but these have a low buoyant density (48). However, the close packing of Gag molecules needed to make particles of high density can be restored by adding back sequences from NC, in

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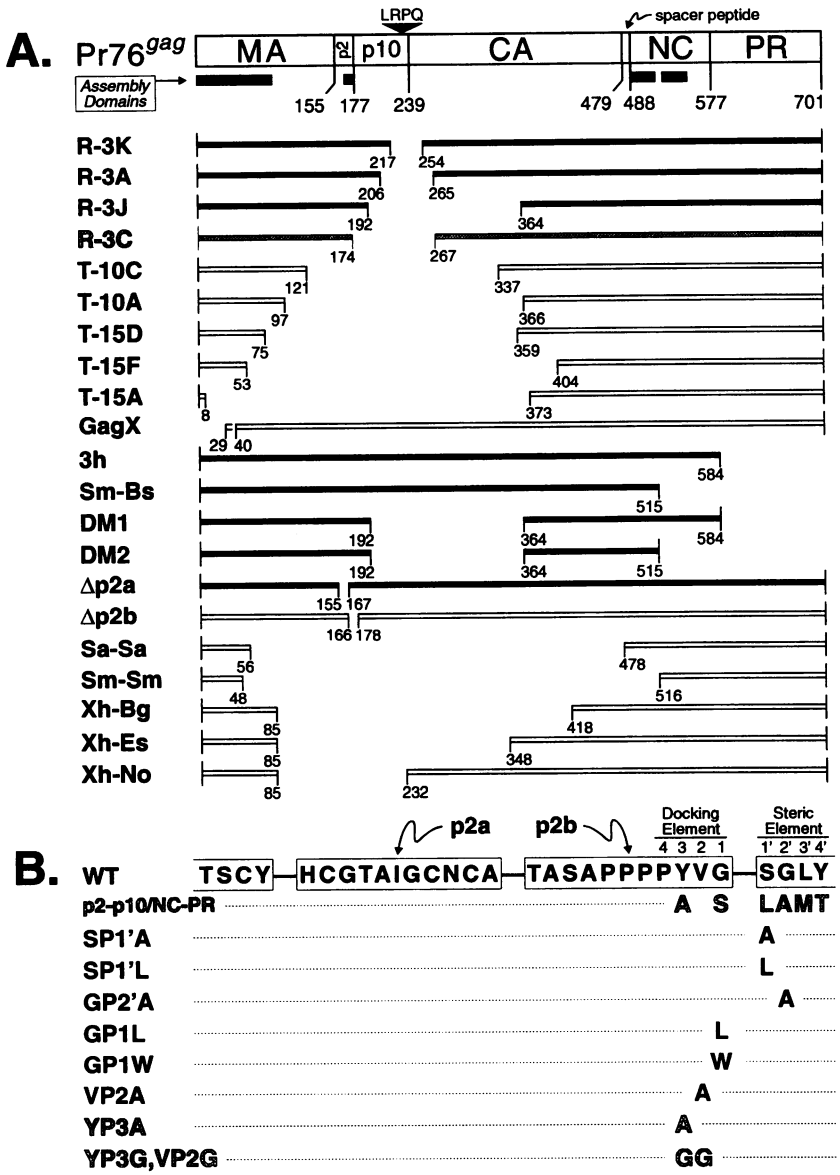

FIG. 1. Alterations of the RSV Gag protein. (A) The wild-type RSV Gag protein, Pr76^{gag}, is shown at the top. The vertical lines represent the viral protease (PR) cleavage sites, which separate the mature Gag products (MA, p2, p10, CA, spacer peptide, NC, and PR), as indicated. Thick solid bars, regions required for budding (assembly domains). Deletions were made in the amino-terminal, carboxy-terminal, and internal regions of the Gag protein. The precise end points of these mutations are listed in Table 1. The amino acid sequence (LRPQ) of the *Not*I linker inserted prior to the making of the BAL 31 deletion mutants is indicated. Thin solid bars, molecules which retain the ability to make dense particles at the same rate as that for the wild-type Gag protein. Open bars, molecules which are trapped in the cells and not released into the growth medium. Mutant R-3C retains the ability to release particles but at a slow rate compared with Pr76^{gag}. (B) The wild-type (WT) amino acid sequences of p2a and p2b are boxed. Horizontal lines separating the boxes represent PR cleavage sites at the MA-p2a, p2a-p2b, and p2b-p10 junctions. At the p2b-p10 junction, the position of each amino acid in the docking element, P4 to P1, and the steric element, P1′ to P4′, is indicated above the sequence. A series of mutations that change specific amino acids of the p2-p10 sequence are listed below. Changes which do not affect particle release are boldfaced, while mutations which block particle assembly are lightfaced.

the absence of virtually all of the CA sequence (48). Moreover, the function associated with AD3 has been found within the Gag proteins of other retroviruses by making chimeras. For example, the NC sequence of the HIV Gag protein has been found to contain two copies of AD3, either of which can substitute for those of the RSV Gag protein (1). In contrast, the Gag protein of murine leukemia virus appears to contain only one such domain (3). AD3s do not correspond to Cys-His boxes, because these can be mutated without affecting particle density (21).

AD2 has been less well characterized and is the primary focus of this report. The existence of an assembly domain located between AD1 and AD3 was suggested previously (48, 49) by a large internal Gag deletion mutant, named T-10C (Fig. 1). This mutant does not produce particles even when the membrane-binding domain from the amino terminus of the Src

protein is present; thus, it appears not to be an AD1 mutant. Moreover, T-10C contains the AD3 sequences that are needed for the efficient release of dense particles (1, 48). Because the p10 and CA sequences were found to be largely dispensable for budding, we hypothesized that the p2 sequence of the RSV Gag protein (Fig. 1) might contain AD2. This idea was strengthened by two further observations. First, temperature-sensitive mutants of RSV Gag were identified within the adjacent p10 sequence (17). Although this region is not required for the production of particles (48), it seems reasonable to suppose that these temperature-sensitive lesions influence the folding of a nearby assembly domain. Second, we discovered that deletions within the second half of MA have little effect on the production of infectious particles (31). Thus, there appears to be a dispensable region between AD1 and the p2 region, just as there is between p2 and AD3.

A few years ago, it was shown that the p2 sequence is cleaved during virus maturation to release two smaller peptides named p2a and p2b (35). In the present report, we show that p2a is dispensable for budding and that AD2 maps to the p2b sequence, near the p2-p10 junction. A detailed mutational analysis of this region suggests that the function of AD2 is needed late in the budding process but not on every Gag molecule in the population.

MATERIALS AND METHODS

DNAs and cells. The wild-type *gag* gene lies between nucleotides (nt) 380 and 2485 in the RSV Prague C genome (41). The mammalian expression plasmids pSV.Myr0 and pSV.Myr1 have been described previously (2, 13, 48, 50, 51), and these were used to express the *gag* gene in simian (COS-1) cells. The product of pSV.Myr0 is the wild-type Gag protein, while that of pSV.Myr1 is a chimeric, myristylated form in which the first 10 amino acids are replaced by those of $p60^{v\text{-}src}$. Mutants carrying the D37S defect lack protease activity because of the replacement of the aspartic acid in the active site with serine (13), while those carrying the D37I substitution have isoleucine at this site (13, 43, 50). Standard protocols were used for all DNA manipulations (39). Recombinant plasmids were propagated in *Escherichia coli* DH-1 or DH5α grown in Luria-Bertani medium containing 100 μg of ampicillin per ml. COS-1 cells were grown in Dulbecco's modified Eagle medium (GIBCO BRL) supplemented with 3% fetal bovine serum and 7% bovine calf serum (HyClone, Inc.).

Mutagenesis of the *gag* gene. The BAL 31 deletion mutations were introduced into pSV.Myr1. For this, a single *Not*I linker (5′-TTGCGGCCGCAA) was first inserted into the *Rsa*I site at nt 1071 to create pSV.Myr1.RNot. This unique restriction site was then cut with *Not*I and digested with BAL 31 by previously described methods (39). The resulting clones were first characterized by restriction endonuclease mapping to eliminate those which contained either no or extremely large deletions. The plasmids from the remaining clones were transfected into COS-1 cells (see below) to determine which expressed Gag antigens and hence contained in-frame deletions. The precise endpoints of the deletions were determined by sequencing the double-stranded plasmid DNAs.

Six of the mutants (Sm-Bs, Sm-Sm, Sa-Sa, Xh-Bg, Xh-Es, and Xh-No) were constructed by removing fragments of the *gag* sequence in pSV.Myr1.NotI with various restriction endonucleases. The names of these mutants reflect the enzymes used: Sm, *Sma*I; Bs, *Bss*HII; Sa, *Sac*II; Xh, *Xho*I; Bg, *Bgl*II; Es, *Esp*I; No, *Not*I. The *Not*I site is the same one introduced for the BAL 31 experiments. Removal of the *Sma*I or *Sac*II DNA fragment of *gag* left in-frame deletions (producing mutants Sm-Sm and Sa-Sa), but for the other restriction fragment deletions it was necessary to modify the DNA ends with the Klenow fragment of DNA polymerase prior to ligation to realign the *gag* reading frame on the downstream side. Duplicate clones of each of the restriction fragment deletion mutants were characterized to ensure that undesirable alterations had not occurred elsewhere.

DM1 and DM2 are double mutants in which the large internal deletion of R-3J has been combined with the carboxy-terminal deletion of 3h or Sm-Bs, respectively. These recombinations were accomplished by replacing the wild-type *Sac*I-*Bgl*II fragment of mutant 3h or Sm-Bs with that of R-3J, which contains the large internal deletion.

Oligonucleotide-directed mutagenesis of the RSV *gag* gene was carried out with uracylated, single-stranded MGAG DNA, as described previously (27). This M13mp19 recombinant contains the *gag* gene within the *Sac*I-*Hin*dIII fragment (nt 255 to 2740) from the RSV genome. The sequences of the mutagenic oligonucleotides used are as follows: Δp2a, GTTGGCA CAT CCT GCT ATA CAG CCT CGG CTC CTC CT CCT CCT TAT; Δp2b, ATTGGCTGTAATTGCGCCAGTGGTTTGTA TCCTTCCCTGGCGGGG; p2-p10/NC-PR, CCTCGGCTC C TCCTCCTCCTGCCGTCTCGCTAGCGATGACACCTTC CCTGGCGGGGGTGGG; SP1′A, CCTCCTTATGTGGGC GCCGGTTTGTATCCTTCC; SP1′L, CCTCCTTATGTGGG GCTCGGTTTGTATCCTTCC; GP2′A, CCTTATGTGGGG AGTGCACTGTATCCTTCCCTG; GP1L, CCTCCTCCTTA TGTGCTCAGTGGTTTGTATCCT; GP1W, CCTCCTCCT CCATATGTGTGGAGTGGTTTGTATCCTTCCC; VP2A, CCTCCTCCTCCTTATGCCGGGAGTGGTTTGTAT; YP3A, TC GG CT CC TC CT CC TC CC GC GG TG GG GA GT GG T; YP3G,VP2G, GCTCCTCCTCCTCCTGGCGGCGGGAGT GGTTTGTAT. All presumptive clones were sequenced to confirm the presence of the desired change. Moreover, two independent (and presumably identical) clones from each mutagenesis experiment were characterized to reduce the possibility of misinterpretations as a result of unwanted mutations elsewhere in the *gag* gene.

Mutations created with oligonucleotides fell between the *Sac*I and *Bgl*II sites in MGAG replicative-form DNA. This segment was transferred to the pSV.Myr0 expression plasmid, and the recombinants were screened by restriction endonuclease mapping and verified by DNA sequencing. To subclone the PR (D37S) mutation into these recombinants, the plasmid DNAs were cut with *Eco*RI (nt 2319) and *Bgl*II and the fragments were exchanged.

Transfection of cells. COS-1 cells were transfected by the DEAE-dextran-chloroquine method as described previously (1, 13, 50, 51). Prior to transfection, the plasmid DNAs were cut with *Xba*I and ligated at a concentration of 25 μg/ml. This removes the bacterial plasmid sequence and joins the 3′ end of the *gag* gene with the simian virus 40 late polyadenylation signal for high-level expression. Typically, 1 μg of DNA was applied to each monolayer (in 35-mm-diameter plates); for cotransfections, the cells received 0.5 μg of each DNA.

Metabolic labeling and immunoprecipitations. COS-1 cells were metabolically labeled with L-[^{35}S]methionine (50 μCi, >1,000 Ci/mmol) approximately 48 h after transfection, as previously described (1, 13, 50, 51). The duration of the labeling was 2.5 h except in the case of the pulse-chase experiments and the sucrose density gradient experiments. The cells and growth medium from each labeled culture were mixed with lysis buffer containing protease inhibitors, and the Gag proteins were immunoprecipitated at 4°C with a rabbit antiserum against whole RSV (reactive with the MA, CA, NC,

and PR products) and subjected to electrophoresis (1, 13, 50, 51).

To follow the kinetics of budding and Gag processing, pulse-chase experiments were performed. For this, sets of identically transfected cells (in 35-mm-diameter plates) were incubated in methionine-free medium for 30 min, pulse-labeled with [^{35}S]methionine for 20 min, and then incubated in serum-free medium containing an excess of unlabeled methionine. One plate of each set was placed on ice immediately following the pulse and fractionated into medium and lysate samples. The remaining plates were processed at the indicated times.

SDS-polyacrylamide gel electrophoresis. Immunoprecipitated proteins were separated by electrophoresis in sodium dodecyl sulfate (SDS)–12% polyacrylamide gels, as described before (1, 13, 50, 51). Afterwards, the gels were fixed in a solution of 5% methanol and 7% acetic acid. The radiolabeled proteins were detected by fluorography using Fluoro-Hance (Research Products International, Inc.) and Kodak X-Omat AR5 film at −80°C. Overnight exposures were typically required. Quantitation of the fluorograms obtained from the pulse-chase experiments was carried out by densitometry using the bioanalysis program on the Sci-Scan 5,000 (U.S. Biochemicals). In the lysate lanes only the precursor band (Pr76^{gag}) was measured, but in the medium lanes all of the Gag cleavage products were quantitated.

Sucrose density gradient analysis. To measure the density of mutant particles, transfected COS-1 cells were labeled with [^{35}S]methionine for 3 h and chased with unlabeled methionine for 1 h. Immediately, the growth medium was collected and centrifuged at 15,000 × *g* for 1 min to remove any cells that might be present. A sample of unlabeled, authentic RSV was mixed with the labeled particles, and the samples were centrifuged through 10 to 50% sucrose gradients (in phosphate-buffered saline) at 26,000 rpm for 12 to 16 h in an SW41 rotor (85,000 × *g*). Fractions were collected, and those containing the mutant particles were identified by scintillation counting. Fractions containing RSV were identified by assaying for reverse transcriptase activity, as described previously (13, 48). Similar methods were used to measure the density of particles produced in the cotransfection experiments, except that murine leukemia virus was used as the internal control (1); moreover, Gag proteins in each of the fractions were collected by immunoprecipitation. The resulting autoradiograms were quantitated by densitometry.

RESULTS

To pinpoint the location and characterize the function of the presumptive AD2 domain of the RSV Gag protein, we made use of a transient expression system in which RSV-like particles are efficiently produced in mammalian cells. Our earliest experiments (50), using a line of CV-1 cells, suggested that the wild-type RSV Gag protein (designated Myr0) produced particles at a reduced rate relative to Gag derivatives bearing the membrane-binding domain from the Src protein (designated Myr1). More recently, we demonstrated that, in COS-1 cells (used here) and in other lines of CV-1 cells, the level of particles produced by the Myr0 protein rivals that of Myr1 (51). In all cases, the particles produced by mammalian cells are virtually identical to authentic RSV from avian cells with regard to their morphological appearance (47), their density (1, 48, 51), and the electrophoretic mobilities of their mature Gag cleavage products (2, 14, 50, 51). Moreover, when the RSV *pol* gene is included in the expression vector at its normal location downstream from the *gag* gene, particles that contain the normal distribution of Pol cleavage products and reverse transcriptase activity are produced (13). The speed with which Gag mutants can be analyzed in this system makes it ideal for studying the mechanism of particle assembly.

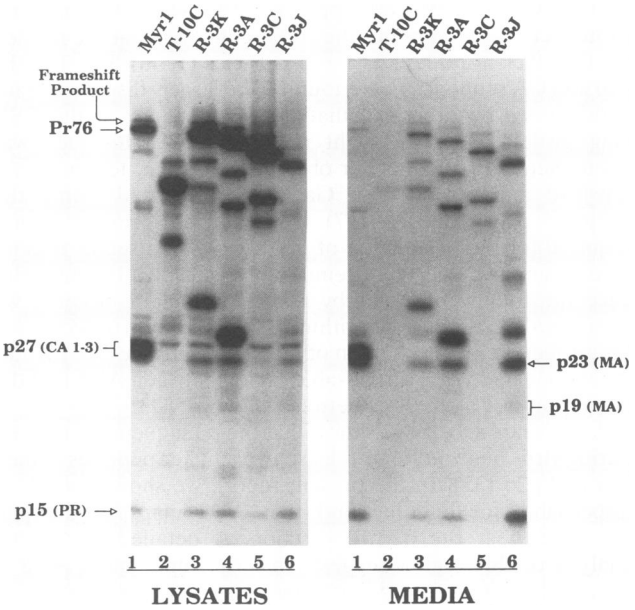

FIG. 2. BAL 31 deletion mutants competent for budding. Cells were metabolically labeled for 2.5 h with [^{35}S]methionine 48 h after transfection with the indicated expression vector. Gag proteins associated with cells or in the culture media (as indicated) were immunoprecipitated with polyclonal anti-RSV serum, resolved by SDS-polyacrylamide gel electrophoresis, and visualized by fluorography, as described in Materials and Methods. Four deletion mutants that are capable of releasing virus-like particles are represented in lanes 3 to 6. For comparison, an assembly-incompetent deletion mutant is shown in lanes 2. Myr1 is a derivative of the Gag protein in which the first 10 residues have been exchanged with those of p60^{src}. The position of Pr76^{gag} and its cleavage products are indicated on the left. Only two of the three previously described CA-related cleavage products (p27) are apparent. Of the MA-related species, p23 is a processing intermediate which includes the p2a-p2b sequence whereas p19 corresponds to MA alone. The NC cleavage product cannot be detected in these experiments because it contains only one methionine and comigrates with PR (p15).

Randomized, large internal deletions of the RSV Gag protein. Our previous studies suggested that AD2 lies to the left of the p10 and CA sequences (Fig. 1), both of which appeared to be largely dispensable for the production of dense particles (48). To pursue the location of assembly domains in a manner independent of previous approaches, we made a random set of internal deletion mutants of the RSV Gag protein. To this end, we first inserted a 12-bp linker containing a unique *Not*I site into the *Rsa*I site within the p10-coding sequence (nt 1071) to create pSV.Myr1.RNot. This mutation results in the insertion of 4 amino acids, LRPQ, in the distal portion of the p10 sequence (Fig. 1). Expression of the altered protein in COS-1 cells revealed that it was fully capable of budding. In fact, when transferred into the complete RSV genome, this *gag* mutation was found to have no effect on virus infectivity, whether the transfection was carried out at 37 or 41°C (data not shown).

To make the internal *gag* deletions, pSV.Myr1.RNot was cut at the unique *Not*I site and the ends were trimmed with the exonuclease BAL 31. The DNA molecules were ligated and clones were obtained after transformation of *E. coli*. Those

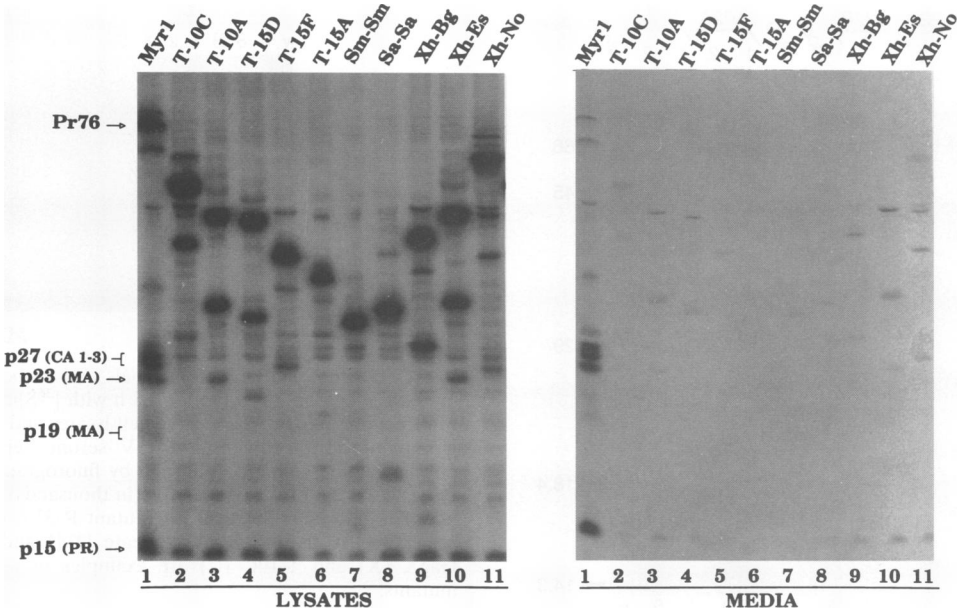

FIG. 3. Deletion mutants defective for budding. Viral proteins from the cell lysates and media were analyzed as described in the legend to Fig. 2. Deletion mutants were created by BAL 31 digestion (lanes 2 to 6) or removal of a restriction fragment (lanes 7 to 11). The migration of the wild-type viral proteins is indicated on the left.

containing the wild-type plasmid were identified and discarded after screening for survival of the *Not*I site. Each of the remaining clones had a one-in-three chance of containing an in-frame *gag* deletion, and mutants of this type were identified by transfecting COS-1 cells to determine which expressed Gag antigens.

Nine clones were obtained and further characterized. Three of the mutants, R-3K, R-3A, and R-3J, produced Gag proteins which were readily detected in the growth medium of the transfected cells in spite of an obvious deletion in their precursor species (Fig. 2, lanes 3, 4, and 6, respectively). Subsequent experiments using protease-defective derivatives of these mutants revealed that each was released with about the same efficiency as (i.e., no less than 90% of) the full-length, control molecule (data not shown). A fourth mutant, R-3C, was found to consistently release protein at a reduced rate (Fig. 2, lanes 5); nevertheless, this level was always higher than those of the other five mutants, T-10C, T-10A, T-15D, T-15F, and T-15A, all of which contain even larger deletions and were severely impaired for release (Fig. 3, lanes 2 to 6, respectively). Mutant T-10C encodes the largest Gag polyprotein of this defective group, but it is still smaller than R-3J, the smallest member of the budding-competent group (Fig. 2, lanes 2).

The precise endpoints of the nine in-frame deletions were mapped by DNA sequencing (Fig. 1 and Table 1). We were particularly interested in R-3J because of its large deletion. We found that 172 of the 701 amino acids of Gag (25%) are missing from this mutant, including most of the p10 and half of the CA sequence. The smaller deletions of R-3K and R-3A fall entirely within the limits of R-3J, while that of R-3C overlaps the left boundary of R-3J and extends just into the p2b coding region. Those mutants which were found to be incapable of being released into the medium (T-10C, T-10A, T-15F, T-15D, and T-15A) lack both segments of the p2 region and different parts of the MA sequence. In the other direction, they lack all of p10 and different parts of the CA sequence; however, these regions have been found to be dispensable in independent experiments (48) and in mutant R-3J. Collectively, the BAL 31 deletion mutants support the idea that the right boundary of AD2 is located near the p2-p10 junction and that CA and p10 sequences are dispensable.

Because the internal deletion mutants were initially con-

TABLE 1. Endpoints of the *gag* deletions

Mutant	Deletion		Foreign residue(s)[b]
	Nucleotides[a]	Amino acids	
R-3K	1032–1139	218–253	
R-3A	999–1172	207–264	
R-3J	957–1469	193–363	
R-3C	903–1175	175–266	W
T-10C	742–1386	122–336	
T-10A	671–1474	98–365	
T-15D	604–1452	76–358	
T-15F	538–1587	54–403	
T-15A	403–1494	9–372	
Sa-Sa	547–1809	57–477	
Sm-Sm	523–1923	49–515	
Xh-Bg	635–1630	86–417	
Xh-Es	635–1420	86–347	
Xh-No	635–1072	86–231	SRPQ
Δp2A	845–877	156–166	
Δp2B	878–910	167–177	
3h	2132–2485[c]	585–701	L
Sm-Bs	1924–2485[c]	516–701	ALFSRERQALMR
DM1	957–1469, 2132–2485[c]	193–363, 585–701	L
DM2	957–1469, 1924–2485[c]	193–363, 516–701	ALFSRERQALMR

[a] The RSV nucleotide sequence is numbered starting at the first base of the RNA genome; *gag* begins at nt 380.

[b] Some of the mutations introduced extra residues at the site of deletion. The extra residues for DM1 and DM2 correspond to the second deletion.

[c] The *gag* stop codon (ending with nt 2485) was deleted in these mutants, allowing readthrough into the flanking sequence.

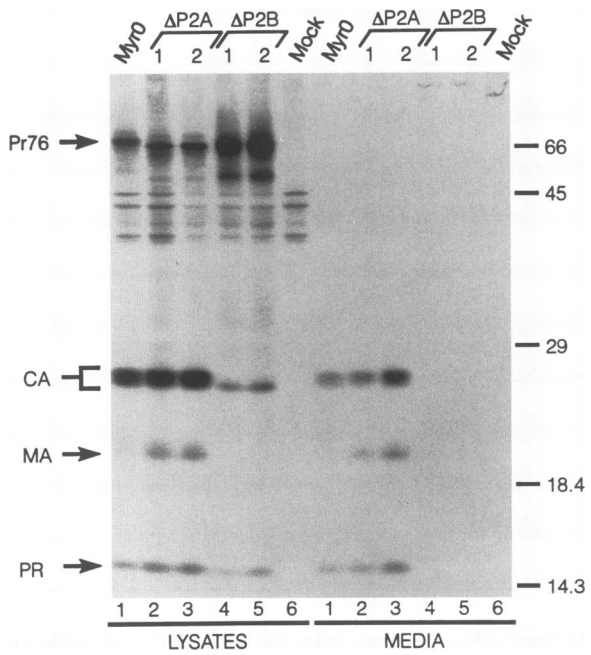

FIG. 4. Deletion mutants lacking the p2a or p2b sequence. Mutant Gag proteins were analyzed as described in the legend to Fig. 2. Two independent clones of deletion mutant ΔP2A are shown in lanes 2 and 3. This deletion does not affect particle release. In contrast, two clones of mutant ΔP2B are unable to direct particle assembly. The positions of the Gag cleavage products are indicated on the left, and the positions of the protein size standards (in kilodaltons) are indicated on the right.

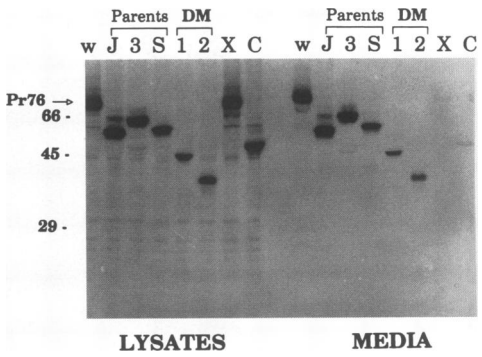

FIG. 5. Combinations of internal- and carboxy-terminal-deletion mutants. Cells were labeled for 2.5 h with [^{35}S]methionine 48 h after transfection. Gag proteins in the cell lysates and media samples were immunoprecipitated with anti-RSV serum, separated in an SDS-polyacrylamide gel, and visualized by fluorography. The positions of $Pr76^{gag}$ (w) and protein standards (in thousands) are indicated on the left. The internally deleted Gag mutant R-3J (J) was combined with either 3h (3) or Sm-Bs (S) to create DM1 and DM2, respectively. GagX (X) and T-10C (C) are examples of assembly-incompetent mutants.

structed in the Myr1 derivative of the Gag protein, it was important to determine whether the Src membrane-binding domain had provided a function necessary for particle release. Accordingly, we transferred the largest deletion that had not affected budding, that of mutant R-3J, into the wild-type Gag molecule, Myr0. The resulting Myr0.R-3J protein was found to be released in exactly the same manner as the Myr1.R-3J derivative (data not shown).

Deletions of the p2a or p2b sequences. To locate the left boundary of AD2, we made precise deletions of the p2a and p2b sequences by oligonucleotide-directed mutagenesis of the *myr0* (wild-type) allele of the RSV *gag* gene. Two clones of each type were identified by DNA sequencing and expressed in COS-1 cells to analyze their ability to release Gag protein into the medium. The results were unequivocal (Fig. 4). Mutants lacking the p2a region (lanes 2 and 3) released particles at the same high rate as the wild-type, parental molecule (Myr0, lanes 1), but those lacking the p2b region were not released at all (lanes 4 and 5). In all cases, the presence of the deletions in the Gag molecules could be detected in the faster migration of the precursor species in the lysate samples. We conclude that a very important function lies within the 11 amino acids of the p2b sequence.

We also constructed a set of internal-deletion mutants by dropping out various DNA segments using convenient restriction endonuclease sites. The deletions of mutants Xh-Bg, Xh-Es, and Xh-No fall within the limits of the defective BAL 31 deletion mutants; however, those of Sm-Sm and Sa-Sa include sequences further towards the carboxy terminus (Fig. 1). All five members of this group lack the p2 sequence, and as predicted, all were found to be defective for budding (Fig. 3).

Combinations of mutations and particle density. The discovery that the assembly functions of the RSV Gag protein are limited to small regions (AD1, AD2, AD3), separated by large dispensable regions, has been surprising to us. To further explore the tolerance of this Gag molecule to deletions, we constructed double mutants DM1 and DM2 (Fig. 1). In DM1, the internal deletion of R-3J has been combined with the carboxy-terminal deletion of mutant 3h, which removes almost all of the PR sequence (47). The resulting molecule is predicted to contain just 413 of the 701 residues (59%) of the Gag protein. In DM2, the R-3J mutation is combined with the still-larger carboxy-terminal deletion of mutant Sm-Bs. The resulting molecule is predicted to contain only 344 (49%) of the Gag residues.

The DM1 and DM2 proteins were expressed in COS-1 cells to examine their ability to be released into the medium. The parental constructs (mutants R-3J, 3h, and Sm-Bs) were included in the experiment to provide positive controls for budding. For negative controls, mutants T-10C (an AD2 mutant) and GagX (an AD1 mutant [50] [Fig. 1]) were included. In all of the constructs, the protease was either deleted (by the 3h or Sm-Bs mutation) or inactivated by a point mutation (D37I) so that in each instance only a single species of Gag protein would be produced. The results demonstrated that DM1 and DM2, like their parents containing single large deletions, were indeed capable of being released into the medium (Fig. 5). Although the intensity of the bands representing these mutants decreased with size because of the loss of sites for incorporation of [^{35}S]methionine, the ratio of labeled protein inside the cell to that outside the cell was approximately the same in every case.

The tolerance of the RSV Gag protein to large deletions has been unexpected. For example, we anticipated that the CA sequence would play an important role in budding because it is the largest component of Gag. Moreover, the CA sequence of murine leukemia virus has been reported to be sensitive to even small deletions and point mutations (19). This discrepancy would become irrelevant if our mutant proteins were released into the medium by a mechanism other than budding, as is the case for Gag proteins expressed in some systems (37). For these reasons, it was important to ascertain whether the

large deletion mutants were released in soluble form or in particles of a density similar to authentic RSV. Simple centrifugation experiments revealed that all of the protein released into the medium by mutants R-3J, 3h, Sm-Bs, DM1, and DM2 was in a particulate form, which readily sediments like authentic virions (data not shown). Subsequent experiments revealed that each type of Gag molecule is released in particles of a uniform density, in a manner similar to that of the full-length Gag control and to authentic RSV (Fig. 6). The slightly higher particle density observed for the RSV Gag protein when expressed alone in COS-1 cells is not understood but has been noted previously (1, 48, 51).

Protease activity of AD2 mutants. All of the AD2 mutants exhibited Gag processing in the absence of budding (Fig. 3 and 4). This surprised us because proteolysis of the RSV Gag protein is thought to be regulated (i.e., tightly coupled to particle assembly). Premature processing would presumably separate the Gag assembly domains from one another and thereby abort budding. Two lines of evidence have supported this idea. First, RSV particles harvested immediately after their release are enriched for uncleaved Gag polyproteins (12, 44). Similar observations have been made for murine retroviruses (16, 29). Second, mutations that disrupt the AD1 function in the MA sequence result in reduced levels of processing and an accumulation of intact Gag polyproteins, suggesting that membrane binding is a prerequisite for protease activation in vivo (50, 51). A similar situation exists for murine leukemia viruses that are blocked for myristylation and membrane binding (36, 40). With these observations in mind, we considered the possibility that AD2 is involved in protease regulation.

Substitutions at the p2-p10 junction. Previous studies indicated that peptides containing the p2-p10 cleavage site are competitive inhibitors of the RSV protease in vitro, in agreement with a role in regulation (11). In contrast, peptides containing the NC-PR cleavage site are processed efficiently in vitro (10), in agreement with the hypothesis that this site is among the first to be cleaved during particle maturation (8). If the p2-p10 junction serves to delay activation of PR in vivo, then its replacement with the NC-PR junction should allow premature activation of PR, destabilization of $Pr76^{gag}$, and a concomitant reduction in viral particles released from cells. To test this prediction, we constructed mutant p2-p10/NC-PR, in which the seven amino acids at the p2-p10 junction are substituted with those present at the junction of NC and PR (Fig. 1B). This mutation resulted in a greater than 10-fold decrease in the amount of viral proteins detected in the cell medium relative to that for the wild type (Fig. 7A; compare lanes NC-PR and WT). This loss was not due to poor expression, since equivalent amounts of viral polyproteins were detected in the cell lysates (Fig. 7) and in pulse-chase experiments (data not shown). In addition, Gag cleavage products are apparent in the lysate samples, and thus, mutant p2-p10/NC-PR behaves like the AD2 deletion mutants described above. The low yield of particles in the medium and the presence of cleavage products in the lysates suggested that the Gag proteins are prematurely processed and subsequently degraded by the cell.

If premature activation of the viral protease were to collapse the internal structure of the nascent bud, thereby aborting particle release, then mutations that eliminate PR activity should restore budding. To test this, the catalytic aspartic acid at position 37 of the RSV protease was changed to serine (D37S) in the p2-p10/NC-PR mutant. As expected, the resulting protein was not processed (compare Fig. 7A and B, lanes NC-PR); nevertheless, the amount of protein released was still far less than that for wild-type Gag. Indeed, in the medium

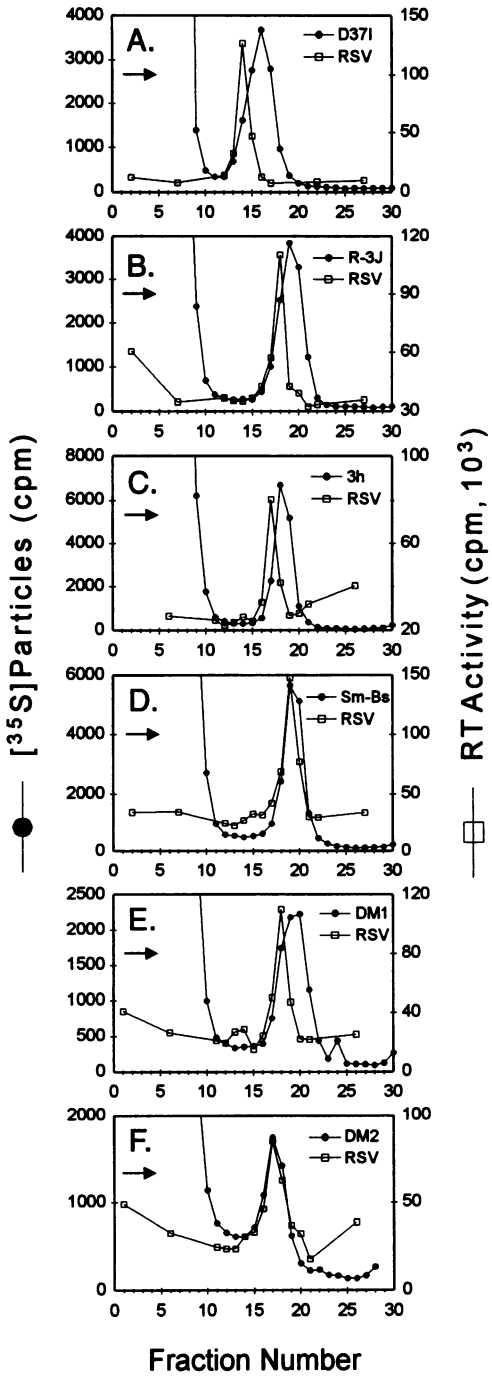

FIG. 6. Density of particles released by internal- and carboxy-terminal-deletion mutants. Cells were transfected with the indicated *gag* derivatives and labeled with [^{35}S]methionine for 8 h immediately prior to removal of the growth medium and addition to it of a sample of unlabeled, authentic RSV. The mixtures were then centrifuged to equilibrium in sucrose gradients for 12 to 16 h, and fractions were collected. Those containing the mutant particles were identified by scintillation counting, and those containing RSV were identified by assaying for reverse transcriptase (RT) activity. Samples A and B were centrifuged in 15 to 60% gradients, while samples C to F were centrifuged in 10 to 50% gradients. Arrows, direction of sedimentation. The large peak of radioactivity at the top of the gradients represents the unincorporated [^{35}S]methionine from the labeling medium.

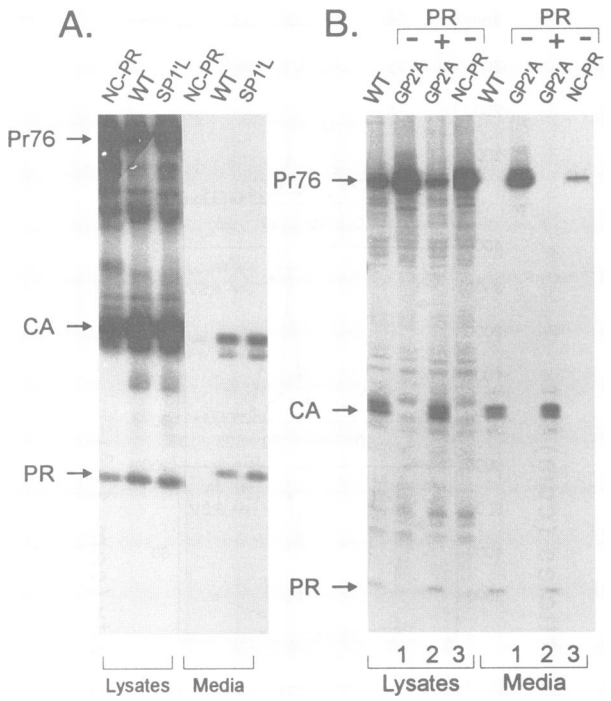

FIG. 7. Effects of steric-element mutations at the p2-p10 junction. Viral proteins from the cell lysates and media were analyzed as described in the legend to Fig. 2. (A) The NC-PR substitution changes the p2-p10 cleavage site of the wild-type (WT) RSV Gag protein to that present at the NC-PR junction. Mutant SP1'L changes the P1' amino acid of the p2-p10 junction from serine to leucine. (B) In mutant GP2'A, the P2' amino acid of the p2-p10 junction is changed from glycine to alanine. PR+ and PR−, wild-type protease and protease containing the D37S mutation, respectively. The positions of the Gag precursor and cleavage products are indicated.

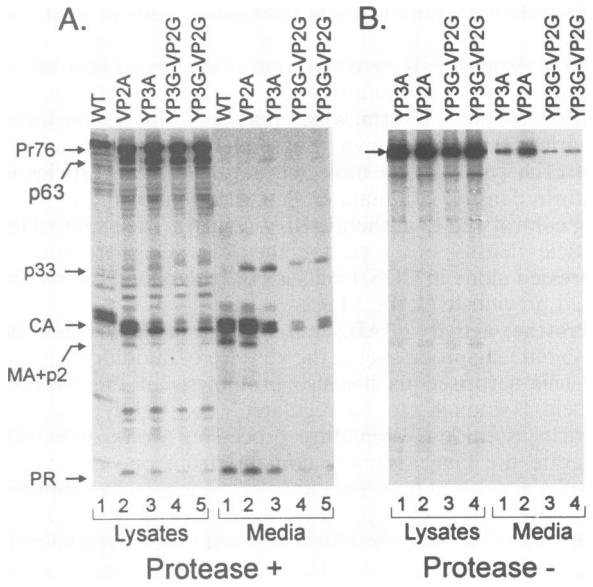

FIG. 8. Effects of docking-element mutations at the p2-p10 junction. Viral proteins from the cell lysates and media were analyzed as described in the legend to Fig. 2. The sequences of the mutant polypeptides are given in Fig. 1. Two independently isolated clones are shown for the YP3G,VP2G *gag* allele. The mutants were analyzed in the context of a wild-type PR (A) and a PR inactivated with the D37S mutation (B). The positions of viral proteins are indicated on the left. Arrow in panel B, position of the uncleaved precursor.

samples from these experiments, there was more radioactivity detected just in the bands of wild-type CA protein than in the band containing the entire uncleaved, mutant polyprotein. Direct comparisons of the D37S forms of the parental protein and the NC-PR protein (data not shown) confirmed that the mutant is defective for particle release and not protease regulation. Thus, this mutant seems to be defective for an assembly domain (AD2) but exhibits a leaky amount of budding, reminiscent of mutant R-3C (see above).

Before abandoning the idea that the p2-p10 cleavage site is involved in protease regulation, we made a variety of single and double amino acid substitutions in this region in an attempt to separate that function from the assembly function of AD2. Previously, it had been shown that the inhibitory properties of the p2-p10 peptide in vitro require three elements (11). First, the serine at the P1′ position of the peptide (Fig. 1) was implicated in an interaction with one of the two catalytic aspartic acid residues of PR. Second, the availability of this residue required conformational freedom provided by residues in the P2′ and P3′ positions, which were termed the "steric element". Third, a "docking element", shown to reside at positions P1 to P4, was required to provide tight binding of the peptide to the protease. The loss of any one of these three elements is sufficient to destroy the inhibitory properties of the p2-p10 peptide.

Gag mutants in which the inhibitory P1′ serine was changed to alanine (SP1′A) or leucine (SP1′L) or the steric element P2′ glycine was changed to alanine (GP2′A) were constructed (Fig. 1). If these changes were to cause a loss of protease regulation, then we would have expected to see a reduction in particle release and perhaps an increase in cell-associated Gag processing. Instead, the wild-type phenotype was observed. (Data for the SP1′L and GP2′A mutants are shown in Fig. 7A and B, respectively). Moreover, inactivation of the protease (with the D37S mutation) did not alter the outcome of the experiment for any of the three mutants, including GP2′A (Fig. 7B). Since all of these changes fall on the p10 side of the p2-p10 cleavage site, they do offer further support for the idea that AD2 is located within the p2b sequence of the Gag protein.

Substitutions were also made within the docking element, and these were of two types. In the first, alterations which are predicted to strengthen the binding of the p2-p10 sequence to PR were made. According to the protease regulation model, these might alter the efficiency of Gag processing but should have no effect on the efficiency of budding. Two mutants of this type, GP1L and GP1W (Fig. 1), were examined, and neither was found to affect Gag release or processing (data not shown).

The second set of substitutions in the docking element were designed to weaken interactions with the protease: VP2A, YP3A, and YP3G,VP2G (Fig. 1). A steady-state kinetic analysis of p2-p10 peptides containing these substitutions (in vitro) showed that VP2A binds to the protease with a K_i of 100 μM while YP3A and YP3G,VP2G bind with K_is in the millimolar range (data not shown). Under identical conditions, the wild-type p2-p10 peptide binds with a K_i of 20 μM (11). If any of these mutations destroyed the regulation of PR, without also affecting AD2, they should exhibit premature processing defects; however, budding should be restored by second-site mutations that inactivate the protease. All three mutants expressed Gag proteins at the wild-type level in COS-1 cells (Fig. 8A). Mutant VP2A (lanes 2), which had the smallest

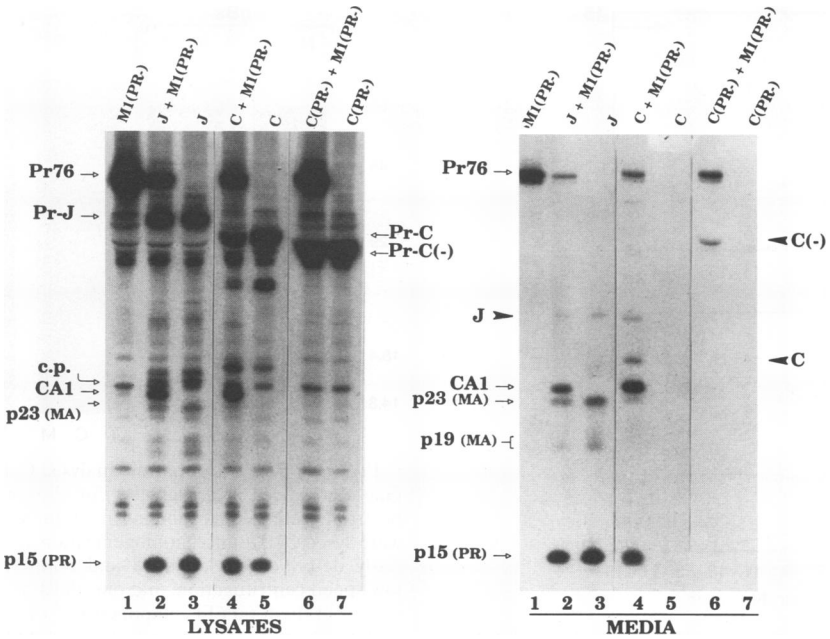

FIG. 9. Rescue of AD2 mutants by complementation. Viral proteins from the cell lysates and media were analyzed as described in the legend to Fig. 2. The myristylated RSV Gag derivative, Myr1.D37I [M1(PR−)] and R-3J (J) were expressed alone or in combination [J + M1(PR−)]. The full-length products, Pr76 and Pr-J are indicated, as are the cleavage products which result from the protease activity of R-3J: CA1, p23(MA), p15(PR), and an R-3J specific product, J. T-10C with an active PR (C) or with the inactivating D37I substitution [C(−)] was expressed alone or in combination with M1(PR−). The positions of the T-10C precursor (Pr-C) and cleavage (C) product and the position of the T-10C(PR−) precursor [Pr-C(−) in panel A and C(−) in panel B] are indicated. c.p., a nonspecific cellular protein.

effect on peptide binding, was found to release particles into the medium at the same level as the wild type (lanes 1). In contrast, mutant YP3A (lanes 3) and two clones of mutant YP3G,VP2G (lanes 4 and 5) were found to be reduced 4- and 10-fold, respectively. However, these defects appear to affect the assembly function of AD2, rather than just protease regulation, because inactivation of protease did not restore budding to wild-type levels (Fig. 8B).

Although our mutations did not reveal a regulatory function in the AD2 region, they do seem to alter interactions with the protease in the manner we had predicted. In particular, all three of the destabilizing mutants released a novel processing intermediate, p33, which has a mobility consistent with a product containing the MA, p2, and p10 sequences (Fig. 8A). This intermediate is normally not observed, because the p2-p10 site tends to be cleaved rather quickly during particle maturation. In contrast, the site between MA and p2 is normally slow to be processed, resulting in the appearance of a 23-kDa intermediate ($p23^{MA+p2}$), which persists in the medium for several hours (2). Mutations which reduce the binding of the p2-p10 cleavage site to the protease would be expected to reduce the amount of $p23^{MA+p2}$, with a concomitant increase in the amount of p33. The VP2A mutant is predicted to be the most efficiently bound of the three mutants and appears to be the most efficiently processed (Fig. 8A, media, lanes 2). In this case, p33 represents a smaller fraction of the total protein observed in the medium (but more than that observed for the wild type), and this product was cleaved to release $p23^{MA+p2}$. In contrast, $p23^{MA+p2}$ was difficult to detect with the other two mutants, YP3A and YP3G,VP2G, which are predicted to have a greater reduction in binding (Fig. 8A, media, lanes 3 to 5). Moreover, the p33 species represents a greater proportion of the total released protein for these two mutants.

Rescue of nonleaky AD2 mutants by complementation. Having located the position of AD2 and several of its critical residues, it was important to learn more about its function. The first question we addressed was whether AD2 is needed on every Gag molecule that is packaged into a particle. The answer was obtained with a complementation assay (Fig. 9). For this experiment we chose mutant T-10C, which is not leaky for particle release when expressed alone (lanes 5). In an attempt to rescue this molecule into particles, we used a protease-deficient form of the full-length Gag protein, mutant D37I. This mutant, designated M1(PR−), can make particles independently, but these contain only the uncleaved Gag protein (lanes 1).

Coexpression of T-10C and M1(PR−) resulted in the appearance of Gag cleavage products in the medium of the cotransfected cells (lanes 4). These could have come about only by the release of particles containing both species of Gag molecules. Control experiments with mutant R-3J, which is capable of budding on its own (lanes 3), showed that the efficiency of *trans* processing of the rescuing molecule is no greater than that observed with T-10C (compare lanes 2 and 4). The efficiency of rescue for mutant T-10C was confirmed with a protease-deficient form, which is also defective for budding and considerably smaller than the full-length Gag molecule (lanes 7). When the two were coexpressed, uncleaved T-10C molecules were readily detected in the medium (lanes 6). Moreover, all of these molecules were contained within dense particles (Fig. 10). This is an important finding because it suggests that AD3, which is needed for the production of dense particles (1, 48), need not work in *cis* with AD2. Particles containing the rescued AD2 mutants were reproducibly denser than the controls, but the explanation for this is not known. We speculate that only a few molecules of wild-type Gag need to mix with the T-10C molecules to release the block to budding.

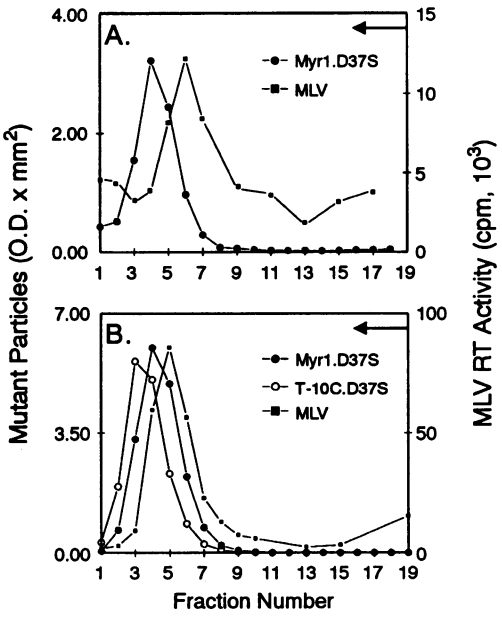

FIG. 10. Density of particles containing rescued AD2 mutants. Cells transfected with Myr1.D37S or Myr1.D37S and T-10C.D37S were labeled for 8 h with [^{35}S]methionine 2 days after transfection. Following metabolic labeling, the sample was spiked with authentic murine leukemia virus (MLV) and then loaded onto a 10 to 50% sucrose gradient. Following centrifugation for 16 h, fractions were collected and analyzed for the presence of RSV Gag proteins by immunoprecipitation and SDS-polyacrylamide gel electrophoresis or for MLV by reverse transcriptase (RT) assays, as described in Materials and Methods. The optical density (O.D.) of the bands on the resulting fluorograms was determined by densitometry and is indicated on the left; the RT activity associated with each fraction is indicated on the right. Fractions 1 and 19 correspond to the bottom and top of the gradient, respectively. Arrows, direction of sedimentation.

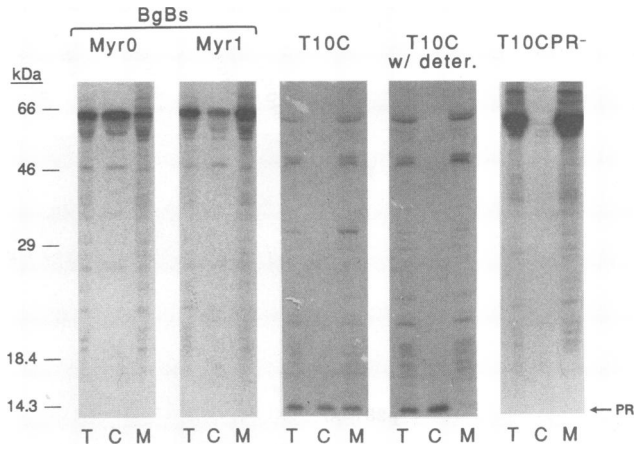

FIG. 11. Cell fractionation analysis. Cells expressing the indicated Gag derivatives were labeled for 2 h with [^{35}S]methionine 48 h posttransfection. The labeling medium was discarded, and the cells were lysed by Dounce homogenization in hypotonic buffer, as previously described (51). Nuclei and unbroken cells were removed by low-speed centrifugation, and one-third of the sample was set aside to represent the total (T) components of the unfractionated sample. The remainder of the lysate was fractionated by ultracentrifugation at 250,000 × *g* for 30 min. Gag proteins present in the resulting supernatant (cytosolic [C]) and pellet (membrane [M]) fractions were collected by immunoprecipitation and electrophoresed in SDS-polyacrylamide gels. In one experiment, the cells were homogenized in buffer containing nonionic detergent (deter.) (0.1% Triton X-100). The position of the viral protease (PR) is indicated on the right. The positions of the protein size standards are indicated on the left.

Association of AD2 mutants with membrane fractions. At what step in the budding pathway are AD2 mutants blocked? The complementation experiments clearly indicate that the block comes after the Gag molecules begin to interact. When do Gag proteins first interact? Electron microscopy has revealed that dense aggregates of Gag proteins can be seen only on the inner face of the plasma membrane (45); however, such low-resolution experiments do not exclude the possibility that Gag interactions might begin much sooner, in a cytosolic compartment. Nevertheless, the observation of proteolytic processing noted above for AD2 mutants (in contrast to AD1 mutants in which membrane binding and proteolysis are both reduced) suggested that the block might occur just prior to particle release. If so, then molecules with AD2 defects would be expected to pellet with membranes in simple cell fractionation experiments, and this possibility was examined.

Cells expressing AD2 mutant T-10C were labeled with [^{35}S]methionine, osmotically lysed, and centrifuged to obtain the cytosolic and membrane fractions according to standard protocols (51). As shown in Fig. 11, virtually all (>95%) of the T-10C precursor (which migrates just below the 66-kDa marker) pelleted with the membrane fraction. In contrast, the PR product, which is released during proteolysis of Gag, was found in equal amounts in both fractions. No other cleavage products were detected in the cytosolic fraction, but as noted above, the NC protein comigrates with PR and is difficult to detect. When the viral protease was rendered inactive (mutant T-10C.PR−), no Gag cleavage products were observed and all of the molecules were associated with the membrane fraction. The results from these two experiments are consistent with the idea that AD2 mutants are targeted to the plasma membrane, where they associate in stable complexes, but these are destabilized when the protease begins to cleave within them. To explore this further, the fractionation was repeated in the presence of 0.1% Triton X-100, which solubilizes cell membranes. This variation of the experiment was based on the fact that immature virions (containing uncleaved Gag molecules) are stable in this concentration of nonionic detergent whereas mature virions fall apart (14, 43). We found that virtually all of the full-length T-10C molecules still pelleted after extraction with Triton X-100, but all of the PR product was released into the supernatant fraction (Fig. 11). Although the data shown here are for the Myr1 form of T-10C, identical results were obtained with the Myr0 form (data not shown). Thus, the fractionation results are consistent with a block very late in the budding pathway, perhaps after the formation of immature particles but before particle release.

As a control in our cell fractionation experiments, we utilized mutant Bg-Bs, which lacks the major region of Gag-Gag interaction, AD3 (1, 47, 48). We previously reported that the Myr0 form of this mutant poorly associates (30%) with the membrane fraction, even though AD1 and AD2 are present (Fig. 11) (51). Dramatically stronger membrane associations (75%) are obtained for this mutant when AD1 is replaced with the excellent membrane-binding domain from the Src oncoprotein (Fig. 11, Myr1). Taken together with the data for AD2 mutants, we speculate that the membrane-binding domain of the RSV Gag protein is rather weak in the context of individual Gag molecules and that cooperative interactions (mediated by

AD3) provide a mechanism for tight membrane binding. However, cooperative interactions with the membrane via AD1 and AD3 are still insufficient for particle release in the absence of AD2.

Kinetic analysis of leaky AD2 mutants. To further explore the hypothesis that AD2 mutants are blocked at a late step in budding, we utilized one of our leaky AD2 mutants, YP3G,VP2G, which is released at a rate 10-fold less than that for the wild type during our standard 2.5-h labeling period (Fig. 8). If this molecule accumulates at a late step in budding, then activation of the protease would be expected to collapse the incomplete structures, thereby aborting particle release. Mutations in the active site of the protease would not eliminate the AD2 defect but might prolong the half-life of the incomplete particle, giving it a greater opportunity to be released.

Pulse-chase labeling experiments were performed with mutant YP3G,VP2G in the presence and absence of the D37S protease mutation. In the case of the PR+ derivative, the Gag precursor disappeared with a half-life of less than 1 h (Fig. 12A). This rate of disappearance is similar to that previously observed with the Myr1 form of the RSV Gag protein (1, 2), except in this case the protein was released from the cells very inefficiently (Fig. 12B). After 5 h of chase, only 8% of the pulse-labeled molecules were detected in the growth medium and only 5% remained uncleaved within the cell. Importantly, it appears that few additional particles would have been released with longer chases since the curve clearly reached a plateau.

When the protease of YP3G,VP2G was inactivated with the D37S substitution, the rate of release into the medium was still much slower (half-life, >5 h) than that previously measured for the RSV Gag protein because of the defective assembly domain. However, after 5 h of chase 33% of the starting molecules had been released into the medium, compared with just 8% for the PR+ construct (Fig. 12B). Moreover, the release of particles into the medium showed no signs of leveling off but, rather, seemed to remain on a steep rise at the end of the experiment. Furthermore, the Gag molecules remaining within the cell seemed to be very stable, and at the end of the experiment we could account for 85% of the starting population (Fig. 12C). Interestingly, the 15% that was lost disappeared during the first hour of the chase. The simplest interpretation of this experiment is that the mutant AD2 proteins are rapidly transported to a compartment, presumably on the plasma membrane, in which they are sequestered from cell proteases capable of degrading them. In the absence of viral protease activity, exit from this compartment into the medium is very slow but nevertheless continues.

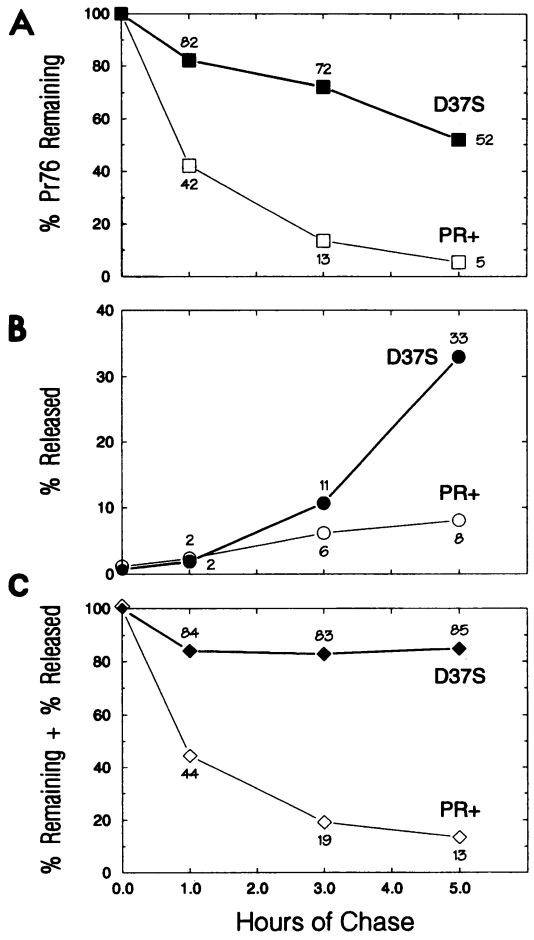

FIG. 12. Pulse-chase analysis of leaky AD2 mutants. Cells were transfected with plasmids that encode the PR+ or the PR− (D37S) form of AD2 mutant YP3G,VP2G. After 48 h, the cells were pulse-chase labeled and the resulting fluorograms were quantified, as described in Materials and Methods. (A) Percentage of $Pr76^{gag}$ at time zero that remains in the cell lysate samples after 0 to 5 h of chasing. The levels of expression of the two Gag derivatives were similar (24.9 arbitrary units for YP3G,VP2G and 19.6 units for the D37S mutant). (B) Release of particles into the medium during the chase, expressed as a percentage of the total labeled precursor in the lysate at time zero. In the case of the PR+ derivative, all of the cleavage products present in the medium were monitored. Note that the slope of the line for the PR− derivative increased throughout the experiment while that of the PR+ derivative had reached a plateau. (C) Sum of the data from panels A and B. Note that 85% of the total PR− protein remains stable throughout the course of the experiment.

DISCUSSION

Gag proteins contain two distinct sets of functions: those needed for the assembly of virions and those needed when the mature virus infects the host cell. We have suggested that the events of budding involve cooperative interactions between Gag molecules and that proteolytic processing disconnects the assembly domains from one another so that they can no longer work together (49). In this way, the internal structural proteins of the virus would be unable to reenter the assembly pathway when infecting a new cell, allowing the mature cleavage products (MA, CA, and NC) to enter alternate pathways needed for a successful infection (i.e., transport to the nucleus [5, 7]). That Gag polyproteins would work in this way is not self-evident. Other enveloped viruses (e.g., influenza virus) assemble the individual internal components of their virions without the use of polyprotein precursors. How the assembly domains of Gag proteins work in concert during budding remains to be elucidated.

Phenotypes of AD mutants. The Gag protein of RSV appears to contain three distinct assembly domains. In this report, we have mapped and characterized the smallest, AD2, which seems to be contained within the 11 residues of the p2b sequence. The other assembly domains are surprisingly small, too. AD1 appears to consist of no more than the first 85 residues of the MA sequence (31) and can be replaced with the small membrane-binding domain of the Src oncoprotein (51). The two copies of AD3 found within the NC sequence (38, 48) can be replaced with an equivalent functional domain found within 29 residues from the HIV NC protein (1). None of the

three assembly domains span cleavage sites; however, AD2 is different from AD1 and AD3 in being part of a protease recognition sequence.

When defects are introduced into each of the RSV assembly domains, distinct phenotypes result. Molecules defective for AD1 bind poorly to membranes and exhibit reduced levels of PR-catalyzed proteolysis (50, 51). Because AD1 mutants can be rescued by complementation, they must retain the functions needed for interactions with other Gag molecules. When rescued in this manner, these mutants exhibit fully efficient processing in that none of their precursor polyproteins are found within the particles released into the medium (51). This further supports the hypothesis that membrane interactions are a prerequisite for activation of the protease in vivo.

AD2 mutants exhibit high levels of proteolytic processing in the absence of budding. They too can be rescued into particles by complementation, indicating that the function required for Gag-Gag interactions is once again unaffected. The experiments presented here suggest that the block to AD2 mutants is encountered on the plasma membrane and, thus, late in budding. This interpretation is reinforced by the observation that the block remains when the membrane-binding domain of the Src protein is present (Myr1 derivatives). This targeting signal is capable of directing heterologous proteins, including the RSV Gag protein, to the plasma membrane (33, 51). Further support for a block late in budding has been obtained from simple membrane fractionation experiments. With either the Myr1 derivative of AD2 deletion mutant T-10C (Fig. 11) or the Myr0 derivative (data not shown), it was found that more than 95% of the molecules pelleted with the membrane fraction. In contrast, only 30% of the Myr0 form of an AD3 mutant was associated with membranes in parallel experiments (51). Although our AD2 mutants have not been examined by electron microscopy, we predict that the molecules will be found in dense regions on the plasma membrane and, perhaps, partially completed buds.

Mutants defective for both copies of the AD3 function sometimes make particles at a readily detected rate and, at first glance, would appear to have no defect. However, the particles are invariably of a density that is much lighter than that for the wild type (48). Moreover, those mutants that are poor producers of particles cannot be rescued from the cells in complementation experiments (3). Thus, we have hypothesized that AD3 promotes the dense packing of Gag molecules within particles and provides the major region of Gag-Gag interaction (1, 48, 49), presumably the one involved in rescuing AD1 and AD2 mutants.

Sequence characteristics of AD2. Although the p2b sequence is unique to RSV, we have noticed that it contains a proline-rich motif, PPP(W/Y)V, that is found near the cleavage site for the MA sequences of a wide variety of retroviruses (Table 2 and Fig. 1). In those viruses in which the tyrosine is replaced by tryptophan, the valine is conserved and there is a second PPPY sequence found nearby. The valine, however, is not conserved among other retroviruses. Further studies will be needed to establish exactly which residues of the p2b sequence are critical for AD2 function. Nevertheless, it is striking that the PPPYV motif is not found in the Gag proteins of lentiviruses, including those of HIV types 1 and 2, equine infectious anemia virus, and feline immunodeficiency virus.

Rates of budding. One of the fundamental, unanswered questions in assembly is why Gag proteins from different retroviruses direct budding at dramatically different rates. In our hands, the Gag protein of RSV is released from the cell with a half-time of about 30 min, whereas that of HIV type 1 requires more than 3 h in the same cells. Because of this slow rate, investigators of HIV typically use labeling times of 12 to 18 h or use immunoblotting to analyze particles collected over long periods of time. In contrast, our standard labeling period for the RSV Gag protein is just 2.5 h. If the delay in budding of RSV AD2 mutants is due to the disruption of the PPPYV motif, then the absence of this sequence from the HIV Gag protein might explain its inherently slower rate of particle release. This is consistent with the properties of recently described Gag chimeras in which the amino-terminal half is from RSV and the carboxy-terminal half is from HIV (1). These chimeras contain the PPPYV sequence and release particles at the same rate as wild-type RSV Gag, rather than at the slow rate of HIV Gag. Thus, the portion of HIV Gag responsible for determining the rate of budding appears to map to the front half of the molecule.

TABLE 2. Proline-rich region of several Gag proteins

Virus[a]	Sequence[b] (↓ cleavage site)
RSV	T A S A P P P P Y V G S G L Y
Mo-MLV	A L A F D P P P W V K P F V H
BLV	A P E E Q P P P Y D P P A I L
GALV	L S E P T P P P Y P A A L P P
M-PMV	P F L T R P P P Y N K A T P S
BaEV	H P L L E P P P Y N S P S P P
SSV	D L A Q S P P P W V P P S A K
HTLV-I	S E P Q I P P P Y V E P T A P
HTLV-II	P E A H V P P P Y V E P T T T

[a] Mo-MLV, Moloney murine leukemia virus; BLV, bovine leukemia virus; GALV, gibbon ape leukemia virus; M-PMV, Mason-Pfizer monkey virus; BaEV, baboon endogenous virus; SSV, simian sarcoma virus; HTLV-I and -II, human T-cell leukemia virus types I and II.

[b] From reference 46. Arrow, cleavage site at the p2-p10 junction in the RSV protein. The highly conserved residues are boxed.

Function of AD2. Although the function of AD2 is unknown, we find it useful to think of it as a molecular hinge or region of movement that is required after Gag proteins have begun to interact with one another on the plasma membrane. Although speculative, this model is consistent with the fact that molecular hinges are often rich in prolines and glycines (15). Moreover, the delayed-budding phenotype associated with our AD2 substitution mutants might be expected if the changes were to stiffen the molecule, and removal of such a hinge would be expected to severely inhibit budding.

The ability of AD2 mutants to be readily rescued by complementation indicates that not all of the molecules in the population have to possess its function. The same can be said for the function of AD1 (membrane binding). These observations indicate that the mechanism of budding does not require the establishment of uniformly organized structures of Gag proteins. Rather, it seems that a threshold of interactions must be established to construct a sufficient network of connections to allow budding to proceed.

AD2 and protease regulation. The mechanism by which retroviruses regulate their proteases is unknown, but it is clear that dimerization of the subunits is absolutely necessary for activity (42). Gag molecules that contain covalently linked protease dimers have increased catalytic activity and exhibit unregulated processing with a loss of particle release from cells (4, 9, 25, 26). Whether dimerization is regulated or there is an inhibitory mechanism to delay Gag processing afterwards remains to be seen. In the case of HIV, and perhaps other lentiviruses, it appears that protease activation can occur well before particle release (23).

We initially envisioned a model for RSV protease regulation

in which the residues of the p2-p10 cleavage site are bound to the active site of PR, thereby preventing its activation until the particle has been released from the cell surface. This idea was based on two observations: the inhibitory activity of synthetic peptides containing this sequence and the continued processing of AD2 mutants in the absence of budding. The experiments described above, however, are not consistent with this model. Amino acid substitutions in the steric element of the p2-p10 peptide that eliminate its inhibitory activity in vitro had no effect on particle release. Moreover, the p2-p10 junction in these Gag mutants was efficiently recognized and cleaved during budding, as indicated by the absence of the 33-kDa intermediate (MA-p2-p10). Nevertheless, we cannot rule out the possibility that the assembly function provided by AD2 coincides with, and is inseparable from, a function needed for protease regulation. The observation that AD2 is the only assembly domain that overlaps a protease cleavage site is particularly interesting from this point of view. Moreover, it is striking that HIV Gag mutants that are defective for membrane binding continue to exhibit PR-mediated processing (6, 22–24, 30, 32, 34) while similar mutants of RSV and murine leukemia virus do not (36, 40, 50, 51). This suggests that the mechanisms of protease regulation might differ among retroviruses.

Is the function of AD2 only involved in budding? If so, then the intracellular processing associated with AD2 mutants cannot be viewed as inappropriate, premature processing. Rather, it may be that the activation of the viral protease of oncoviruses normally occurs well before, instead of after, particle release. In this model, the slow rate of budding associated with the AD2 mutants provides the activated protease with an extended opportunity to cleave Gag proteins in the emerging buds, aborting them before they can pinch off from the cell surface. The D37S mutation negates the effects of protease but does not correct the fundamental defect to budding. The idea that retroviral proteases are activated during budding raises the possibility that they play a role in particle release (e.g., perhaps in severing connections with cellular proteins). This seems unlikely for RSV, because the rate of budding mediated by its Gag protein is no different when the viral protease is inactivated or deleted (2, 13, 43, 47).

In summary, the biological function of the p2-p10 region of the RSV *gag* gene appears to be complex. At the level of the protein, as described here, the p2 region is involved in the events of budding and, perhaps, activation of PR. The same p2-p10 substitution mutations described in this report have been used to demonstrate that, at the level of RNA, the p10 (but not the p2) region is involved in regulation of RNA splicing (20). Thus, this is yet another example of a retrovirus making multiple use of its limited genetic information.

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