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Fine Mapping and Characterization of the Rous Sarcoma Virus Pr76^{gag} Late Assembly Domain

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The p2 region of the Rous sarcoma virus (RSV) Gag polyprotein contains an assembly domain, which is required late in replication for efficient budding of virus-like particles from cells (J. W. Wills, C. E. Cameron, C. B. Wilson, Y. Xiang, R. P. Bennett, and J. Leis, J. Virol. 68:6605–6618, 1994). This domain, referred to as the L domain, was previously mapped to the 11 amino acids of p2b. Through the analysis of a series of deletion and substitution mutations, the L domain has now been fine mapped to a highly conserved amino acid sequence, PPPPYV of p2b. Sequences flanking PPPPYV motif can be deleted without any effect on budding. Defects caused by L-domain deletions can be rescued by placing a wild-type copy of the sequence at several other positions in RSV Gag. A proline-rich P(S/T)APP motif is found in many retroviral Gag polyproteins; the motif found in the p6 region of human immunodeficiency virus type 1 has been implicated in late functions of the virus. Substitution of the RSV L domain with this motif in a 10-amino-acid sequence derived from visna leukemia virus results in wild-type release of virus particles from cells. In contrast, the slightly different sequences from Gibbon ape leukemia virus, Moloney leukemia virus, PSAPP alone, or a proline-rich SH3 binding sequence do not efficiently rescue RSV L-domain mutations.

Translation of the Rous sarcoma virus (RSV) genomic RNA results in the synthesis of two polyprotein precursors, Pr76^{gag} and Pr180$^{gag\text{-}pol}$ (10), which are subsequently processed by the virus-specific protease (PR) to yield the mature proteins found in virions (19). Expression of Pr76^{gag} or the Gag polyprotein (Fig. 1) is both necessary and sufficient to form virus-like particles which bud from the cell surface; neither viral RNA nor *env* and *pol* gene products are required (3, 8, 10, 22, 25–27). Moreover, an extensive deletion analysis has revealed that less than a third of Gag is required to produce virus-like particles. These sequences map to three discrete regions in Gag, referred to as the M domain for its function in membrane binding, the L domain for its function late in the viral assembly, and the I domain for its function in Gag-Gag interactions (2, 16, 23–25). The M domain resides at the amino terminus of MA and contains the membrane targeting signal that directs the precursor polyprotein to the plasma membrane prior to particle assembly. It can be replaced by the heterologous membrane-binding domain from the Src oncoprotein (27) or the human immunodeficiency virus (HIV) Gag protein (2, 17). The I domain maps within the NC coding sequence and is essential for the dense packing of Gag molecules within the particle (2, 23). It can be replaced with functionally equivalent sequences from HIV Gag (2) and probably represents a major region of Gag-Gag interaction.

At the L-domain core is the proline-rich sequence PPPPYV, which is conserved among a wide variety of retroviral Gag polyproteins except for those of lentiviruses (24). This motif is generally located between the MA and CA sequences in Gag. In some viruses, the Tyr is replaced by a Trp and there is a second PPPY sequence nearby. Deletion of the p2b region from RSV Gag leads to a dramatic decrease in the release of virus-like particles from cells even though the Gag proteins are tightly associated with the membrane fraction and contain a high level of protease activity. Furthermore, RSV L-domain mutations can be rescued into dense particles in a genetic complementation assay, indicating that their defect is not due to a conformation distortion of the protein and that the L-domain sequences are not required to be present on every synthesized Gag molecule for budding to occur (24). An amino acid sequence near the carboxyl terminus of HIV type 1 (HIV-1) Gag in p6 previously has been shown to be required for budding of HIV-1 (12). This sequence, as well as one found in the p9 region at the end of equine infectious anemia virus (EIAV) Gag, can functionally substitute for the RSV L domain in vivo (16). This is despite the fact that neither has a primary sequence homology to the RSV L domain. The HIV-1 L-domain equivalent is proline rich, containing the sequence PTAPP at its core, while the EIAV L-domain equivalent is not. The role of the PTAPP sequence in HIV-1 p6 for budding of virus particles from cells has recently been confirmed in studies using a full-length molecular clone of HIV-1 (14).

The RSV L domain was initially detected (24) through analysis of a large deletion placed into the *gag* allele, called T10C, which removed amino acids 122 to 336 and resulted in the complete block to release of virus-like particles from COS-1 cells (Fig. 2). A smaller deletion, which removed only 11 amino acids from the Gag allele, p2b, also results in a block to budding (24); however, we have found that this mutant can sometimes be leaky and release virus-like particles at a level up to 10% of the control level (Fig. 2B, lane 6). In addition to the budding defect, the p2b deletion alters the processing of a spacer peptide between the CA and NC proteins. This is seen by the single CA band in Fig. 2A, lane 6. This band represents the CA protein with a nine-amino-acid sequence on its carboxyl terminus ending at Met-488 (9, 18) and is referred to as CA1. In the wild-type case, a triplet which represents this band and two more slowly migrating bands which have been processed, leaving Met-476 or Ala-479 at the carboxyl terminus

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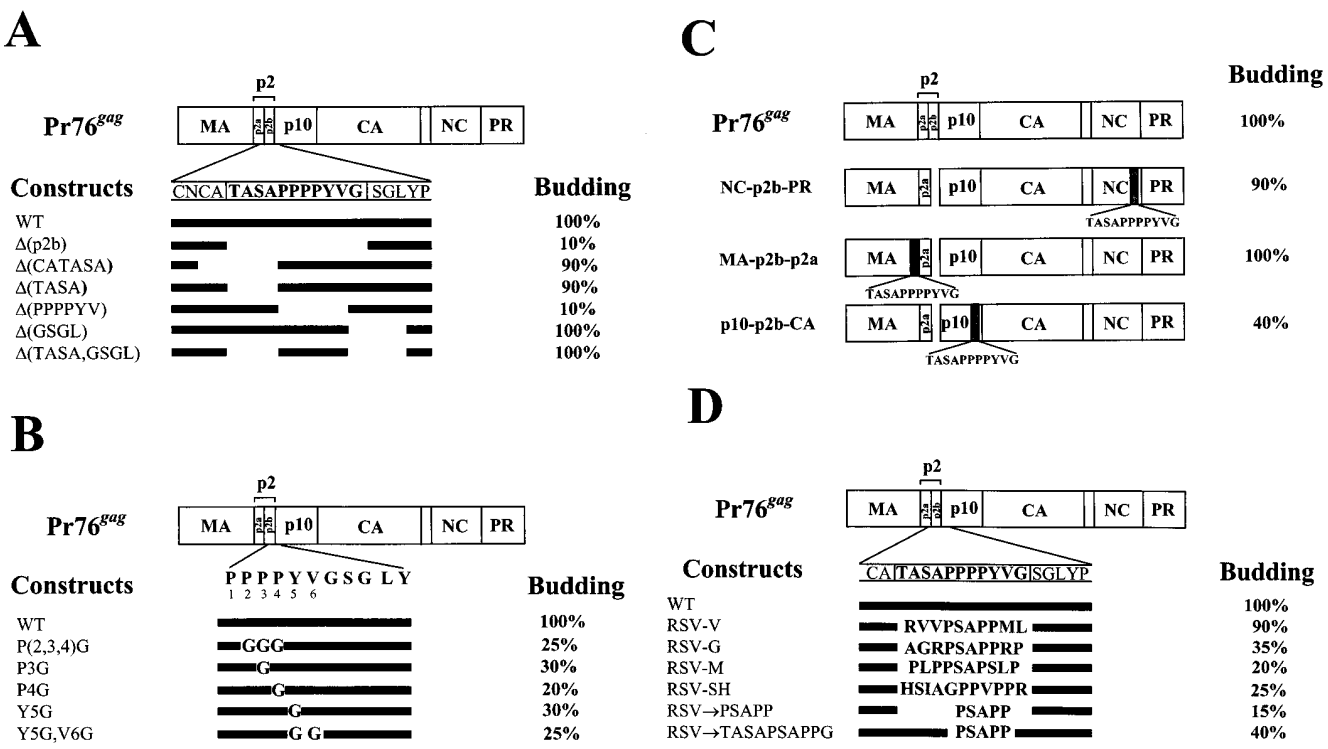

FIG. 1. Mutations in the RSV Gag polyprotein. The RSV Gag polypeptide is represented by rectangular boxes. The amino acid sequence of p2b (boldface letters) and its flanking sequence is expanded below as shown. The vertical lines represent the viral PR cleavage sites between the different sequences, which are indicated inside the box. The p2 region, which is 22 amino acids in length, is divided in half, and the halves are referred to as p2a and p2b. A series of mutations that change Pr76^{gag} are listed below the boxes and were constructed by overlap extension mutagenesis (1) using the oligodeoxynucleotides listed in Table 1. The name of each mutant is on the left, and the effects of these mutations on budding of virus-like particles compared with the wild type (WT) are summarized on the right. (A) Deletion mutations in the p2b region. The full-length black line represents wild-type Gag. The positions and size of the deletions are indicated by the gaps in the black line and align to the expanded p2b sequence. (B) Amino acid substitutions to the conserved PPPPYV sequence. The amino acid substitutions are indicated below and are aligned with the expanded sequence as in panel A. (C) Mutations that move p2b to other sites in Pr76^{gag}. In each mutation, the p2b sequence has been deleted (break in the solid line) and a wild-type copy of p2b has been inserted (by substitution) into another position in Gag (shaded box) as indicated. (D) Gag chimeras. The p2b sequence (boldface letters) has been substituted with sequences derived from the Gag polyprotein from visna virus (RSV-V), gibbon ape leukemia virus (RSV-G), or Moloney leukemia virus (RSV-M). The p2b sequence also has been substituted with a mouse SOS1 protein SH3 binding domain (RSV-SH), PSAPP, and TASAPSAPPG sequences, as indicated.

(18), is observed. These bands are referred to as CA2 and CA3, respectively.

The assembly domain in RSV p2 maps to the conserved PPPPYV sequence. To examine the significance of the proline-rich motif in p2b, a series of deletion mutations were introduced into the region as illustrated in Fig. 1A. The oligonucleotides used to make the mutations are listed in Table 1. The deletions removed just the conserved sequence or small groups of amino acids flanking either the amino terminus or the carboxyl terminus of the PPPPYV motif. As shown in Fig. 2B, lane 3, removal of the conserved amino acids resulted in the same extent of defect observed with the Δ(p2b) mutation (compare lane 3 with lane 6 in Fig. 2B). In contrast, when the TASA or the CATASA sequence on the amino-terminal side of the PPPPYV motif was deleted, there was little effect or small detectable effects on release of virus particles from cells or on processing of the CA, as seen by the appearance of the triplet bands (Fig. 2B, lanes 1 and 2). In a similar fashion, when the GSGL sequence flanking the carboxyl terminus of the PPPPYV motif was deleted, no defect on particle release or processing of CA was observed (Fig. 2B, lane 4). The only difference in the viral polyprotein products observed with the Δ(GSGL) mutant and wild type was the appearance of a 33-kDa band (referred to as p33). This band represents a processing intermediate that contains the MA, p2, and p10 proteins, and its appearance is expected since the deletion of the GSGL sequence mutates the PR cleavage site between the p2 and p10 proteins, slowing its rate of cleavage (5, 6, 13). More importantly, combining the deletion of the TASA and the GSGL sequences into one construct had no effect on particle release or on CA maturation (Fig. 2B, lane 5). Taken together, these results demonstrate that the L domain maps within the conserved PPPPYV motif.

To further probe the significance of the PPPPYV sequence, we made a series of mutations in which a glycine residue was substituted for the tyrosine or some of the individual proline residues in the PPPPYV motif (Fig. 1B). For each of the Gly substitution mutants, there was a decrease in release of particles and a defect to maturation of the carboxyl terminus of CA, detected by the accumulation of the CA1 band compared with the wild type. The reduction ranged between 70 and 80%, slightly less than observed by removal of the complete motif with the Δ(p2b) mutant (data not shown). These results are consistent with the report of Bowles et al. (4) with the exception that L-domain mutations in our hands are not efficiently rescued by a protease-inactivating mutation.

The assembly domain is able to function independent of position in Gag. Previously, it was shown that the p2b sequence will promote budding of particles when moved to the carboxyl terminus of the Gag polyprotein (16), a site where the equiv-

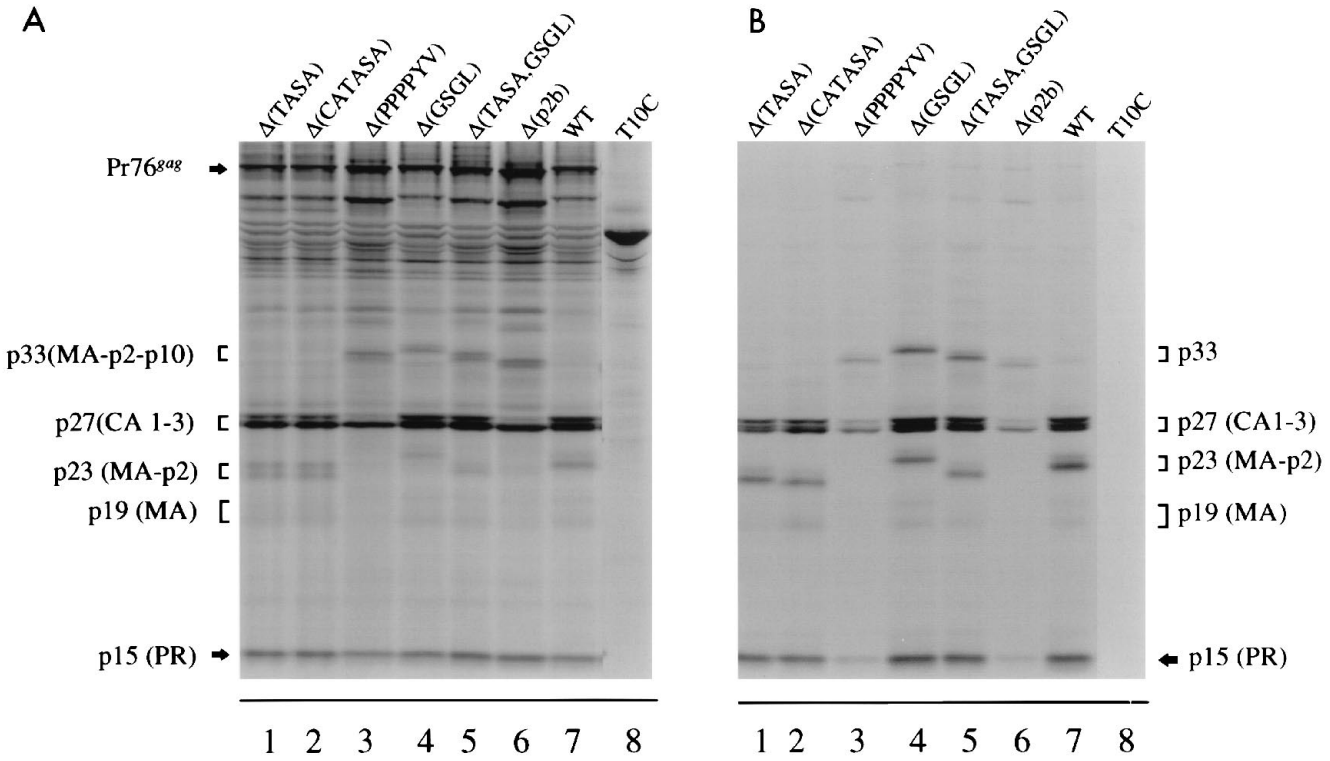

FIG. 2. Fine mapping of the RSV L domain. Cells were metabolically labeled for 2.5 h with [^{35}S]methionine 48 h after transfection with the indicated simian virus 40-based, pSV.Myr0, Pr76^{gag} expression vector in COS-1 cells. RSV proteins associated with cell lysates (A) or in the culture media (B) were immunoprecipitated with polyclonal anti-RSV serum, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized by fluorography. The positions of migration of Pr76^{gag} and its cleavage products are indicated to the left of each panel. The viral proteins detected in the medium are contained in virus-like particles released from the cell surface (26, 27). Budding of these particles is independent of proteolytic processing (8, 26, 27); if an active PR is present, the particles contain mature viral proteins. Under these labeling conditions, wild type (WT) shows three species of CA, MA, a MA precursor, p23$^{MA\text{-}p2}$, and PR. The MA-p2 band is the predominant form of MA detected under these conditions as a result of the slow cleavage rate of the MA-p2 cleavage site (6). Also, MA-containing bands are seen as doublets as a result of serine phosphorylation of the protein (15). NC is not observed since it has only one methionine and migrates near PR under these conditions. Deletions to the Gag allele are indicated above the lanes and are depicted in Fig. 1A. T10C represents a Gag allele with a deletion of amino acids 122 to 136 (24). Viral precursor and mature proteins are indicated at the sides of the panels. In every case, two independent clones for each mutation were constructed and analyzed, though the results from only one clone are shown. These experiments were repeated two or three times, and the results shown are representative.

alent L domain of HIV-1 or EIAV is normally located. To investigate whether the RSV motif can function at positions in Gag not normally associated with L-domain function, the wild-type p2b sequence was moved from its normal position to near the MA-p2 junction (MA-p2b-p2a), near the p10-CA junction (p10-p2b-CA), or near the NC-PR junction (NC-p2b-PR) (Fig. 1C). The names of the mutants indicates the relative order of the proteins in the scrambled sequence. The first construct moves the assembly domain 11 amino acids to the amino-terminal side of its original position, the second construct moves it approximately 60 amino acids to the carboxyl-terminal side of its original position, while the last construct moves it several hundred amino acids to the carboxyl terminus of its original position (Fig. 1C). All of the constructs contain a wild-type PR and do not introduce large deletions into Gag.

The locations for the p2b replacements were chosen because each is near a Gag polyprotein PR cleavage site and would presumably be exposed so that a budding domain would be sterically available to function. The MA-p2b-p2a mutation changes the MA-p2 cleavage junction to that of the p2-p10 cleavage sequence, while the p10-p2b-CA and NC-p2b-PR mutations leave the respective wild-type cleavage junctions intact. The insertions of p2b in both of these instances were made five amino acids to the amino-terminal side of the respective cleavage junctions. In each case, budding of virus-like particles, as well as normal processing of the CA protein, was observed (Fig. 3). The p2b sequence functions as well as or nearly as well as the wild type in the MA-p2 or NC-PR position, respectively. It partially replaces the assembly functions when placed near the p10-CA junctions. In this latter case, the rescue is of the same magnitude as placing p2b at the end of Gag (16). None of these mutations perturbed the overall conformation of Gag since normal cleavage of the polyproteins was observed, with the exception that the p10-p2b-CA and NC-p2b-PR mutations showed the appearance of the p33 band as a result of disruption of the p2-p10 cleavage junction. The two bands migrating in the position between MA-p2 and MA in the RSV MA-p2b-p2a mutant represent the MA-p2b products, as expected. Taken together, these results indicate that the p2b sequence can function as an assembly domain independent of position in Gag.

Another proline-rich sequence can functionally replace the RSV PPPPYV sequence. The predominant characteristic of the RSV L domain is its richness in proline residues. We therefore tested whether other proline-rich sequences, especially those found in other viral Gag polyproteins, would substitute for the PPPPYV sequence. A proline-rich P(S/T)APP motif is found in lentivirus Gag polyproteins. We substituted the RVVPSA PPML sequence representing amino acids 432 to 442 from visna virus Gag polyprotein or the AGRPSAPPRP sequence

TABLE 1. Mutagenic oligodeoxynucleotides

Mutant	Sequence of mutagenic oligodeoxynucleotide	New restriction site(s)[a]
Δ(CATASA)	5′ ACAGCTATTGGCTGTAATCCTCCTCCTCCTTATGTGGGG 3′	None
Δ(TASA)	5′ GCTATTGGCTGTAATTGTGCACCTCCTCCTCCTTATGTGGGG 3′	*Apa*LI
Δ(PPPPYV)	5′ TGCGCCACAGCCTCAGCTGGGAGTGGTTTGTATCCTTCCCTG 3′	*Pvu*II
Δ(GSGL)	5′ GCTCCTCCTCCTCCATATGTGTATCCTTCCCTGGCGGGGGTGG 3′	*Nde*I
Δ(TASA,GSGL)	5′ GCTATTGGCTGTAATTGTGCACCTCCTCCTCCATATGTGTAT 3′	*Apa*LI, *Nde*I
Y5G	5′ TGCGCCACAGCCTCGGCTCCTCCTCCTCCCGGGGTGGGGAGTGGTTTG 3′	*Sma*I
P4G	5′ TGCGCCACAGCCTCGGCTCCTCCTCCCGGGTATGTGGGGAGTGGTTTG 3′	*Sma*I
P3G	5′ TGCGCCACAGCCTCGGCTCCTCCCGGGCCTTATGTGGGGAGTGGTTTG 3′	*Sma*I
P(2,3,4)G	5′ TGCGCCACAGCCTCGGCTCCCGGGGGGGGGTATGTGGGGAGTGGTTTG 3′	*Sma*I
MA-p2b-p2a	5′ GCCACACCTAAAACCGTTGGCACAGCATCCGCGCCACCACCCCCGTACGTAGGCTCCGGCTTATACCC GATTGGCTGTAATTGCGCCA 3′	*Sna*BI
p10-p2b-CA	5′ CTTGCGAGTACTGGTCCGCCCACAGCATCCGCTCCTCCTCCCCCGTACGTAGGCGTGGTGGCCATGCC TGTAGTG 3′	*Sna*BI
NC-p2b-PR	5′ GGGCCGTGGCCCGGCCCTGAGACAGCATCCGCGCCACCACCCCCGTACGTAGGCCCACCTGCCGTCTC GTTAGCG 3′	*Sna*BI
RSV-V	5′ GCTATTGGCTGTAATTGCGCCCGCGTGGTACCTAGCGCTCCTCCTATGCTCAGTGGTTTGTATCCTTCC CTG 3′	*Kpn*I
RSV-G	5′ GCTATTGGCTGTAATTGCGCCGCAGGTCGACCTAGTGCTCCTCCTAGACCTAGTGGTTTGTATCCTTCC CTG 3′	*Sal*I
RSV-M	5′ GCTATTGGCTGTAATTGCGCCCCTCTTCCTCCTAGTGCACCTTCTCTTCCTAGTGGTTTGTATCCTTCC CTG 3′	*Apa*LI
RSV-SH	5′ GCTATTGGCTGTAATTGCGCCCACAGCATCGCCGGACCTCCTGTCCCGCCGCGGAGTGGTTTGTATCC TTCCCTG 3′	*Sac*II
RSV→PSAPP	5′ ATTGGCTGTAATTGCGCCCCTAGTGCACCGCCGAGTGGTTTGTATCCTTCC 3′	*Apa*LI
RSV→TASAPSAPPG	5′ GCTATTGGCTGTAATTGCGCCACAGCCTCGGCTCCTAGTGCACCTCCTGGGAGTGGTTTGTATCCTTCC CTG 3′	*Apa*LI
NC-T3	5′ CGAGACGGCAGGTGG 3′[b]	
MAEA1	5′ GGCAGATATCGATTAATGGAAGCCGTCATA 3′[b]	

[a] A unique restriction enzyme site was introduced with the mutation to facilitate selection of clones containing the desired mutation.
[b] Used as PCR primer for constructing mutants as described in reference 1. Oligodeoxynucleotides used for construction of Y5G, V6G are described in reference 24.

representing amino acid 112 to 122 from the gibbon ape leukemia virus Gag polyprotein for the RSV PPPPYV sequence in the Gag allele (Fig. 1D). As shown in Fig. 4, comparison of the amounts of PR in particles released into the media shows that substitution of the visna virus sequence conferred wild-type particle release from cells as well as proper CA maturation. Substitution of the gibbon ape leukemia virus sequence resulted only in partial rescue of the L-domain mutation. Again, all of these mutations maintained the overall conformation of Gag, since all of the known cleavage sequences were properly used during the processing.

To determine whether the PSAPP motif itself can substitute for the PPPPYV domain, the five residues were inserted into the Gag protein in place of the 11 amino acids of p2b (Fig. 1D). The PSAPP substitution did not significantly increase the release of virus particles from cells compared with the RSV Δ(PPPYV) mutant (Fig. 4B, lane 8), suggesting that flanking sequences are important. The addition of a TASA sequence to the amino terminus of the PSAPP sequence resulted in an increase in budding of virus particles (Fig. 4B, lane 9) to the level observed by substitution of the gibbon ape leukemia virus sequence. These results indicate that the PSAPP sequence by itself is insufficient to substitute for the PPPPYV domain. To further investigate whether any proline-rich sequence can replace the PPPPYV function, the amino acid sequence PLPPSA PSLP, derived from positions 107 to 117 from the murine leukemia virus Gag polyprotein, was substituted for the RSV sequence. The murine leukemia virus sequence did not effectively restore the late budding functions to Gag (Fig. 4B, lane 6). An SH3 binding motif, HSIAGPPVPPR, derived from the mouse SOS1 protein (positions 1285 to 1295), was also substituted for the RSV sequence and again failed to effectively replace L-domain function (Fig. 4B, lane 7). The size differences of the MA-p2-p10 products in the different mutants analyzed here are caused by the different amino acid sequences placed in p2.

The mapping of a biologically functional domain to such a small sequence is a surprise, raising interesting questions as to how it functions in RSV assembly. One possibility is that the L domain can influence the conformation of $Pr76^{gag}$ and it is this conformation that is somehow required for the budding of virus particles. This is not likely since the L domain can function at multiple positions in Gag, including near the RSV MA-p2, p2-p10, p10-CA, and NC-PR junctions (this report) and at the end of $Pr76^{gag}$ (16). Alternatively, the L domain could specifically interact with some cellular protein(s) to facilitate the budding process. For instance, HIV-1 Gag polyproteins have been reported to interact with human cyclophilins A and B (11). Moreover, these interactions are dependent on proline residues in HIV-1 $Pr55^{gag}$. However, cyclophilin A is not likely to be the cellular protein that interacts with the L domain since the HIV-1 Gag-cyclophilin binding site can be deleted without affecting particle release from cells and HIV-2 does not package cyclophilins (11).

A recent observation in the *yes* oncogene field strongly suggests that L domains may function in particles release by mediating an interaction with a specific host protein at the membrane. Yes is a tyrosine kinase located on the cytoplasmic face of the plasma membrane, the same surface to which Gag proteins are targeted. It interacts with Yap (Yes-associated pro-

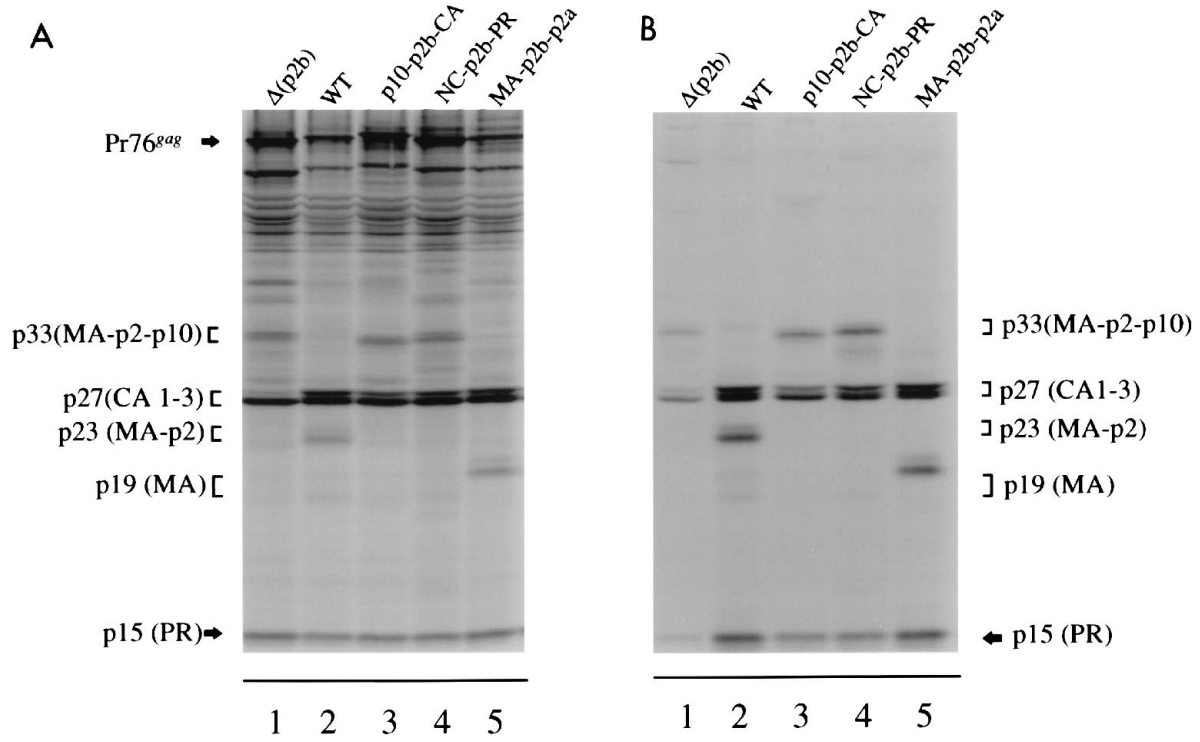

FIG. 3. The RSV L domain can function at different positions in Pr76^{gag}. Mutations that move the p2b sequence to other locations in Pr76^{gag} are shown in Fig. 1C and are indicated above the lanes. In each case, the p2b sequence was deleted from its wild-type (WT) position and inserted into other positions of Gag. Viral proteins from the cell lysate (A) and media (B) were analyzed as described in the legend to Fig. 2. Migration positions of viral proteins are as indicated.

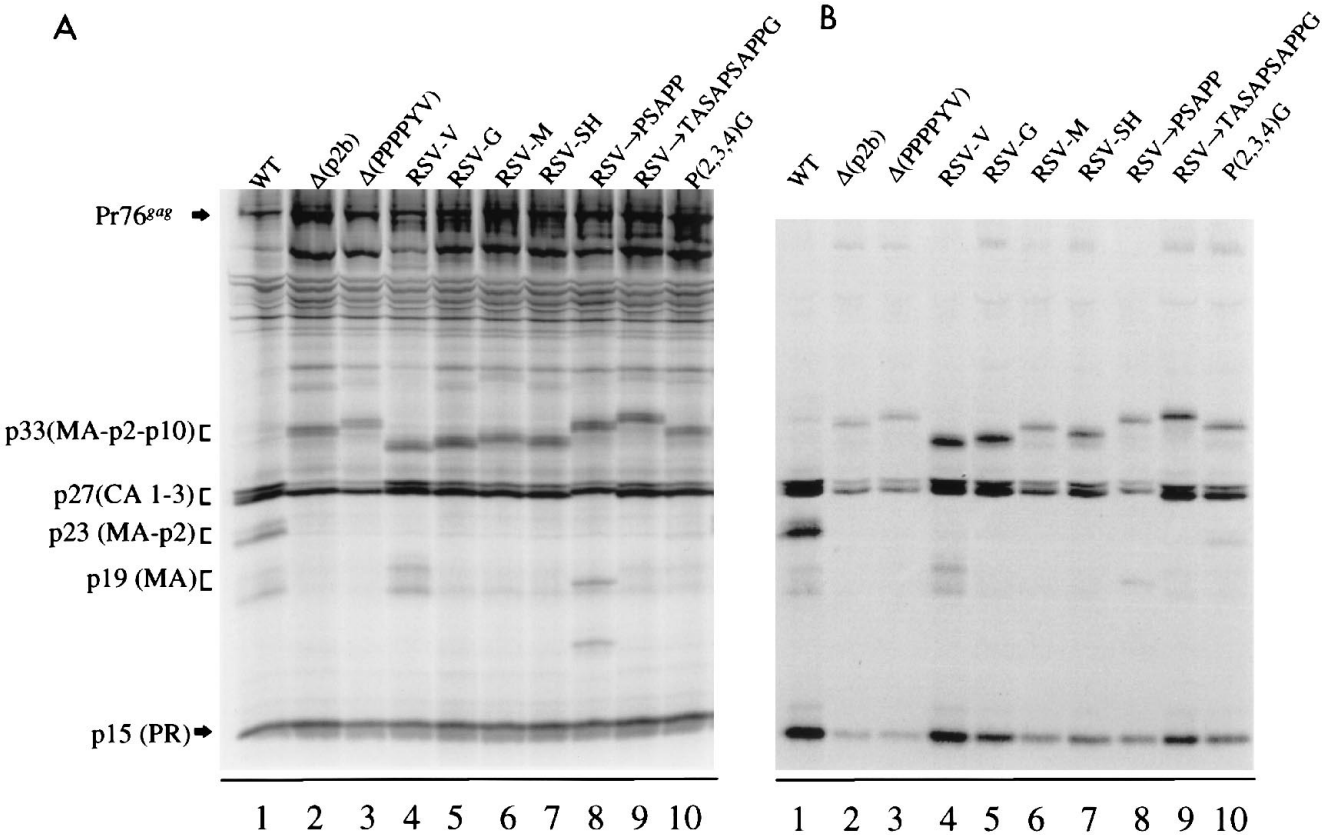

FIG. 4. Substitution of the RSV L domain with proline-rich sequences. Chimera constructs are as listed in Fig. 1D and are indicated above the lanes. Viral proteins from the cell lysate (A) and media (B) were analyzed as described in the legend to Fig. 2. Migration positions of viral proteins are indicated on the left.

tein) during signal transduction (20). Yap contains a WW motif, a sequence of about 38 amino acids in length containing two widely spaced Trp residues (20, 21). This motif is found not only in signaling molecules like Yap but also in certain cytoskeletal elements. This finding is intriguing since the latter have long been proposed, but never unequivocally demonstrated, to be involved in budding. By using a glutathione *S*-transferase–WW fusion protein to screen a cDNA expression library, two Yap-binding proteins (WP-1 and WP-2) which contain different amino acid sequences except for the presence of a five-amino-acid sequence, PPPPY, which has been shown to interact with the WW motif (7), were identified. Remarkably, this target sequence is identical to the RSV L-domain sequence, PPPPYV. This observation, in combination with the location at which L-domain mutants accumulate, provides a compelling argument for the involvement of a cellular protein, one with a WW motif, in the late stages of budding for RSV. Because the L domain of RSV can be replaced with the sequences from HIV and visna virus Gag and by the unrelated sequence from the p9 region of EIAV Gag, there are likely to be at least two other cellular proteins that can provide this function but in a manner that may not involve the WW protein. This hypothesis is now being tested.

Finally, in analyzing all of the L-domain mutations reported here, we found a correspondence between the severity of the budding defect caused by an L-domain mutation and the extent of defect observed in CA maturation (Fig. 3 and 4). For instance, when there is a severe defect to budding, there is a corresponding strong defect to processing of CA and vice versa. The relationship between budding of virus particles and CA maturation is also suggested by the fact that substitution of heterologous amino acid sequences for the RSV L domain results in the concomitant rescue of budding of virus particles and CA maturation. In addition, mutations introduced into the M domain at the amino terminus of MA, which prevent particle release from cells, show CA maturation defects (15a). Altering the MA-p2 cleavage site of RSV Gag so that it is cleaved 200 times more efficiently than the wild-type site results in premature processing of Gag, loss of virus particles released from cells, and the same CA maturation defect (28). Huang et al. (14) have also observed the altered CA maturation during viral replication with mutations to the p6 region of HIV-1 Gag. The mechanism of how CA maturation is tied to budding is not understood. However, it is possible that activation of the PR occurs only at or during budding and that the Gag precursor is in a specific conformation at this time needed for correct processing of CA.

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