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Positionally Independent and Exchangeable Late Budding Functions of the Rous Sarcoma Virus and Human Immunodeficiency Virus Gag Proteins

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The Gag proteins of Rous sarcoma virus and human immunodeficiency virus (HIV) each contain a function involved in a late step in budding, defects in which result in the accumulation of these molecules at the plasma membrane. In the Rous sarcoma virus Gag protein (Pr76^{gag}), this assembly domain is associated with a PPPY motif, which is located at an internal position between the MA and CA sequences. This motif is not contained anywhere within the HIV Gag protein (Pr55^{gag}), and the MA sequence is linked directly to CA. Instead, a late assembly function of HIV has been associated with the p6 sequence situated at the C terminus of Gag. Here we demonstrate the remarkable finding that the late assembly domains from these two unrelated Gag proteins are exchangeable between retroviruses and can function in a positionally independent manner.

All retroviruses encode Gag proteins (28). These are synthesized on free ribosomes in a cytosolic compartment and are subsequently transported to the cytoplasmic face of the plasma membrane. They interact to form complexes of 2,000 to 3,000 molecules (24), which collectively direct the budding process whereby enveloped particles emerge from the cell surface. Very late in or immediately after budding, a virus-encoded protease cleaves the Gag protein into its mature products, MA (matrix), CA (capsid), and NC (nucleocapsid) (Fig. 1); however, proteolytic processing is not a prerequisite for particle release. Moreover, none of the viral products other than Gag are required for budding. This includes the viral glycoproteins, the viral RNA genome, and the enzymatic activities of protease, reverse transcriptase, and integrase (28).

Although the relative order in which MA, CA, and NC are linked is invariable, there is little conservation of amino acid sequence among different Gag proteins (28). The differences are underscored by the presence of additional cleavage products in distantly related Gag proteins (Fig. 1). For example, in the Gag protein of human immunodeficiency virus (HIV) (and other lentiviruses), MA and CA are linked directly (10), whereas in Rous sarcoma virus (RSV), they are separated by three additional sequences, p2a, p2b, and p10 (20). The HIV and RSV Gag proteins also differ dramatically at their C termini. In RSV, this is the location of the viral protease (PR) (3); however, in HIV, a protein named p6 is found at this position (10). The p6 sequence has been implicated in a late step in budding (8).

Because of the lack of sequence conservation, it has been necessary to empirically map the domains required for budding within the Gag proteins of each retrovirus. Studies of RSV Gag have revealed that most of its residues are dispensable for budding (6, 7, 16, 25–30) and that the essential regions or assembly domains (AD) map to three functional modules (Fig. 1). The membrane binding/targeting function is contained in AD1, which is located at the N terminus of Gag (16, 30). A major region for interactions between Gag proteins is supplied by AD3, of which there are two copies contained within the NC sequence (2, 7, 26). A function that is needed late in budding, after the Gag proteins have been targeted to the membrane and have begun to interact, is provided by AD2 (27). This function is located within the RSV p2b sequence and is associated with a PPPY/W motif, which is found in all retroviruses except the lentiviruses. In those viruses that possess this motif, it is always located at a position between MA and CA.

To locate the functional equivalents of AD1, AD2, and AD3 in unrelated retroviruses, we have been making RSV Gag chimeras. These studies have revealed that AD1 can be replaced by the HIV membrane-binding domain (2, 17) and also the small membrane-binding domain of the Src oncoprotein, pp60^{src} (30). Likewise, functional equivalents of AD3 have been found in the Gag proteins of HIV (2), murine leukemia virus, and human foamy viruses (14). However, the construction of functional AD2 chimeras, using segments from other Gag proteins containing the PPPY/W motif, we have found to be more difficult. Presumably the problem relates to the necessity of grafting foreign peptides into the internal position of Gag without affecting its ability to properly fold and function. Then, it occurred to us that RSV AD2 mutants (26, 27) have a phenotype analogous to that of HIV Gag mutants from which the C-terminal p6 sequence has been deleted (8). In particular, the block to budding is at a very late step, and the Gag molecules appear to accumulate on the membrane (27). This observation, coupled with the fact that HIV lacks the internal PPPY/W motif, suggested that the p6 sequence might be supplying the exact same function as RSV AD2, except from a different location within the Gag protein.

Here we report that the late budding function of RSV Gag can be replaced with the C-terminal p6 sequence of HIV Gag

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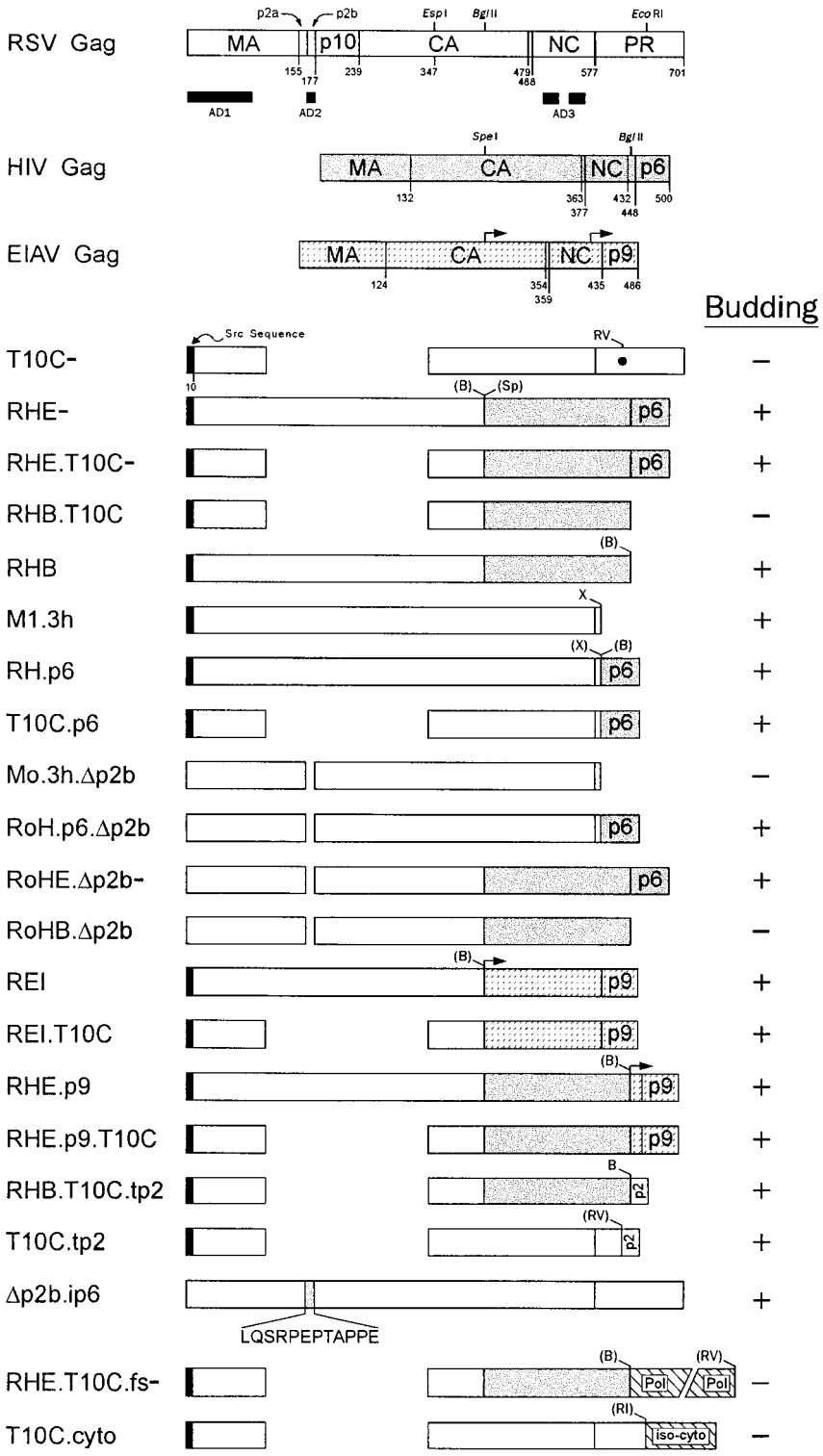

FIG. 1. Schematic diagrams of wild-type and chimeric Gag proteins. The Gag proteins of RSV (open boxes), HIV (darkly shaded boxes), and EIAV (sparsely shaded boxes) are illustrated at the top and are aligned at the sites in capsid (CA) used for making chimeras. The names of the Gag cleavage products are indicated, and the numbers refer to amino acid residues. The black bars beneath the RSV protein mark positions of assembly domains (AD1 to AD3). The positions of relevant restriction endonuclease sites, relative to the corresponding *gag* genes, are indicated with parentheses around sites that were destroyed during cloning. S, *Sst*I; B, *Bgl*II; RV, *Eco*RV; Sp, *Spe*I; RI, *Eco*RI; X, *Xba*I. The arrows above the EIAV protein indicate the relative positions of upstream primers used during PCR amplification. Black boxes present at the N termini of some of the constructs represent the first 10 amino acids from $pp60^{v\text{-}src}$, which were substituted for those of RSV Gag. With the exception of Δp2b.ip6, all of the constructs either lack the coding sequence for the viral protease or have an active-site mutation that eliminates its activity: D37I (black dot in mutant T10C−) or D25S (in the *pol* sequences of RHE− and RoHE−; not shown). In Δp2b.ip6, amino acids 1 to 12 of p6, shown below the darkly shaded box, replace the 11 residues of the p2b sequence of RSV Gag. Mutant T10C.cyto contains the protein iso-1-cytochrome *c* (iso-cyto; indicated by cross-hatching) fused to the RSV Gag protein at residue 647. Mutant RHE.T10C.fs− has the first 294 amino acids of the HIV Pol protein fused after the NC sequence. The ability of each chimera to be released by budding is indicated in the column to the right.

or the similarly positioned, but nonhomologous, p9 sequence from the Gag protein of equine infectious anemia virus (EIAV). Moreover, we show that the late functions of RSV and HIV can work at either an internal or C-terminal position.

MATERIALS AND METHODS

Previously constructed *gag* alleles. Several of the constructs used in this study (Fig. 1) have been described previously: pSV.M1.3h (25), pSV.T10C (26, 27), pSV.MoΔp2b (27), pSV.RH.p6 (2), pSV.RHE− (2), pSV.RHB (2), and pSV.RHE.fs− (2). Two of these, pSV.RHE− and pSV.RHE.fs−, contain a portion of the downstream HIV *pol* gene (to nucleotide 2976 in HIV type 1 strain HXB2; not illustrated in Fig. 1) but encode an inactivated protease (D25S) (2).

Newly constructed RSV-HIV *gag* alleles. All DNA manipulations were carried out by using standard methods (22). Each of the *gag* alleles made by oligonucleotide-directed mutagenesis or PCR amplification was sequenced to confirm the presence of the desired mutations. Furthermore, at least two presumably identical clones of all constructs were tested to ensure that unanticipated mutations had not arisen outside the sequenced region during the constructions.

pSV.RHE.T10C− was made by subcloning the T10C deletion into pSV.RHE−, using the *Sst*I site just upstream of the RSV *gag* gene and the *Esp*I site located immediately after the deletion. pSV.RHB.T10C was derived from pSV.RHB using the same fragment exchange. pSV.T10C.p6 was constructed from pSV.RH.P6 by replacing its *Sst*I-*Bgl*II fragment with one containing the T10C deletion. pSV.Mo.3h.Δp2b was derived from pSV.M1.3h and pSV.MoΔp2b by a fragment exchange utilizing the *Kpn*I site located just upstream of the *gag* gene and the downstream *Bgl*II site. pSV.RoHp6.Δp2b was generated by inserting the *Sst*I-*Bgl*II fragment containing the p2b deletion into pSV.RH.p6. pSV.RoHE.Δp2b− and pSV.RoHB.Δp2b were made by substituting the *Sst*I-*Esp*I fragment containing the p2b deletion.

pSV.RHE.p9 was created by PCR amplification of a fragment from the infectious EIAV proviral clone PV 19-2 (19), using 5′-GCAATGCAGAAGGATCCCAAAAAACGGGAAGCAA as the upstream primer and 5′-GTAGGCCTGCGCGCTAGATTATATGTTACT as the downstream primer. Following digestion with *Bam*HI and *Bss*HII, the PCR product was ligated into the *Bgl*II-*Bss*HII sites of the pSV.RHB parent. Construction of pSV.REI utilized the same downstream PCR primer along with upstream primer 5′-CAGAAGGCATCAAGGATCCGATTGGAAAACCTA on the PV 19-2 EIAV template. The resulting fragment was cut with *Bam*HI and *Bss*HII and ligated into the *Bgl*II-*Bss*HII sites of pSV.Myr1.D37S (5).

pSV.RHB.T10C.tp2 was created by PCR amplification of the p2 coding sequence, using upstream primer 5′-TGGCACATCCAGATCTGGCATTGCGGAACAGCTATT and downstream primer 5′-TTACTACAAACCACTCCCCACATAA. The PCR product was made blunt with Klenow enzyme and then cut at the 5′ end at the *Bgl*II site (underlined in the primer sequence). This sequence was ligated into the pSV.RHB parent after being digested with *Bss*HII, treated with Klenow enzyme, and cut at the *Bgl*II site located between the NC and p6 coding sequences; this produced the intermediate construct, pSV.RHB.tp2. The T10C deletion was then inserted by using the *Sst*I and *Bgl*II sites to give pSV.RHB.T10C.tp2. To make pSV.T10C.tp2, intermediate pSV.RHB.tp2 was digested with *Bgl*II, treated with Klenow enzyme, and digested with *Sst*I; the *Sst*I-*Eco*RV fragment from pSV.T10C− containing the T10C deletion was inserted. Oligonucleotide-directed mutagenesis was used to create pSV.Δp2b.ip6, using a single-stranded DNA template, as previously described (29, 30). The mutagenic primer has the sequence 5′-GCTATTGGCTGTAATTGCGCCCTTCAGAGTAGACCAGAGCCAACAGCCCCACCAGAAAGTGGTTTGTATCCTTCCCTG. The resulting mutation was transferred into pSV.Myr1 (29) by using *Sst*I and *Esp*I.

pSV.RHE.T10C.fs− was created by digesting RHE.T10C− with *Bgl*II, treating with Klenow enzyme, and religating as previously described for RHE.fs.D25S (2), which does not contain the T10C deletion. In this chimera, p6 is replaced with the first 294 residues of the Pol protein as a result of a frameshift mutation at the *Bgl*II site, and the protease within this sequence is inactivated by an amino acid substitution (D25S). pSV.T10C.cyto was derived by placing the T10C deletion into pSV.MyCY, which contains the iso-1-cytochrome *c* sequence fused to protease (25, 26). This fragment exchange was accomplished by using *Sst*I and *Esp*I.

Transfection, labeling, immunoprecipitation, and analysis of Gag proteins. COS-1 cells were grown in 35-mm-diameter dishes in Dulbecco's modified Eagle medium (GIBCO BRL) supplemented with 3% fetal bovine and 7% bovine calf serum (HyClone, Inc.). They were transfected with *Xba*I-digested and ligated plasmids by the DEAE-dextran-chloroquine method and metabolically labeled for 2.5 h with L-[^{35}S]methionine (50 μCi, >1,000 Ci/mmol) approximately 48 h after transfection as previously described (25, 29). The cells and growth medium from each labeled culture were separated and mixed with lysis buffer containing protease inhibitors, and the Gag proteins were collected by immunoprecipitation at 4°C. In most cases, a rabbit antiserum against whole RSV (reactive with the MA, CA, NC, and PR products) was used (25). For the RSV-EIAV chimeras, rabbit antiserum specific for p9 (15) was used to confirm that the EIAV Gag sequences were properly fused to RSV Gag. Previously reported experiments demonstrated the presence of the HIV p6 sequence on the RSV-HIV chimeras (2). The immunoprecipitated proteins were separated by electrophoresis in sodium dodecyl sulfate (SDS)–12% polyacrylamide gels and detected by fluorography. Quantitation of the fluorograms obtained from the pulse-chase experiments was carried out by densitometry.

Particle analysis. To ensure that the chimeric Gag proteins were released from the cells by the process of budding, several of the key constructs were tested for the ability to produce virus-like particles by centrifugation of the samples through sucrose density gradients as previously described (2, 26). The tested constructs included RHE.T10C−, T10C.p6, RoH.p6.Δp2b, and RoHE.Δp2b−, all of which were found to be released in particulate form (data not shown). Many of the other constructs have been analyzed previously and found to be released into particles, including the wild-type RSV Gag protein (26, 30), RHE− (2), RHB (2), M1.3h (26), RHE.fs.D25S (2), and even T10C−, if it is rescued into particles by complementation (27).

RESULTS

We hypothesized that the late budding function of RSV Gag could be replaced with the late function of HIV Gag, even though these two sequences have very different relative positions (within the p2b and p6 sequences, respectively; Fig. 1). To test this idea, we took the gain-of-function approach of examining whether p6 could restore budding to RSV AD2 mutants when placed at the C terminus of Gag. The first mutant examined was T10C, which lacks a large segment of the RSV Gag protein, including all of p2a, p2b, and p10 as well as parts of MA and CA (Fig. 1). As previously reported (26, 27), this mutant was completely defective for particle release (Fig. 2A, lanes 8). The T10C deletion was introduced into a previously described (2) chimera of the RSV and HIV Gag proteins, RHE− (Fig. 1). Both the RSV p2b and the HIV p6 sequences are present in RHE−, and when expressed, RHE− produces particles identical to those of authentic RSV in rate of release (Fig. 2A, lanes 2) and density (2). To remove the p2b (AD2) sequence, and thereby test whether p6 can serve in its place, the T10C deletion was introduced into this chimera to create RHE.T10C− (Fig. 1). To our surprise, this recombinant was released into the medium with the same efficiency as the wild type (Fig. 2A, lanes 3 and 4). The particulate nature of the proteins released into the medium was confirmed by pelleting the mutant virions through sucrose gradients (data not shown). When p6 was removed, leaving neither of the late budding functions (Fig. 1, RHB.T10C), the release of particles into the medium was abolished (Fig. 2A, lanes 6 and 7). Budding was restored when the RSV AD2 sequence was added back to this construct (Fig. 1, RHB; Fig. 2A, lanes 5).

We next addressed whether the HIV sequence upstream of p6 is required for the late function. For this, we replaced the RSV protease, which is not needed for budding (Fig. 1, M1.3h; Fig. 2B, lanes 2), with p6. This chimera, RH.p6 (Fig. 1), was released into the medium (Fig. 2B, lanes 4) even after the RSV AD2 function was removed (Fig. 1, T10C.p6; Fig. 2B, lanes 5 and 6). While the level of release was far greater than that of T10C (lanes 3), it was approximately 1/10 the amount of the earlier mutant, RHE.T10C−, which included the upstream HIV sequences. This difference was not seen with another AD2 mutant (see below) and may be due to a less than optimal junction between the RSV and HIV sequences. In any case, there is not an absolute requirement for the upstream HIV sequence.

To make sure that mutant T10C is not a special case, we examined the ability of p6 to suppress a second AD2 mutant, Δp2b (Fig. 1, Mo.3h.Δp2b). As shown previously (27), the precise deletion of all 11 amino acids of p2b severely impaired the late function of RSV (Fig. 2C, lanes 2). When p6 was added to the C terminus of this mutant, budding was fully restored, and the level of release was the same in this case whether the flanking sequences from HIV-1 were included or not (Fig. 1, RoH.p6.Δp2b and RoHE.Δp2b−; Fig. 2C, lanes 3

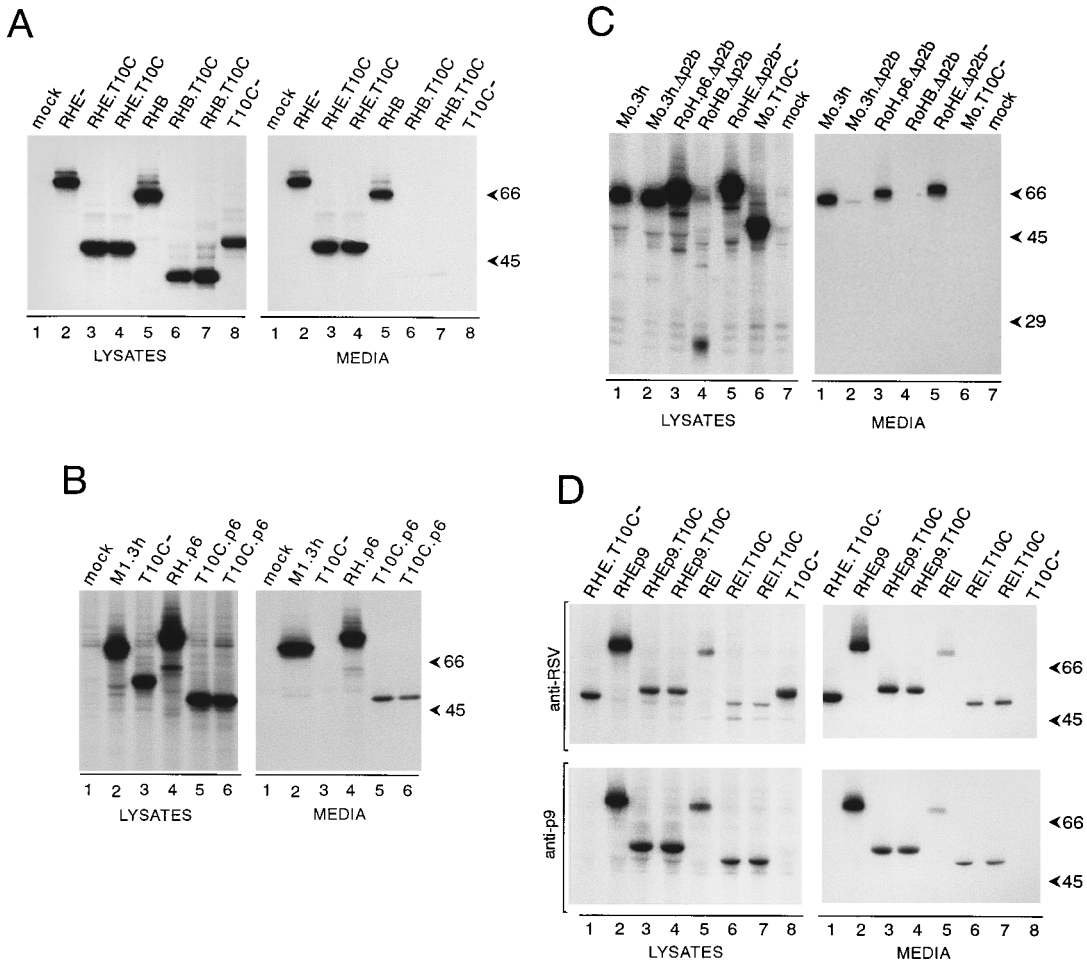

FIG. 2. Exchangeability of late budding functions. COS-1 cells were transfected with the indicated DNAs and labeled with L-[^{35}S]methionine for 2.5 h, and then the Gag proteins present in the cell lysates and media were immunoprecipitated with rabbit antibodies against RSV, resolved by SDS-polyacrylamide gel electrophoresis, and visualized by fluorography. The positions of molecular weight standards (in kilodaltons) are indicated to the right. Identically labeled lanes represent independently isolated clones. (A) Suppression of large AD2 deletion mutant T10C with the C-terminal sequences of HIV. (B) Sequences upstream of p6 are not absolutely required for suppression. (C) Suppression of small AD2 deletion mutant Δp2b with the HIV p6 sequence. (D) Analysis of RSV-EIAV chimeras. Proteins were immunoprecipitated with rabbit antibodies against RSV (top) or the p9 sequence (bottom).

and 5, respectively). Again, the upstream sequence alone was unable to provide the late function, as indicated by the failure of double mutant RoHB.Δp2b (Fig. 1) to be released (Fig. 2C, lanes 4). This mutant, which lacks both of the late budding domains, was somewhat unstable, as indicated by the breakdown product running slightly faster than the 29-kDa marker and the low intensity of the band corresponding to the full-length product (running near the position of the 66-kDa marker). This was somewhat unexpected given that the larger AD2 deletion of RHB.T10C resulted in no apparent degradation (compare Fig. 2A, lanes 3 and 4, with Fig. 2C, lanes 4). To test the possibility that differences at the N termini of these two proteins might account for their distinct behaviors, the membrane-binding domain of the Src protein (present on RHB.T10C) was placed onto RoHB.Δp2b; however, this change did not increase the stability of the RHB.Δp2b molecule (data not shown). Whatever the explanation for the instability of this nonfunctional chimera, it is clear that the addition of the C-terminal p6 sequence restores budding to mutants containing the p2b deletion.

To test whether HIV is unique among lentiviruses in having a late function linked to the C terminus of NC, we examined the terminal peptide from another lentivirus, p9 from EIAV (11, 15). This sequence, along with different amounts of flanking sequence, was placed at the C terminus of mutant T10C (Fig. 1, REI.T10C and RHE.p9.T10C). Although the sequence of p9 is very different from those of p6 and p2b (see Discussion), it restored budding in both constructs (Fig. 2D, lanes 6 and 7 and lanes 3 and 4, respectively). Thus, p9 appears to be a second example of a C-terminal, late budding domain.

Since the terminal p6 and p9 sequences can substitute for the function of the upstream p2b sequence in RSV, it seemed possible that late budding functions might work independently of their position. To test this idea, we first examined whether p2b could function at the C terminus of Gag, and we found that it could. Placement of the p2b sequence (flanked by the sequence of p2a and 3 residues of p10) at the end of RHB.T10C restored budding to this assembly-defective molecule (Fig. 1, RHB.T10C.tp2; Fig. 3A, lanes 4 and 5). Remarkably, the level of release was only 50% lower than wild-type levels. The p2 sequence also functioned at this position when the CA and NC sequences from HIV-1 were replaced with those of RSV (T10C.tp2; not shown).

After finding that p2 can function at the end of Gag, it was

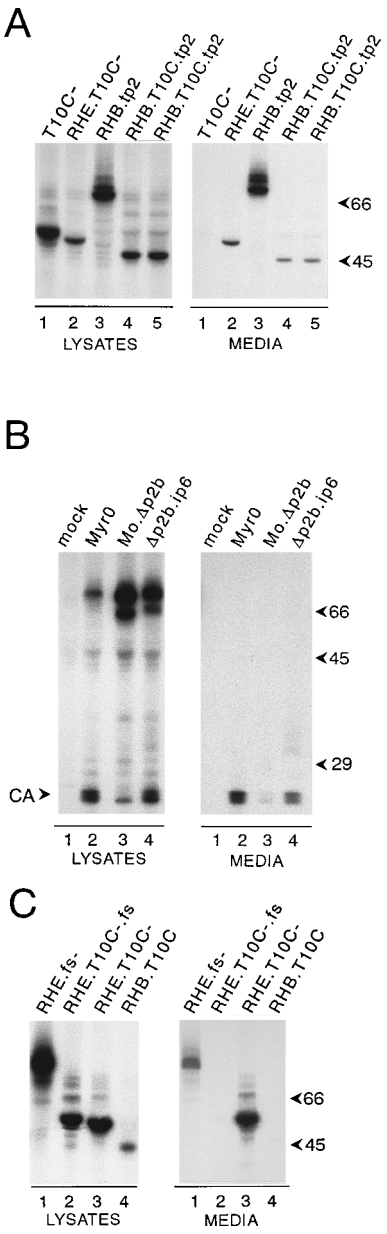

FIG. 3. Positional independence of late budding functions. Gag proteins were analyzed as described in the legend to Fig. 2. (A) The p2b sequence functions at a C-terminal position. (B) The first 12 residues of p6 restore budding when placed between MA and CA in RSV Gag. (C) Not all sequences suppress AD2 deletion mutants.

of interest to test whether p6 could replace the late budding function in RSV when positioned between MA and CA. The portion of p6 most important for budding has been mapped to the first 12 residues, including the prolines at positions 10 and 11 (8), and these were substituted precisely for p2b (Fig. 1, Δp2b.ip6). This internal chimera directed budding with 36% of the efficiency of the wild type (Fig. 3B; compare lanes 2 and 4). In this experiment, the CA protein rather than the intact Gag precursor was detected in the medium because the RSV protease was not inactivated. From all of the experiments described above, we conclude that the p2 and p6 sequences are interchangeable and serve identical functions, even though they are not conserved in primary sequence or position within the Gag precursor.

Not every sequence placed at the C terminus of an AD2 mutant enhances budding. For example, a portion of the *pol* frameshift product of HIV did not restore budding to T10C (Fig. 1, RHE.T10C.fs−; Fig. 3C, lanes 2), although the full-length protein that contains the AD2 function buds efficiently (RHE.fs−; lanes 1), as reported previously (2). Furthermore, a nonretroviral protein, iso-1-cytochrome *c* from *Saccharomyces cerevisiae*, was unable to function as a late assembly domain when placed at the end of mutant T10C (Fig. 1, T10C.cyto; data not shown), even though the full-length construct does bud (25, 26).

DISCUSSION

We do not yet understand how the p6, p9, and p2b sequences function late in budding. They have no primary sequence homology, and while p2b and p6 are proline rich, p9 is not (Fig. 4). Furthermore, the number and arrangement of proline residues in p2b and p6 are quite different. Because the functionally important parts of these sequences are very short (11 amino acids for p2b and 12 for p6), they are likely to be conformationally simple. Structural data to support this inference exist for p6; in this case, circular dichroism and nuclear magnetic resonance spectrum analyses have shown that it does not adopt a rigid conformation in solution (23). It may be that the lack of structure is important for the function of the late budding domain.

How could unrelated, unstructured sequences provide similar functions? One possibility is that they do so through interactions with a host protein that recognizes other proteins in a sequence-independent manner. Well-known examples of proteins with this capability are the chaperonins, which recognize the unstructured regions of many different proteins and assist in their folding (1, 4, 9). Thus, the late assembly domains of Gag molecules might enhance budding by recruiting a host factor(s) into a macromolecular complex much in the way that transcriptional enhancers stimulate RNA synthesis by binding transcription factors.

The involvement of a host factor(s) in enhancing particle release might explain why some groups have not observed a reduction in particle release for p6 mutants (12, 13, 18, 21). The individual cell lines and cell types used, the methods of transfection, or perhaps some of the expression vectors employed (e.g., baculovirus and vaccinia virus) might account for differences in the expression of cellular proteins. Altered levels of host factors might in turn reduce the specific need for p6 or

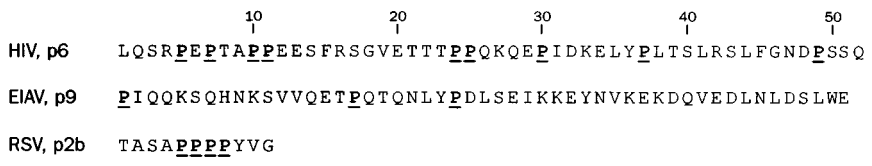

FIG. 4. Comparison of peptides containing late budding functions. The sequences are aligned beginning at their N termini. Proline residues are underlined.

provide alternative mechanisms of particle release. In any case, the data presented here, obtained through an independent line of investigation with a different retrovirus, provide compelling evidence that p6 indeed contains a late budding function.

As the molecular mechanisms of late functions are pursued, three points should be kept in mind. First, it is not certain where along the Gag transport pathway they exert their effects. Although it is attractive to imagine events at the site of budding, it also conceivable that the late domains provide a function that is needed prior to transport of Gag proteins to the membrane. That is, events early in the pathway may influence the actual pinching-off step that occurs very late in budding. Second, it is possible that late domains from different Gag proteins work in different ways (perhaps through different host proteins, for example) even though the end result is the same. Finally, we do not know to what extent the dramatic changes of position of late functions in linear space result in changes within the three-dimensional structure of the emerging virion. For example, if the C terminus of the RSV Gag protein happens to lie near the p2b sequence during budding, then linkage of the p6 sequence to this end would place it near the normal position of the RSV late function.

In conclusion, we have now identified the functional equivalents of all of the RSV assembly domains within the Gag protein of HIV. These findings strongly suggest that the functions of Gag proteins are highly conserved, even though their amino acid sequences and relative positions in linear space are not. For these reasons, we have adopted a new nomenclature for the assembly domains of all Gag proteins in which the membrane-binding domains (formerly AD1 in RSV), the late domains (AD2), and the interaction domains (AD3) are more descriptively named the M, L, and I domains, respectively.

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