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Rescue of internal scaffold-deleted Mason-Pfizer monkey virus particle production by plasma membrane targeting

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Abstract

The Mason-Pfizer monkey virus (M-PMV) Gag protein follows a morphogenesis pathway in which immature capsids are preassembled within the cytoplasm before interaction with and budding through the plasma membrane. Intracytoplasmic assembly is facilitated by sequences within the p12 domain of Gag that we have termed the Internal Scaffold Domain (ISD). If M-PMV utilizes an ISD then what provides the equivalent function for most other retroviruses that assemble at the plasma membrane? To investigate the possibility that the membrane itself fulfills this role, we have combined functional deletion of the ISD with a mutation that disrupts intracellular targeting or with a plasma membrane targeting signal. By either modification, targeting of ISD-deleted Gag to the plasma membrane restores particle production. These results provide support for a model in which the plasma membrane and the D-type ISD provide an interchangeable scaffold-like function in retrovirus assembly.

Keywords: M-PMV; Retrovirus; Assembly; Gag; Immature capsid; Internal Scaffold Domain; Plasma membrane

Introduction

Retroviruses assemble in a complex process involving an intermediate immature capsid that upon budding from the plasma membrane undergoes a morphologically dramatic maturation step to yield the infectious particle. This transformation is mediated by the viral proteinase, which cleaves polyprotein precursors allowing the liberated components to reassemble into the core structures of the mature virion. The exception to this is the Spumaviruses, which undergo limited proteolytic processing (reviewed in Swanstrom and Wills, 1997). Mature cores take different forms characteristic for each retrovirus, such as cones, cylinders, or spheres for human immunodeficiency virus (HIV), Mason-Pfizer monkey virus (M-PMV), or murine leukemia virus, respectively (reviewed in Vogt, 1997). The structure of the HIV core, in particular, has been the subject of intensive scrutiny. Not only has the core of budded particles been analyzed in detail by electron (Welker et al., 2000) and cryo-electron microscopy (Benjamin et al., 2005; Briggs et al., 2003; Yeager et al., 1998), but several in vitro assembly systems have been developed that imitate cone formation by the assembly of tubes (Campbell and Vogt, 1995; Ehrlich et al., 1992; Gross et al., 1997; Lanman et al., 2003) and even morphologically authentic cones (Ganser et al., 1999). Analysis by cryoelectron microscopy of these structures produced in vitro has led to the development of a fullerene model for the HIV core (Briggs et al., 2003; Li et al., 2000), which by simple rearrangement of pentagonal elements, can be modified to resemble the spheres and cylinders seen as the cores of other retroviruses (Ganser-Pornillos et al., 2004). Thus, despite gross morphological differences, the arrangement of mature subunits in the mature virion is likely similar in most retroviruses.

The immature virion among retroviruses is likewise similar in both morphologic and biochemical characteristics. It is composed primarily of polyprotein precursors and appears in thin section electron microscopy as a particle composed of concentric rings of varying density surrounding an electron lucent center (reviewed in Vogt, 1997). These immature particles, like their mature counterparts, can vary greatly in mass and diameter, reflecting a variability in the number of structural precursor molecules (Briggs et al., 2004b; Fuller et

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al., 1997; Kingston et al., 2001; Parker et al., 2001; Vogt and Simon, 1999; Wilk et al., 2001; Yeager et al., 1998). The pleomorphic nature of immature retrovirus particles has made traditional crystallographic atomic-level structural analysis impossible. However, cryoelectron microscopy has revealed a radial arrangement of densities within the primary structural protein Gag (Briggs et al., 2004a, 2004b; Fuller et al., 1997; Kingston et al., 2001; Parker et al., 2001; Wilk et al., 2001; Yeager et al., 1998). The order of densities from exterior to interior corresponds with the Gag domains in their N to C terminal positions within the precursor protein: N-matrix (MA), the two domains of capsid (CA), and nucleocapsid (NC) -C. The correspondence of primary elements in immature particles to the Gag polyprotein precursor is consistent with the widely confirmed result that for most retroviruses Gag is the only viral gene product necessary for such particles to assemble and bud from the cell, with the exception being the Spumaviruses where Env is required (reviewed in Swanstrom and Wills, 1997). The recent systems using purified fragments of Gag (Campbell and Vogt, 1997; Campbell et al., 2001; Gross et al., 1998, 2000; von Schwedler et al., 1998; Yu et al., 2001) or Gag synthesized in vitro (Sakalian et al., 1996, 2002) to form immature particle-like structures have further confirmed Gag as the only essential protein of the assembled immature particle.

Despite the structural similarity of immature particles, the location of assembly differs by retrovirus genus. While the majority assemble at a membrane, typically though not always the plasma membrane (Ono and Freed, 2004; Pelchen-Matthews et al., 2003), the Betaretroviruses assemble their immature particles inside the cytoplasm without any apparent membrane involvement. Historically, with the exception of the Spumaviruses, retroviruses that assemble at the plasma membrane were designated C-type while those that assemble in the cytosol were designated as B- or D-type. Of the Betaretroviruses (the B- and D-type viruses), the most extensively studied with regard to its assembly and morphogenesis is the Mason-Pfizer monkey virus (M-PMV). An 18-amino acid sequence within the MA domain of M-PMV Gag, termed the cytoplasmic targeting/retention signal (CTRS) (Choi et al., 1999; Rhee and Hunter, 1990a; Yasuda and Hunter, 2000), directs nascent Gag polypeptides to a pericentriolar region of the cell where assembly of immature capsids occurs (Sfakianos et al., 2003). These particles are then actively transported (Weldon et al., 1998) by either an Env/Gag interaction on recycling endosomes or by a less efficient and as yet undescribed default pathway to the plasma membrane where they bud (Sfakianos and Hunter, 2003). Thus, unlike most retroviruses that assemble and simultaneously become enveloped at the plasma membrane, the processes of assembly, envelopment, and budding are spatially and temporally separated for M-PMV.

Numerous mutants of M-PMV Gag have been described that display altered transport, assembly, or both (Rhee and Hunter, 1987, 1990a, 1991; Sommerfelt et al., 1992; Stansell et al., 2004; Yasuda and Hunter, 2000). We have described the use of an in vitro synthesis and assembly system based upon coupled transcription and translation in a rabbit reticulocyte lysate (Sakalian et al., 1996) to distinguish between defects in intracellular transport and the process of assembly itself for two *gag* gene mutants (Sakalian and Hunter, 1999). The matrix domain mutant R55W redirects Gag to the plasma membrane where assembly subsequently occurs and was the first evidence for a CTRS in M-PMV Gag (Rhee and Hunter, 1990a). We have shown that R55W can assemble in both the in vitro translation-assembly system and within inclusion bodies in bacteria and thus has retained the capacity to assemble in the cytoplasm. In contrast, deletions within the p12 domain of M-PMV Gag appear to affect the efficiency of particle formation such that under low-level expression conditions Gag fails to assemble (Sommerfelt et al., 1992). We have demonstrated that the efficiency of assembly in the in vitro system mirrors that seen in cells under expression conditions similar to that of an infection (Sakalian and Hunter, 1999). However, overexpression of Gag, either in bacteria (Sakalian and Hunter, 1999), or in COS-1 cells or CV-1 cells by the vaccinia/T7 system (Sommerfelt et al., 1992), can compensate for the effect of the deletion and allow Gag to assemble. These results argue that the p12 domain functions as an "Internal Scaffold Domain" (ISD), much like the scaffold proteins of numerous bacteriophage, which as an aspect of their function increase the efficiency of particle assembly, and plays a critical role in the membrane-independent assembly of immature capsids. We have also shown that fusing M-PMV p12 to HIV Gag enables the chimera to efficiently assemble in the in vitro translation system (Sakalian et al., 2002).

The idea that an ISD serves to modulate the efficiency of capsid assembly leads to the question of what performs this function for retroviruses that do not contain it. After all, the processes of assembly cannot be fundamentally different if it is possible to convert a virus that assembles in the cytoplasm into one that assembles at the plasma membrane by means of a single amino acid substitution (Rhee and Hunter, 1990a; Yasuda and Hunter, 2000). We hypothesized that for many retroviruses the plasma membrane itself may provide the scaffold-like function for assembly. To test this hypothesis, we examined M-PMV Gag variants containing a deletion of ISD function combined with a mutation to target Gag to the plasma membrane. Consistent with the hypothesis that a membrane can function to assist Gag assembly, we found that targeting Gag to the plasma membrane restores particle production for ISD-deleted Gag.

Results

The Internal Scaffold Domain deletion phenotype is dominant in an in vitro assembly system

To test the hypothesis that the plasma membrane can substitute for the ISD if Gag proteins are directed there, we constructed a series of mutant M-PMV *gag* genes that contain the R55W mutation, an ISD deletion (Δ1–25), or both (Fig. 1). The combination of these two mutations (R55W/Δ1–25) within the D-type Gag molecule would be expected to render

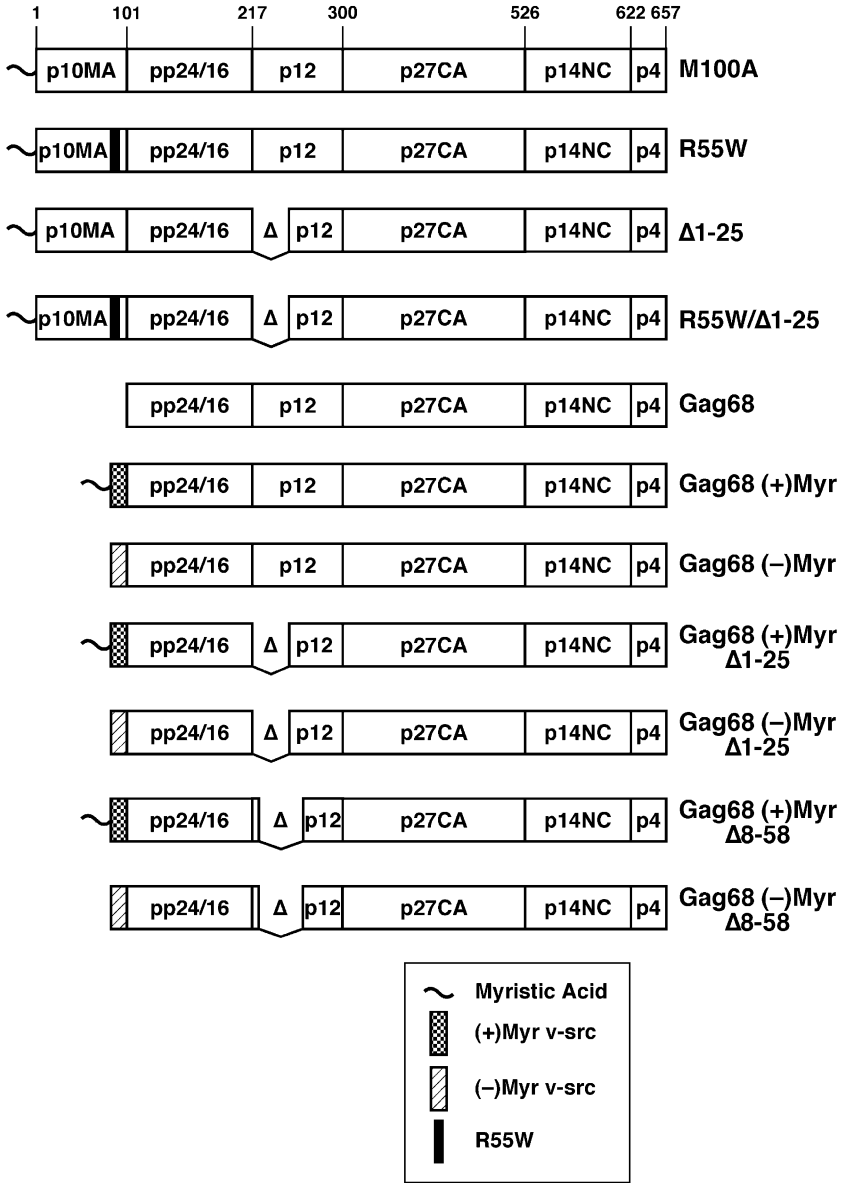

Fig. 1. Schematic illustration of M-PMV Gag variants. M100A contains a methionine to alanine change at amino acid 100 of Gag to prevent translation initiation at this position. For purposes of this study, M100A represents the wild type. R55W contains an additional single amino acid change at position 55 that inactivates the CTRS. The deletion in Δ1–25 removes a region critical to the functioning of the ISD. Gag68 was created by mutation of the first methionine codon to that for alanine, thus forcing translation to initiate at the second methionine codon, number 100. The result is a Gag precursor precisely deleted of the MA domain. Versions of Gag68 were created containing either the first 10 amino acids of src, so that the N-terminus is myristylated, or an altered version with glycine two changed to alanine to prevent myristylation. Further derivatives of these were made by incorporating two ISD defective deletions, Δ1–25 and Δ8–58. Numbers at the top indicate the starting residue within Gag for each domain as well as the last residue of the polyprotein.

that precursor functionally equivalent to a C-type Gag. Examination of the morphogenesis of capsids assembled from this Gag variant was then performed in vitro and in cells. We also examined in parallel an M-PMV Gag species, Gag68, deleted of its MA domain, and thus, devoid of the transport and membrane binding elements of the precursor (Fig. 1). In addition to the mutations under analysis in this study, all constructs that include MA express the M100A substitution in order to prevent initiation of translation at the second AUG in the *gag* sequence. This was done to simplify the analysis by reducing the number of Gag-related species produced in vitro and in cells. The M100A substitution has no discernable effect upon virus replication in culture as has been discussed previously (Sakalian and Hunter, 1999).

Before analyzing the dual mutant Gag in cells, it was necessary to first ascertain its phenotype in the in vitro system. We predicted that since there is no active cellular transport mechanism available within the in vitro system, the Δ1–25 deletion would be dominant. This is indeed what we found; Gag containing both R55W and Δ1–25 failed to assemble into particulate material as examined by sucrose gradient analysis (Fig. 2) and produced a profile of material in the gradient similar to that produced by the single mutant, Δ1–25, species. In contrast, the MA-deleted version, Gag68, produced partic-

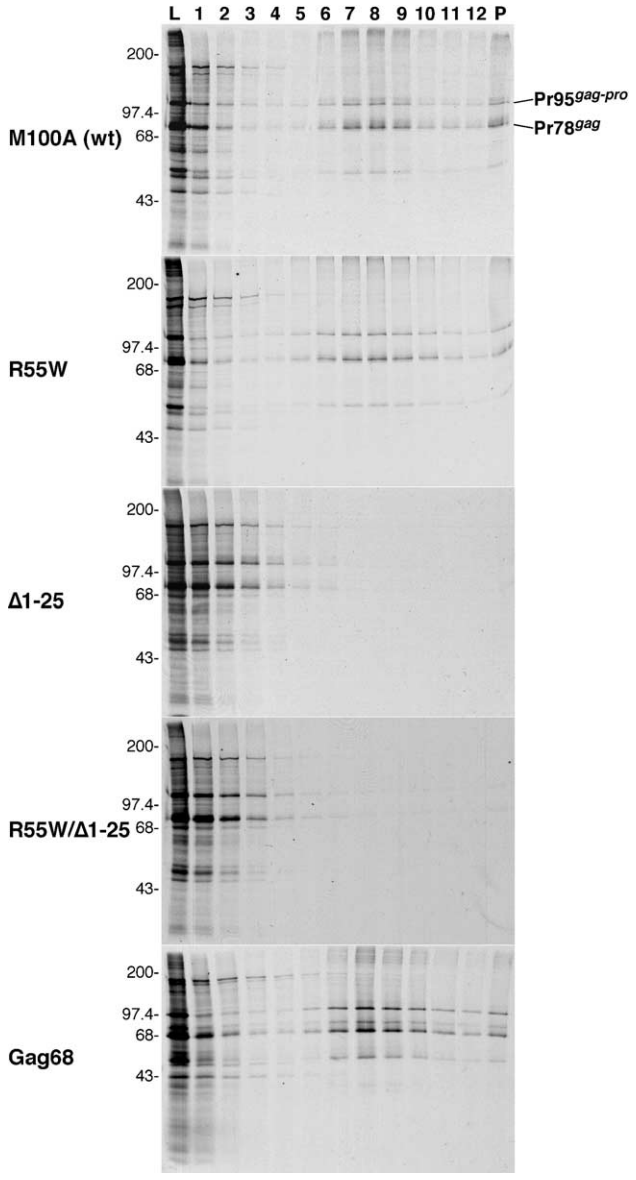

Fig. 2. Comparison of in vitro-synthesized and -assembled Gag species by sucrose gradient analysis. Aliquots of gradient fractions were electrophoresed on an SDS-10% polyacrylamide gel. Lane numbers indicate gradient fractions beginning with the top fraction at no. 1. Lanes L contain an equivalent aliquot of the translation reactions before gradient fractionation. Lanes P contain material remaining as a pellet in the tube after removal of the gradient. Lanes 6–9 contain material indicative of immature capsids at a density of ~1.19–1.21 g/ml. $Pr78^{gag}$ and $Pr95^{gag-pro}$ indicate the positions of the Gag and Gag–Pro precursor polypeptides of M-PMV, respectively.

ulate material with no apparent difference in efficiency from the M100A control or R55W.

The CTRS mutation can compensate for the Internal Scaffold Domain deletion for particle release

To examine the potential effect of transport on an ISD-deleted Gag to the plasma membrane, we next tested expression of the dual R55W/Δ1–25 mutant Gag in mammalian cells. If the plasma membrane functions as a scaffold for the assembly of C-type viruses, then particle production of the ISD-deleted Gag should be restored by the CTRS mutation. Stably transfected HeLa cell lines were created using the pSHRM15 series of plasmids containing the various species of *gag* (Fig. 1) in the context of a complete M-PMV provirus.

Particle production was measured by pulse-chase analysis of these cells (Fig. 3A). The single mutations, R55W and Δ1–25, were tested in parallel with the dual mutant and Gag68. For all constructs examined, three forms of Gag precursor can be seen: Gag, Gag–Pro, and Gag–Pro–Pol. These result from the slippage of the ribosome at two frameshift signals between the *gag* and *pro* and also the *pro* and *pol* reading frames (Jacks et al., 1987, 1988). The ability of each proviral species to produce particles was monitored by the release of matured CA (p27) protein into the tissue culture supernatant (Fig. 3A, lower panel). Since the culture medium was filtered and virus was collected by centrifugation, only assembled particles were examined, and not CA released by secretion or cell lysis.

Both M100A (wild type) and the single R55W mutant released the matured CA protein indicative of virus particle production. Consistent with previously reported data, the single Δ1–25 species failed to release a significant quantity of CA protein (Sommerfelt et al., 1992). Likewise, Gag68 failed to release virus particles. In contrast, the dual R55W/Δ1–25 species released a significant amount of particles, suggesting that interaction with the plasma membrane can, indeed, substitute for the loss of ISD function.

To measure the relative efficiency with which particles were produced, the release of CA as a function of the expression of Gag in cells was determined for each cell line and then compared with that from cells expressing M100A (Fig. 3B). The quantity of mature CA released by each cell line and the amount of Gag synthesized during the pulse labeling was counted. After correction for the number of methionines in each species, the amount of particulate protein in the culture medium was divided by the quantity in the cell to obtain a proportion for particle released. This proportion for each cell line was then divided by that for the line producing the M100A (wild type) species to give a measure of relative release [relative release = $(CA/Gag)_{mutant}$ / $(CA/Gag)_{wild\ type}$, where CA and Gag are the methionine corrected counts for CA in released particles and pulse-labeled Gag, respectively]. The CTRS mutant, R55W, is approximately twice as efficient in particle release as wild type, while the ISD deletion, Δ1–25, is nearly completely defective. The greater efficiency with which R55W produces particles has been noted previously and has been attributed to the more rapidly traversed C-type morphogenesis pathway versus the multi-step B/D-type pathway (Rhee and Hunter, 1990a). In contrast to the single ISD deletion, the double CTRS/ISD mutant released particles at about 60% of wild type. The matrix-deleted species, Gag68, is completely defective for particle production.

Failure to release particles by the Δ1–25 and Gag68 mutants might result from rapid degradation in the cytoplasm rather than from defects in assembly or transport. Previous analysis of R55W (Rhee and Hunter, 1990a; Yasuda and

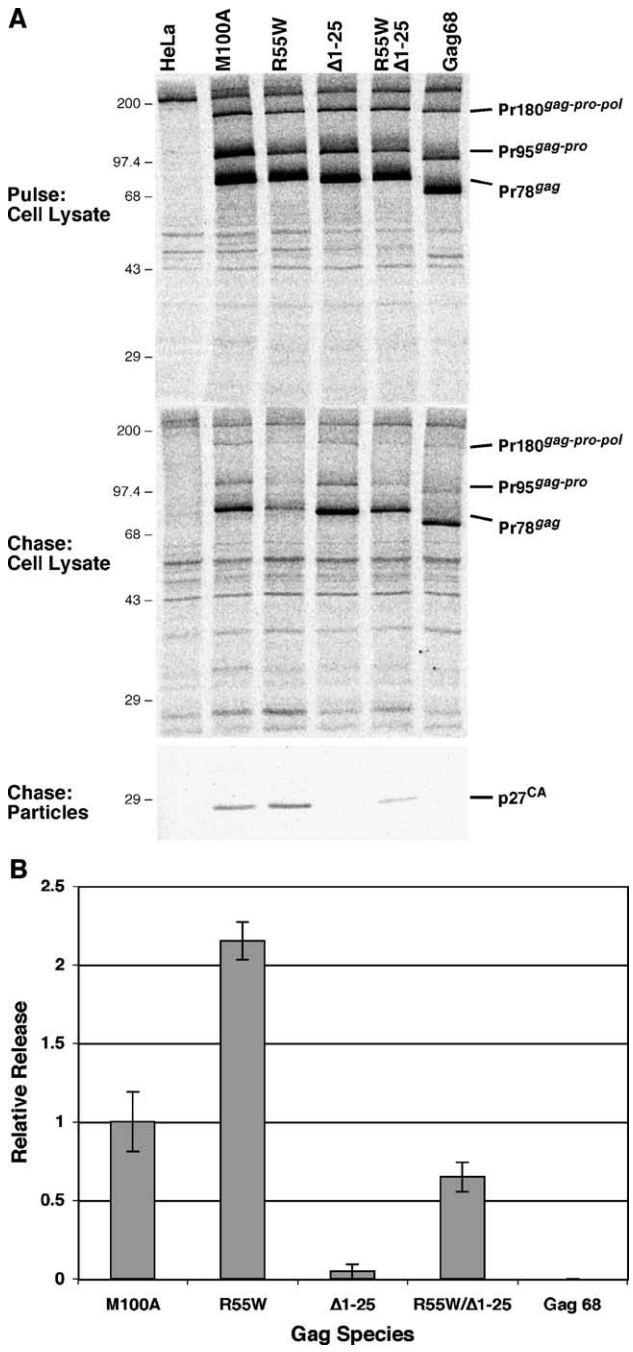

Fig. 3. Pulse-chase analysis of particle production by stably transfected HeLa cell lines expressing M-PMV *gag* species. Cells were labeled for 30 min with [^{35}S]-methionine and then chased overnight. (A, top panel) SDS-12% PAGE of immunoprecipitations of lysed, pulse-labeled cells. (A, center panel) immunoprecipitation of lysed cells after the chase. (A, bottom panel) immunoprecipitation of particles collected after the chase from the cell medium. Labels for each expressed species are given at the top of the gel lanes. HeLa indicates the parental cell line. Pr78^{gag}, Pr95$^{gag-pro}$, and Pr180$^{gag-pro-pol}$ indicate the positions of the Gag, Gag–Pro, and Gag–Pro–Pol precursor polypeptides of M-PMV, respectively. p27^{CA} indicates the position of the proteolytically matured capsid protein. The numbers to the left indicate the position of prestained relative molecular size standards in kilodaltons. (B) Comparative analysis of virus production by quantitation of CA released from cells in a particle form. Each species is shown relative to the M100A variant, which serves as the wild-type control, set to one. Results shown are the average of two experiments with the range of values given by error bars. The Gag68 species failed to produce any measurable CA released as particles.

Hunter, 2000) and also of Δ1–25 (Sommerfelt et al., 1992) showed that these mutants are not significantly less stable than wild-type Gag. To confirm that the various Gags were not destabilized in our analysis, we examined the amount of cell associated Gag remaining after the chase. For each species, the remaining amount was roughly inversely proportional to its relative particle release, with one exception (Fig. 3A, center panel). Consistent with previous findings that deletions in MA destabilize Gag (Rhee and Hunter, 1990b), only approximately 50% of Gag68 remained after the chase even though none was released.

The dual CTRS/ΔISD mutant can assemble at the plasma membrane

To gain a better understanding of the assembly phenotypes of the CTRS and ISD mutations, HeLa cell lines expressing these Gag species were analyzed by thin section electron microscopy. Immature capsid structures were readily identified in cells expressing the M100A M-PMV Gag species (Fig. 4A) with large clusters frequently observed (Fig. 4B). A few immature D-type capsid structures were observed in HeLa cells expressing the CTRS mutant R55W (not shown), but curiously no C-type structures were observed. Previous investigations of this mutant using COS or insect cell overexpression have established its morphogenesis as predominantly C-type (Parker and Hunter, 2000; Rhee and Hunter, 1990a). Our inability to observe such structures compared to past reports is likely due to a lower expression level from the M-PMV promoter than is possible with the overexpression systems used in previous studies. Consistent with the biochemical data (Fig. 3B), for the ISD deletion Δ1–25, only a very few preassembled immature particles were observed in the cytoplasm compared to cells expressing M100A (not shown). Significantly, for the CTRS/ISD double mutation/deletion, several C-type structures were observed (Fig. 4C). The matrix-deleted Gag68 protein also assembled efficiently into immature-like particles. In fact, large clusters of structures were seen (Fig. 4D).

Direct targeting to the plasma membrane rescues particle production for ISD deletions

While the R55W mutation rescues particle production for the ISD mutation using HeLa cells (Fig. 3), the results were somewhat different when analyzed in human osteosarcoma (HOS) cells. In these cells, the R55W substitution and the ISD deletion each contributed to a reduction in particle release, with C-type assembling structures observed in cells expressing both Δ1–25 (Fig. 4E) and R55W/1–25 (Fig. 4F). The different behavior of the mutations in another cell type is not surprising. As was noted in the original description of the mutation, the alteration of transport by R55W is not 100% efficient; some D-type immature particles are still observed in the cytoplasm (Rhee and Hunter, 1990a). Also, in a study of M-PMV particle production from insect cells, wild-type and R55W versions of

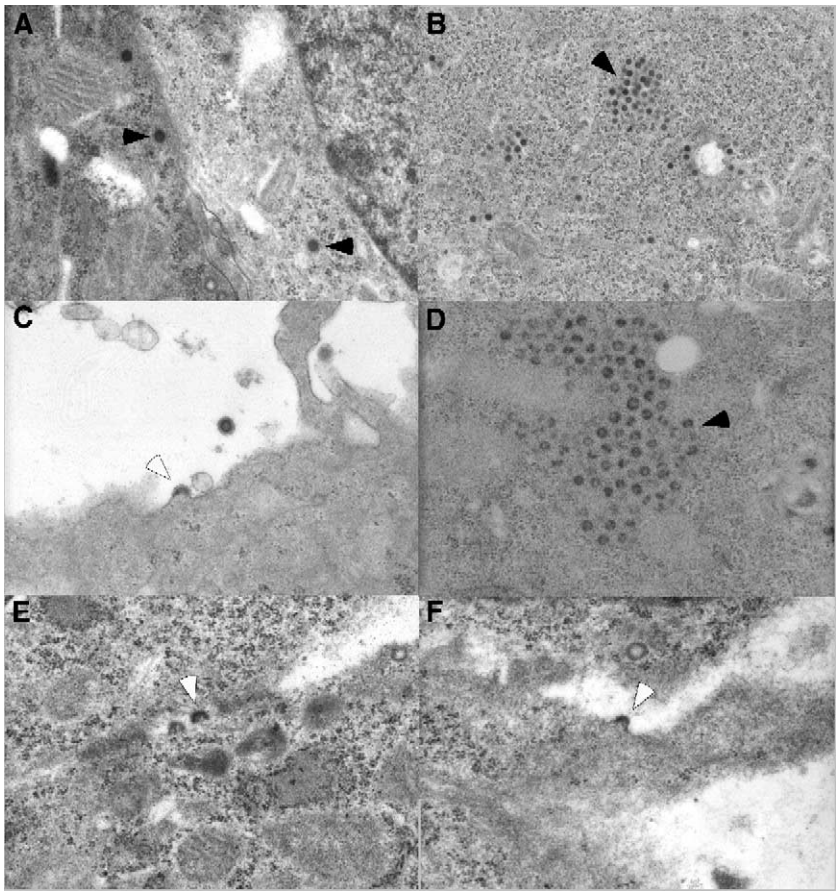

Fig. 4. Representative fields from analysis of M-PMV Gag variants expressed in stably transfected cell lines and examined by thin-section electron microscopy. Solid arrows indicate Gag species that have followed D-type morphogenesis to produce intracytoplasmic immature capsids. The open arrows indicate Gag assembling at the plasma membrane via the C-type morphogenic pathway. (A and B) M100A (wild type) in HeLa cells. (C) R55W/Δ1–25 in HeLa cells. (D) Gag68 in HeLa cells (E) Δ1–25 in HOS cells. (F) R55W/Δ1–25 in HOS cells. All fields are shown at ×20,000 magnification save that in panel B, which is at ×10,000.

M-PMV Gag were found to bud as expected from Sf9 cells, but wild type was specifically impaired in High Five cells (Parker and Hunter, 2000). While R55W could still bud from High Five cells, it was less efficient than from Sf9 cells providing a precedent for cell-type dependent differences in M-PMV mutant particle morphogenesis. Moreover, the R55W mutation inactivates the CTRS, but does not necessarily directly target Gag to the plasma membrane. Whether Gag follows an alternate pathway in the absence of CTRS function or just finds its way to the plasma membrane by default is not yet understood.

To direct the targeting of R55W in HOS cells, we reconstructed our *gag* derivatives to contain the well-characterized src plasma membrane targeting signal. The first 10 codons of the src oncoprotein were inserted into the Gag68 construct thus substituting the entire MA domain in the expressed Gag protein. This short sequence of src is sufficient to direct the myristylation of the nascent polypeptide and target the protein to the plasma membrane (Deichaite et al., 1988; Resh, 1989; Resh and Ling, 1990). Studies of Rous sarcoma virus (RSV) assembly in mammalian cells were greatly facilitated by the appendage of this same signal sequence to the N-terminus of RSV Gag (Wills et al., 1989).

For each variant of Gag analyzed, a myristylation plus and a control myristylation minus version was constructed (see Materials and methods). Stably transfected HOS cell lines were created and pulse-chase analyses were used to measure particle production (Fig. 5). In addition to the Δ1–25 ISD deletion already described, a more extensive Δ8–58 deletion was also analyzed in parallel (Sommerfelt et al., 1992). The M100A and Gag68 versions again served as positive and minus controls for particle production. All the cell lines expressed their respective proteins with both the Gag and Gag–Pro precursors seen in the immunoprecipitations of cell lysates (Fig. 5A). The culture medium from these cells was filtered and virus was collected by centrifugation such that only assembled particles were examined, as before. Again, the ability of each proviral species to produce particles was monitored by the release of matured CA (p27) protein into the tissue culture supernatant. As expected, the M100A cell line produced particles while the Gag68 did not. Also as expected, all the cells expressing the myristylation minus src versions of Gag failed to release particles. In contrast, all the cells expressing the myristylation positive versions released a significant amount of particles; although when quantitated, these amounts were only about 20% of M100A (Fig. 5B). The presence or

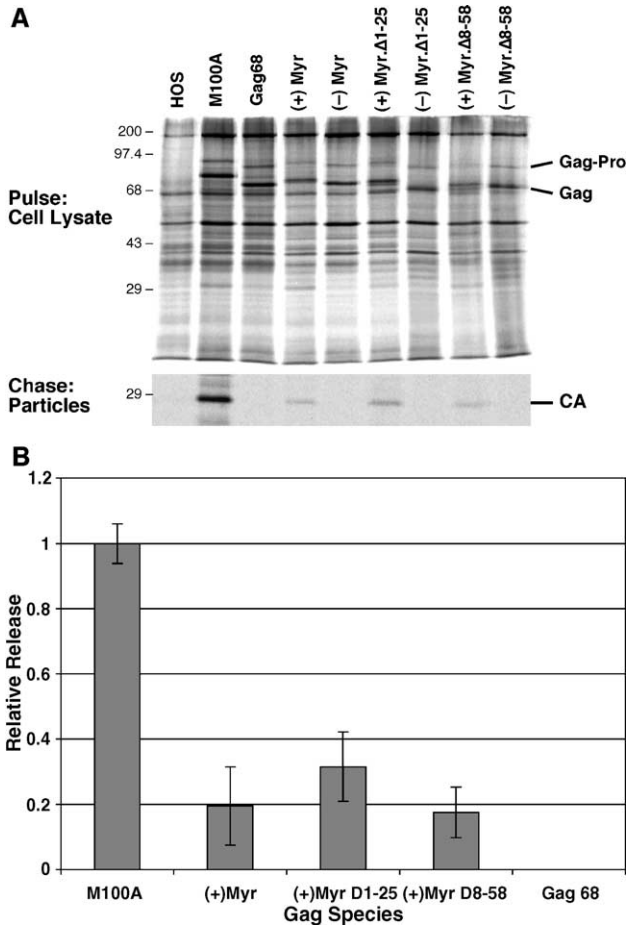

Fig. 5. Pulse-chase analysis of particle production by stably transfected HOS cell lines expressing M-PMV src-directed Gag proteins. Cells were labeled for 1 h with [^{35}S]-methionine and then chased 8 h in complete DMEM. (A, top panel) SDS-12% PAGE of immunoprecipitations of lysed, pulse-labeled cells. (A, bottom panel) immunoprecipitation of particles collected after the chase from the cell medium. Lanes for each expressed species are given at the top of the gel. HOS indicates the parental cell line. The approximate positions of the Gag and Gag–Pro precursor polypeptides are indicated. CA indicates the position of the proteolytically matured capsid protein. (B) Comparative analysis of virus production by quantitation of CA released from cells in a particle form. Each species is shown relative to the M100A variant, which serves as the wild-type control, set to one. Results shown are the average of two experiments with the range of values given by error bars. The Gag68 species and all myristylation minus versions (not shown) failed to produce measurable CA released as particles.

absence of either ISD deletion had no significant effect upon the efficiency of particle production.

Discussion

In previous reports, we have highlighted the role of an internal domain within the M-PMV Gag precursor that functions as a scaffold (ISD) to augment the ability of Gag to multimerize into immature particles (Sakalian and Hunter, 1999; Sommerfelt et al., 1992). We have also further described a domain in MA, termed the cytoplasmic targeting and retention signal (CTRS), that has no effect upon assembly itself (Sakalian and Hunter, 1999), but transports nascent Gag precursors to the pericentriolar region for assembly (Sfakianos et al., 2003). Since a single amino acid substitution R55W can largely disrupt the CTRS and transform the morphogenesis of M-PMV from D-type to C-type, the underlying mechanism of Gag assembly between C- and D-type retroviruses must be fundamentally the same. Therefore, if D-type Gag precursors require an ISD, what provides this function for C-type Gag? We hypothesized that the plasma membrane may play this role since numerous studies have pointed to the critical role membrane interaction plays in the assembly of retroviruses (Bryant and Ratner, 1990; Rein et al., 1986; Spearman et al., 1994; Wills et al., 1991).

To test the idea that the plasma membrane and the ISD play similar roles, we have combined an ISD deletion within M-PMV Gag with two different modifications to direct Gag to the plasma membrane. Use of the R55W mutation resulted in a restitution of particle production in HeLa cells from 5% of the wild-type level for the ISD-deleted form to 65% of that level for the dual CTRS/ΔISD mutant. Directing the ISD-deleted Gag to the plasma membrane restored its ability to assemble efficiently as a C-type virus. In contrast to the results in HeLa cells, in HOS cells the CTRS mutation itself was deleterious and in combination with the ISD deletion was further impaired. Since C-type assembling structures were observed in HOS cells expressing the double mutant, we believe that this cell-type dependent difference is due to variability in cellular transport mechanisms rather than in the ability of ISD-deleted Gag to utilize the plasma membrane.

To more directly examine the ability of the plasma membrane targeting of Gag in HOS cells, we modified Gag by substituting the entire MA domain with the membrane targeting/myristylation sequence of src. The in vitro analysis had already shown the MA domain to be dispensable for assembly. Although all of the src sequence-directed Gags were somewhat impaired versus the wild type, there was no significant difference in particle production between the two variants missing a functional ISD and the one that contained it. In the context of forcing an interaction with the plasma membrane, the presence of the ISD became irrelevant to particle production, consistent with the hypothesis that the plasma membrane can provide a scaffold-like function for the many C-type Gag proteins that are targeted there.

How do the plasma membrane and the ISD both fulfill similar functions? As discussed previously (Sakalian and Hunter, 1999), these elements may serve to concentrate the Gag polyprotein and thereby assist in the process of multimerization that leads to assembly. For both C-type and D-type viruses, these scaffold-like functions are dispensable when Gag is overexpressed in cells (Royer et al., 1991; Sakalian and Hunter, 1999; Sommerfelt et al., 1992) or when Gag or Gag fragments are purified and assembled at high concentration in vitro (Campbell and Rein, 1999; Campbell and Vogt, 1997; Gross et al., 2000; Klikova et al., 1995). For C-type viruses, the plasma membrane may serve to concentrate Gag molecules by simply restricting diffusion to two dimensions. For the D-type virus, the mechanism of ISD function is unknown but may be inferred from the behavior of the matured p12 domain that contains it. p12 possesses an unusual propensity to multimerize

(Knejzlík et al., 2004). Cross-linking experiments with purified soluble recombinant protein identified every possible multimer of p12 on SDS-PAGE, from dimers to irresolvable multimers. Much of this ability to self-associate was attributed to the N-terminal helical portion of p12, which corresponds to the location of ISD function characterized by deletion analysis (Sakalian and Hunter, 1999; Sommerfelt et al., 1992).

In addition to Gag proteins that were targeted to the plasma membrane, we found that a Gag deleted of all targeting signals, Gag68, also efficiently assembled in cells, although these assembled particles were not released. Several studies have shown that the transport signals in MA can be altered without abrogating assembly (Bowzard et al., 2000; Fäcke et al., 1993; Ono et al., 2000). In fact, if the MA domain is replaced with a minimal plasma membrane targeting signal, this region of Gag is dispensable for particle production (Lee and Linial, 1994) and indeed for infectivity (Reil et al., 1998). In addition, studies of M-PMV Gag have shown that while the CA and NC domains are necessary for assembly of particles, the MA domain is not required when Gag is overexpressed in bacteria (Rumlova-Klikova et al., 2000). It is likely that in the absence of transport signals Gag68 simply accumulates at the site of synthesis in the cell, and through the assistance of the ISD, efficiently assembles into particles. The observation of large accumulations of structures by this species is consistent with this scenario.

All D-type viruses possess an ISD, but the other viruses in the genus, at least by sequence analysis, do not appear to contain an analogous leucine zipper-like region in their Gag proteins. However, the mouse mammary tumor virus (MMTV), the prototypical B-type retrovirus, does possess a Gag domain p3 in the position analogous to p12 that contains a sequence of similar charge composition. While the D-type virus contains many glutamic acid residues in its leucine zipper-like region, MMTV contains a similar number of aspartic acids (Moore et al., 1987; Sonigo et al., 1986). Furthermore, in a two-hybrid analysis of Gag–Gag interaction, p3, in combination with the adjacent p8n domains, displayed a modest ability to self-associate, as did the MA-pp21 and the CA regions—a property not shared by the analogous regions of M-PMV (Zábranský et al., 2005). MMTV, therefore, appears to utilize the concerted self-association of multiple Gag domains while M-PMV utilizes the ISD.

If the plasma membrane can substitute for the function of the ISD for a D-type virus, can the converse be true? Can an ISD substitute for the function of the plasma membrane for a C-type virus? Chimeric C-type Gag proteins containing an ISD might assemble efficiently without the need for interaction with the plasma membrane. When a CTRS was inserted into MA of MuLV, D-type morphogenesis did result, but it was inefficient, perhaps because of the absence of an ISD (Choi et al., 1999). Fusing the p12 domain to the HIV Gag precursor enables assembly of the chimera versus wild-type HIV Gag in the in vitro translation system (Sakalian et al., 2002). This suggests that the ISD is a modular domain of the polyprotein whose function can be transferred to the Gag protein of another retrovirus. Can adding a CTRS to such a chimeric Gag result in efficient D-type morphogenesis in cells? These experiments are in progress.

Materials and methods

DNA constructs

Plasmids for expression in vitro were constructed in the pTFCG.M100A vector background (Sakalian and Hunter, 1999). Plasmids pTFCG.R55W.M100A and pTFCG.M100A.Δ1–25 have already been described (Sakalian and Hunter, 1999). Plasmid pTFCG.R55W.M100A.Δ1–25 was constructed by transfer of the *Pac*I to *Bsg*I fragment of pTFCG.M100A.Δ1–25, containing both the M100A and the Δ1–25 mutations, to pTFCG.R55W.M100A. Plasmid pTFCG.Gag68 was constructed by site-directed mutagenesis of plasmid pTFCG to convert the *gag* start codon from that for methionine (ATG) to one for isoleucine (ATT). The result of this mutation is to force translation of *gag* to begin at the second methionine codon located at the end of the MA coding region yielding a protein of approximately 68 kDa in size. Conversion of the ATG to ATT had the added benefit of creating an additional diagnostic *Ssp*I restriction site in the plasmid.

Plasmids for expression in tissue culture cells were constructed in the pSHRM15 vector background (Rhee et al., 1990). Plasmids pSHRM15.M100A, pSHRM15.R55W.M100A, pSHRM15.M100A.Δ1–25, and pSHRM15.R55W.M100A.Δ1–25 were all constructed by transferring the *Pac*I to *Bsg*I fragment from the corresponding pTFCG series plasmid into pSHRM15. The src myristylation sequence-containing plasmids pSHRM15.Gag68.(+)Myr, pSHRM15.Gag68.(−)Myr, pSHRM15.Gag68.(+)Myr.Δ1–25, pSHRM15.Gag68.(−)Myr.Δ1–25 pSHRM15.Gag68.(+)Myr.Δ8–58, and pSHRM15.Gag68.(−)Myr.Δ8–58 were constructed in the pSHRM15.Gag68 and pSHRM15.Gag68.Δ1–25 vector backgrounds. First, the *Dra*III to *Spe*I fragment of pTFCG.68 was transferred to pBluescript-KSII to produce the 3.2 kb plasmid nr-ksvector-1. Primer pairs were designed for insertion of the src sequence into nr-ksvector-1 just upstream of the pp24 coding sequence (Forward- 5′ GTT AAC CCA CAA GTA ATG GGG AGC AGC AAG AGC AAG CCT AAG GAC GCT GCC GTA GCC C 3′; Reverse- 5′ G GGC TAC GGC AGC GTC CTT AGG CTT GCT CTT GCT GCT CCC 3′). For addition of the src sequence without myristylation, similar primer pairs were designed with the initial src glycine codon changed to an alanine (Forward- 5′ GTT AAC CCA CAA GTA ATG GCC AGC AGC AAG AGC AAG CCT AAG GAC GCT GCC GTA GCC C 3′; Reverse- 5′ G GGC TAC GGC AGC GTC CTT AGG CTT GCT CTT GCT GCT GGC CAT TAC TTG TGG GTT AAC 3′). Additional outside primers were designed for the PCR reactions (Forward-5′ GGG TAA AAA ACG TCT AGG CCC CCC G 3′; Reverse-5′ TAC GCA AAC CGC CTC TCC CCG CGC G 3′) for use in combination with the mutagenic primers. PCR was performed by first using pairs of primers (forward mutagenic with outside reverse, reverse mutagenic with outside forward) to produce intermediate products that were purified and then combined in

a second PCR reaction with the outside primer pair to produce a larger fragment containing the mutated sequence. This final PCR product was then cloned into plasmid pCR2.1 (Invitrogen) using the manufacturer's instructions. These intermediate clones were confirmed by sequencing. Fragments including the (+) and (−) myristylation src sequences were then excised from the pCR2.1 vector by digestion with *Pac*I and *Spe*I and inserted into the similarly digested pTFCG.Bam(−), a *Bam*HI to *Bam*HI-deleted version of pTFCG. The *Pac*I to *Sac*I fragment from the resulting (+) and (−) version plasmids was then inserted into the same sites of pSHRM15.Gag68 and pSHRM15.Gag68.Δ1–25.

In vitro analysis of immature capsid assembly

Transcription and translation were performed simultaneously using the TNT Coupled Reticulocyte Lysate System (Promega) in the presence of [^{35}S]-methionine and programmed with the pTFCG series plasmids. Products of these synthesis reactions were analyzed on sucrose gradients. Standard 50 μl reactions were diluted to a total volume of 200 μl with 30% (w/w) sucrose in gradient buffer containing 20 mM Tris (pH 8.0), 100 mM NaCl, 5 mM EDTA, and 0.1% Triton X-100. Diluted samples were then loaded onto 2.2-ml continuous 30–55% (w/w) sucrose gradients. Gradients were centrifuged in a TLS-55 rotor (Beckman Instruments) for 2 h at 55,000 rpm. Approximately 200-μl fractions were taken by hand with a Pipetman (Gilson) from the top of the gradient. The pellet was resuspended in 200 μl of 55% (w/w) sucrose in gradient buffer. Aliquots (5 μl) of each fraction were dissolved in sodium dodecyl sulfate (SDS) sample buffer and then loaded onto an SDS-10% polyacrylamide gel. After electrophoresis, radioactive bands were visualized on a Packard Cyclone Storage Phosphor Screen system.

Pulse-chase analysis of particle production in cultured cells

Tissue culture cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 20 mM L-glutamine, 100 U/ml of penicillin G and 100 μg/ml streptomycin sulfate. Both HeLa and HOS cells were transfected with FuGENE 6 (Roche) reagent according to the manufacturer's instructions. Before transfection, pSHRM15 series plasmids were linearized by digestion with *Fsp*I. Stable cell lines were batch selected and maintained in the presence of 300 U/ml of Hygromycin B.

Cell lines and control cells were pulse labeled in DMEM minus methionine (Specialty Media) supplemented with 50 μCi/ml of [^{35}S]-methionine. The label was chased overnight by replacing the labeling medium with maintenance medium. For analysis of intracellular Gag species, labeled cells were lysed in buffer containing 0.15 M NaCl, 50 mM Tris–HCl (pH 7.5), 1% Triton X-100, and 1% deoxycholate (Lysis Buffer). Cellular debris and nuclei were removed by centrifugation for 1 min at 14,000 rpm in an Eppendorf microfuge (Brinkmann Instruments). The lysate was then adjusted to 0.1% SDS and the M-PMV Gag species were precipitated by the addition of polyclonal anti-Pr78 antiserum (Sakalian et al., 1996) followed by formalin-fixed *Staph. aureus.* For analysis of released virus particles, tissue culture medium was first filtered through a 0.45 μm filter. Particles were then collected by centrifugation through a 20% w/v sucrose cushion at 30,000 rpm for 90 min in a 70.1 Ti rotor (Beckman-Coulter). Pelleted virions were dissolved in Lysis Buffer plus 0.1% SDS and the Gag species immunoprecipitated as for cell lysates. Immunoprecipitates were examined by separation on SDS-10% PAGE gels followed by phosphorimager analysis.

Electron microscopy

For analysis of tissue culture cells, the medium was removed and the plates washed 3× in phosphate buffered saline. Cells were then fixed on the plate for 1 h with a solution of 0.1 M cacodylate (pH 7.4) buffer containing 3% electron microscopy grade (EM grade) glutaraldehyde and 0.8% tannic acid. After three rinses in cacodylate buffer, the cells were postfixed, in the dark, in 0.1 M cacodylate buffer containing 0.1% osmium tetroxide. The samples were again rinsed three times and then dehydrated in a series of 50, 75, 95, and finally 100% ethanol (EM grade) solutions for 5 min each. The 100% ethanol step was repeated two times and the samples were then infiltrated in a 1:1 solution of Polybed 812 resin and 100% ethanol overnight followed by three infiltrations with 100% Polybed resin for 2 h. Embedding was performed overnight in a thin layer of 100% Polybed at 60 °C. The embedded cell layer was pried off the plate and then a small piece was punched out, which was then glued to a prepolymerized block of resin. Ultrathin sections were acquired by using a Rechert-Jung Ultra Cut E ultramicrotome. Sections were poststained for 10 min in a solution of 1% uranyl acetate in 50% ethanol, washed in ~35 °C water, further stained for 5 min in Reynold's lead citrate, and then finally washed again in water. Sections were examined and photographed by using a Hitachi-7000 transmission electron microscope at 75 kV.

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