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A Large Deletion in the Matrix Domain of the Human Immunodeficiency Virus *gag* Gene Redirects Virus Particle Assembly from the Plasma Membrane to the Endoplasmic Reticulum

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Morphogenesis of retroviruses involves assembly of the structural Gag and Gag-Pol polyproteins with subsequent budding of the virus particle from the plasma membrane and proteolytic cleavage by the viral proteinase. The matrix (MA) domain, representing the N-terminal segment of Gag, plays a critical role in this process. We constructed an in-frame deletion in the MA coding region (lacking codons 16 to 99) of the human immunodeficiency virus (HIV) type 1 *gag* gene. Following transient transfection of the complete proviral DNA carrying the deletion, the mutant polyprotein was synthesized and proteolytically processed like the wild-type polyprotein. However, release of virus particles was reduced approximately 10-fold. The extracellular particles that were released did not contain viral glycoproteins and were noninfectious. Electron micrographs revealed budding of virus particles into the endoplasmic reticulum (ER) of transfected cells and large numbers of particles within the ER. These particles were all immature and morphologically indistinguishable from intracisternal A-type particles, a class of murine endogenous retrovirus elements. Budding structures at the plasma membrane were rarely seen and only a few extracellular particles were observed, but in contrast to those in the ER, these particles had the morphology of mature particles, similar to that of wild-type HIV, except for the lack of surface projections.

Morphogenesis of infectious retrovirus particles requires the morphopoetic function of viral core proteins (encoded by the *gag* gene) as well as the incorporation of functional replication enzymes (proteinase [PR], reverse transcriptase [RT], and integrase [IN]; derived from the *pol* gene) and the envelope glycoproteins gp120 (SU) and gp41 (TM; encoded by the *env* gene; for a review of nomenclature of retroviral proteins, see reference 21). The products of the *gag* and *pol* genes are translated as two polyproteins ($Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ for human immunodeficiency virus type 1 [HIV-1]) which are coterminal in their N-terminal segments. Viral assembly is believed to occur by association of uncleaved polyproteins to form the spherical immature core either in the cytoplasm (type B and D oncoviruses and spumaviruses) or at the plasma membrane (type C oncoviruses and lentiviruses [e.g., HIV]) of the infected cell (for reviews, see references 9 and 41). Ultimately, however, either the preformed cores or unassembled polyproteins must migrate to the plasma membrane to initiate the budding and release of the immature virion. The mechanism of membrane targeting is currently not known in any retrovirus system, but N-terminal myristoylation of the Gag and Gag-Pol polyproteins, which is found in most, but not all, retroviruses has been shown to play a role in this process. In their detailed analysis of Mason-Pfizer monkey virus morphogenesis, Rhee and Hunter showed that transport of the structural polyproteins is an active and specific intracellular targeting process and that additional signals, besides N-terminal myristoylation, are required for membrane targeting (34, 35, 37). As a final step in morphogenesis, the condensation of the immature spherical core to the mature core is necessary for the released virion to become infectious and depends on proteolytic processing of the structural polyproteins by viral PR.

The *gag* genes of all replication-competent retroviruses invariably contain the three separate functional domains matrix (MA), capsid (CA), and nucleocapsid (NC), which are always linked in this order but are often interspersed by additional domains of unknown function (for a review, see reference 41). These domains become proteolytically separated by viral PR before or during maturation of the virion (for a review, see reference 19). For HIV-1, the structural polyprotein of the core ($Pr55^{gag}$) can be divided into MA, CA, NC, and a C-terminal p6 domain (Fig. 1). Cotranslational myristoylation of the N-terminal MA domain, which is closely apposed to the inner surface of the lipid envelope of the virus (10), has been shown to be essential for production of extracellular particles (4, 13). Besides its apparent role in the targeting of viral polyproteins, MA is also implicated in the specific incorporation of viral glycoproteins into virions (8, 44). The CA protein (p24) forms the core shell of the mature virus particle, which encases a ribonucleoprotein complex consisting of the dimeric viral RNA genome associated with the NC protein.

While the production of all infectious retroviruses requires the release of virus particles from the plasma membrane of the cell, there is a peculiar class of defective endogenous retroviruses of rodents which appear to have no extracellular phase. These so-called intracisternal A-type particles (IAP), which exhibit exclusively immature (A-type) morphology, assemble at the membranes of the endoplasmic reticulum (ER) and bud into the ER cisternae, where they remain as enveloped spherical particles consisting of unprocessed Gag

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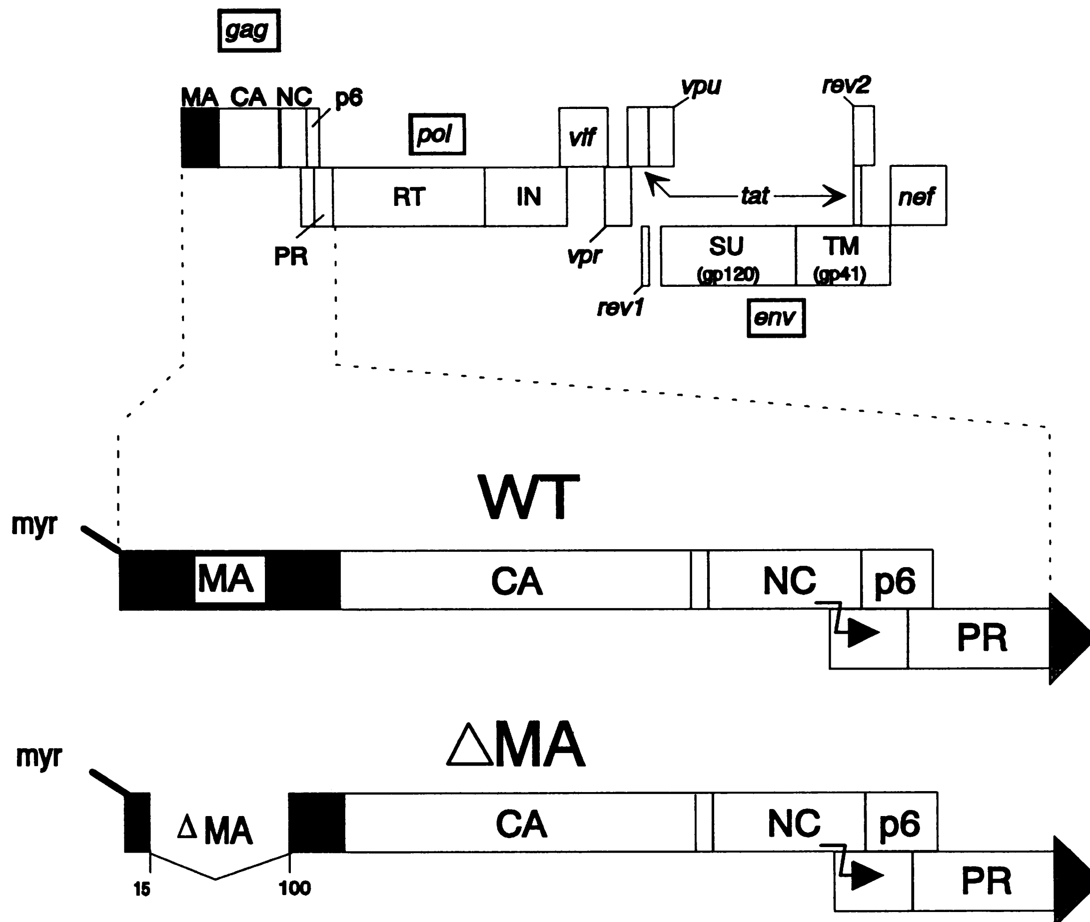


FIG. 1. Schematic representation of the ΔMA deletion. At the top of the figure, the coding region of the HIV-1 genome is shown, with the different open reading frames depicted as open boxes. The N-terminal matrix (MA) domain of the *gag* reading frame is indicated as a black box. In the lower part of the figure, the *gag* region is enlarged and the signal for N-terminal myristoylation and for translational frameshifting giving rise to a Gag-Pol polyprotein are indicated. The ΔMA deletion is depicted at the bottom and the amino acids flanking the deletion are indicated.

and possibly Gag-Pol polyproteins (for a review, see reference 20). IAP are abundant in many murine tumors and derived cell lines but are also found in normal tissues of mice and other rodents. The site of IAP morphogenesis suggests that IAP may be defective in polyprotein targeting. The complete sequences of several IAP elements and partial sequences of the *gag* and *pol* genes of others have been determined recently (7, 24, 26, 33). These reports documented a marked sequence conservation between the different IAP genomes and significant homologies, particularly to type B and D retroviruses which extend through almost the entire *gag-pol* region, with the exception of the N-terminal segment of *gag* corresponding to the MA domain (for a review, see reference 20).

In this report, we demonstrate that a large deletion within the HIV-1 MA domain causes a drastic alteration of particle morphogenesis, leading to immature particles in the ER that closely resemble murine intracisternal A-type particles. Only a few extracellular particles which exhibited mature morphology but lacked viral glycoproteins were observed.

MATERIALS AND METHODS

Cells, transfections, and infections. COS 7 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine. Transfections were performed as described previously (23). Briefly, approximately 5×10^6 cells were suspended in 0.1 ml of phosphate-buffered saline (PBS) and electrotransfected with 20 μg of DNA, using a BioRad gene pulser set at 150 V, 960 μF, and 100 Ω (resistance).

HIV-1 permissive MT-4 cells (16), maintained in RPMI 1640 with the supplements described above, were adjusted to 2×10^5 cells per ml 24 h before infection. Equal amounts of virus particles (normalized for CA antigen), released into the culture medium of transfected COS 7 cells were used to infect the MT-4 cells. Every 2 to 3 days, the cultures were diluted to maintain the cells in rapid growth, and aliquots were removed for analysis by indirect immunofluorescence and enzyme-linked immunosorbent assay (ELISA).

Expression plasmids. A partial deletion of the MA coding region of HIV-1 (strain BH10; 30) was generated in a subviral plasmid by deleting the *Cla*I-to-*Hin*dIII fragment (nucleotides 374 to 629), filling the 5′ protruding ends, and religating the plasmid. This manipulation created an in-frame deletion of 84 codons within the MA domain of HIV-1 *gag* (codons 16 to 99 [Fig. 1]). To introduce the ΔMA deletion into the complete HIV-1 proviral plasmid pNL4-3 (1), a

common *Bss*HII-to-*Sph*I fragment (nucleotides 255 to 987 of BH10) containing the ΔMA deletion was inserted into pNL4-3 to give pNL43-ΔMA. Since the deletion was originally constructed in the BH10 strain of HIV-1, we also constructed a plasmid containing the wild-type BH10 MA sequence in pNL4-3 (pNL43-BH10). Plasmids pNL4-3 and pNL43-BH10 were shown to confer identical phenotypes when analyzed in parallel experiments; only the data obtained with pNL4-3 are shown in this report. The presence of the in-frame deletion was verified in all plasmids by sequence analysis. To normalize for transfection efficiency, the reporter construct pRSV-luciferase (6) was cotransfected in some experiments and luciferase activity was measured in cell extracts.

Radioactive labeling and immunoprecipitation. For labeling of glycoproteins, transfected cells were plated on 6-cm-diameter dishes, washed in PBS at 12 h after transfection, and incubated for an additional 36 h in medium lacking glucose but supplemented with 5 mM fructose and containing 100 μCi of [^{3}H]glucosamine (22 Ci/mmol; Amersham, Braunschweig, Germany) per ml. Labeled cells and particle fractions were lysed in 2% sodium dodecyl sulfate (SDS), diluted to 0.1% SDS in a solution of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, and 1% deoxycholate, and immunoprecipitated with 10 μl of a 1:1 mixture of rabbit anti-gp120 serum and rabbit anti-gp160 serum (kindly provided by V. Bosch). Radiolabeled immune complexes were released by boiling for 2 min in sample buffer and separated on SDS-polyacrylamide gels. Radioactive bands were visualized by fluorography after the gels were soaked in Amplify (Amersham).

Analysis of expression products. For detection of HIV antigens, medium was cleared and appropriate dilutions were analyzed with a commercial ELISA kit (Organon Teknika, Eppelheim, Germany) which detects the cleaved CA protein. Extracellular particles were precipitated from cleared medium by incubation with 7.5% polyethylene glycol 6000 (PEG 6000)–1 M NaCl for 60 min at 0°C and centrifuged as previously described (23). Alternatively, cleared medium was centrifuged through a cushion of 20% sucrose at 120,000 × *g* for 2 h at 4°C.

For protein analysis, cell or particle extracts were separated on SDS-polyacrylamide gels containing 17.5% polyacrylamide (200:1 ratio of acrylamide/*N,N*-methylenebisacrylamide) for detection of *gag* gene products and 8% polyacrylamide (40:1 ratio) for detection of *env* gene products. For immunoblot analysis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) by electroblotting and reacted with polyclonal antiserum against HIV-1 CA protein (dilution, 1:500) and alkaline phosphatase-conjugated second antiserum (Jackson Immunochemicals Inc.).

For indirect immunofluorescence, infected MT-4 cells were collected by low-speed centrifugation, air dried on glass slides, and fixed with acetone-methanol (1:1) at −20°C for 10 min. Fixed cells were incubated with serum from an HIV-1-infected person (dilution 1:200; kindly provided by M. Pawlita) and with fluorescein isothiocyanate-labeled anti-human immunoglobulin G (1:100; Dianova, Hamburg, Germany).

Electron microscopy. Transfected cells were grown as monolayers on overhead projection transparency sheets which had been washed with acetone and sterilized with ethanol. At 48 h after transfection, cells were fixed with 2.5% glutaraldehyde in 50 mM sodium cacodylate (pH 7.2) for 30 min at room temperature and were embedded in 1% low-melting-point agarose in 50 mM sodium cacodylate (pH 7.2). The remaining steps were performed essentially as described by McDonald (22), except that the buffer employed was 50 mM sodium cacodylate (pH 7.2), the cells were treated with 1% OsO_4 plus 0.8% $K_3Fe(CN)_6$ for 30 min at room temperature, and the preparations were dehydrated in a graded ethanol series. The cells were embedded in Epon by inverting filled BEEM capsules (Plano, Marburg, Germany) over the fixed and dehydrated monolayers which were still attached to the plastic sheets. Following polymerization at 60°C, ultrathin sections were cut with a LKB ultramicrotome and were poststained with uranyl acetate and lead citrate. Sections were viewed and photographed on a Zeiss EM 902 electron microscope.

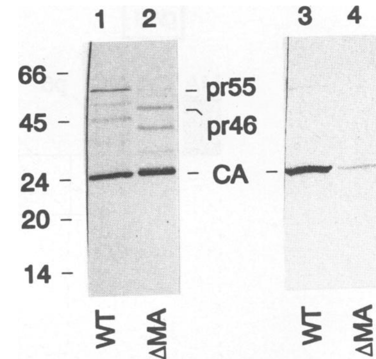


FIG. 2. Western blot analysis of *gag* gene products after transient transfection. COS 7 cells transfected with pNL4-3 (WT) (lanes 1 and 3) or pNL43-ΔMA (ΔMA) (lanes 2 and 4) and media were harvested 48 h after transfection. Lysates of transfected cells (lanes 1 and 2) and viral particles precipitated with PEG 6000 (lanes 3 and 4) were resolved by SDS-polyacrylamide gel electrophoresis, and Western blots were reacted with rabbit polyclonal antiserum against CA. Note that 2.5 times more of the total protein recovered after transfection was loaded in the particle fractions compared with the cell lysates. Molecular mass standards (in kilodaltons) are indicated on the left, HIV-specific precursor proteins and cleaved products are indicated between the panels. The mutant Gag polyprotein is designated Pr46.

RESULTS

Construction of MA deletion mutant and analysis of protein expression and particle release. To analyze the importance of the N-terminal MA domain of the HIV-1 Gag and Gag-Pol polyproteins for protein transport, particle assembly, and release, we created an in-frame deletion mutant removing 84 of the 132 codons of the MA domain (Fig. 1) (pNL43-ΔMA). The resulting polyproteins should retain the N-terminal 15 amino acids and the C-terminal 33 amino acids of MA.

Expression of HIV proteins was analyzed 48 h after transfection of COS 7 cells with plasmids pNL4-3 and pNL43-ΔMA. Western blot (immunoblot) analysis of transfected cells using an antiserum against CA showed a similar but slightly higher amount (ca. 1.5-fold) of immunoreactive material in mutant-virus-transfected cells (Fig. 2, lanes 1 and 2). Expression of pNL4-3 (WT) yielded mostly processed CA protein with small amounts of the Gag polyprotein remaining ($Pr55^{gag}$) and an intermediate containing the MA and CA domains (MA-CA; also reactive with antiserum against MA; 23). For the MA deletion mutant (ΔMA), a truncated Gag polyprotein (Pr46; corresponding to 415 amino acids) and a truncated ΔMA-CA intermediate were

observed, in addition to completely processed CA (Fig. 2, lane 2). Similar results were obtained following immunoprecipitation of radioactively labeled transfected cells (data not shown), indicating that the large deletion within MA had no major effect on protein stability or processing by HIV PR. Immunoprecipitation with a monoclonal antibody against the C terminus of MA (whose epitope should be present in ΔMA; 25) also yielded the truncated Pr46 polyprotein and the ΔMA-CA intermediate but only minute quantities of a protein of approximately 6 kDa, which is the expected size for the completely cleaved mutant MA protein (data not shown). It appears likely, therefore, that the ΔMA protein is rapidly degraded after cleavage from the polyprotein. Labeling of transfected COS 7 cells with [^{3}H]myristate revealed that both the wild-type and mutant polyproteins, as well as their MA-CA intermediates, were myristoylated (data not shown).

To analyze the production of virus particles, culture media were harvested 48 h after transfection and extracellular particles were collected by PEG 6000 precipitation or sedimentation through a sucrose cushion. Western blot analysis of PEG 6000 precipitates using antiserum against CA indicated that considerably more particulate antigen was released from wild-type-virus-transfected cells than from mutant-virus-transfected cells (Fig. 2, lanes 3 and 4), although slightly less intracellular antigen was found in this case (Fig. 2, lanes 1 and 2). Both particulate fractions contained almost exclusively processed proteins (Fig. 2). To obtain a quantitative estimate for the antigen concentrations in the particulate fractions, we used a CA-specific ELISA and normalized for transfection efficiency by using a reporter plasmid. In four independent experiments, using either PEG 6000 precipitation or sedimentation through a sucrose cushion, particle fractions from ΔMA-transfected cells were shown to contain 10- to 20-fold less antigen than those from wild-type-virus-transfected cells. An estimation of the total amounts of Gag-derived proteins (both intracellular and particulate) indicated that slightly less total antigen (approximately 80% of wild type) was recovered for the MA deletion mutant. As for wild-type HIV, particle release was abolished when an additional mutation of the myristoylation signal was introduced into the ΔMA mutant (data not shown).

Effect of ΔMA deletion on glycoprotein incorporation and viral infectivity. Since MA is closely apposed to the lipid envelope in the mature virion (10) and has been shown to be cross-linked to the TM glycoprotein in another retrovirus (Rous sarcoma virus [8]), it is possible that the mutant virus may be defective in glycoprotein incorporation into virus particles. To test this hypothesis, transfected COS 7 cells were labeled with [^{3}H]glucosamine and cell extracts (Fig. 3, lanes 1 to 4) and particle fractions (normalized for equal amount of CA antigen) (Fig. 3, lanes 5 and 6) were immunoprecipitated with a mixture of antisera against gp120 and gp160. Incorporation of HIV glycoproteins was readily detected in wild-type virus but not in the ΔMA mutant virus (Fig. 3, lanes 6 and 5), even after prolonged periods of exposure. Similar results were obtained by Western blot analysis of particle fractions (data not shown). Since the intracellular accumulation and processing of *env* gene products were very similar in wild-type- and mutant-virus-transfected cells (Fig. 3, compare lanes 4 and 2), it is likely that the MA deletion caused a defect in glycoprotein incorporation into virus particles or resulted in the rapid loss of glycoprotein spikes.

To assay for viral infectivity, permissive MT-4 cells (16) were infected with equal amounts of wild-type and mutant viruses (normalized for CA antigen), and the course of the infections was followed by determination of the amount of newly synthesized HIV-1 antigen released into the medium at different time points as well as by indirect immunofluorescence. Infection with wild-type virus resulted in very rapid infection kinetics with the culture being completely infected at 6 days postinfection (Fig. 4). In contrast, virus released from ΔMA-transfected cells was completely noninfectious (Fig. 4), even after prolonged periods of incubation with the CD4$^+$ cell line. This lack of infection may be explained by the defect in glycoprotein incorporation into virus particles, but there may also be additional reasons.

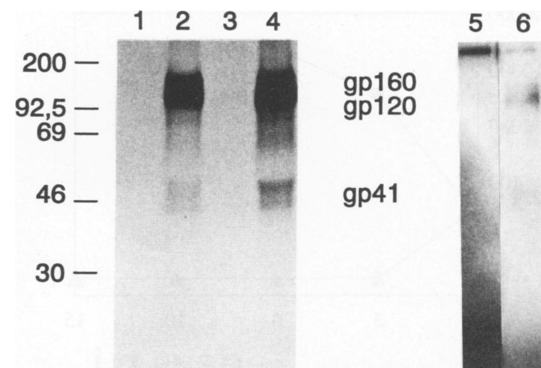
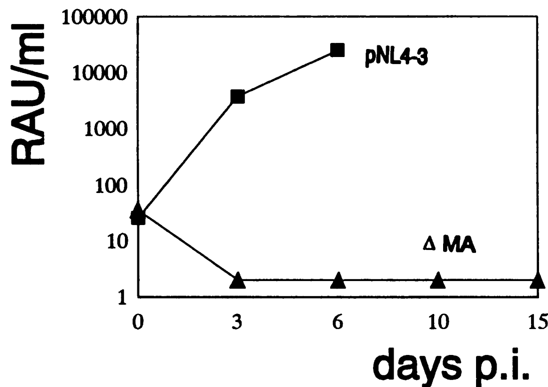


FIG. 3. Immunoprecipitation of radiolabeled HIV-1 *env* gene products. COS 7 cells transfected with pNL4-3 (WT) or pNL43-ΔMA (ΔMA) were labeled with [^{3}H]glucosamine from 12 to 48 h after transfection. Cells (lanes 1 to 4) and PEG 6000 precipitates (lanes 5 and 6) of cleared media were immunoprecipitated with preimmune serum (lanes 1 and 3) or with a mixture of rabbit anti-gp120 serum and rabbit anti-gp160 serum (lanes 2 and 4 to 6). PEG 6000 precipitates were adjusted to contain equal amounts of CA antigen before immunoprecipitation. Radiolabeled immune complexes were separated on SDS-polyacrylamide gels, and radioactive bands were visualized by fluorography. Lanes 1 and 2 and lane 5 correspond to ΔMA-transfected cells and PEG 6000 precipitates, respectively. Lanes 3 and 4 and lane 6 correspond to WT-transfected cells and PEG 6000 precipitates. Molecular mass standards (in kilodaltons) are indicated on the left, HIV-specific glycoproteins are indicated between the two gels.

Electron microscopic analysis of wild-type- and mutant-virus-transfected cells. The decrease in extracellular particle production with the ΔMA mutant might be due to a defect in intracellular transport or in particle assembly, budding, or release. To address this question directly, we performed electron microscopy analysis of COS 7 cells transfected with pNL4-3 and pNL43-ΔMA. In cells transfected with the wild-type provirus, production of virus particles was found at the plasma membrane (Fig. 5A). Budding particles had a crescent-shaped layer of electron-dense material adjacent to the plasma membrane, typical of lentivirus morphogenesis (9). Extracellular particles contained the typical electron-dense core structures of mature viruses, which were cone-shaped or round, depending on the plane of sectioning (Fig. 5A and B). Immature particles were observed very rarely. No virus particles were observed within the cytoplasm, in the ER, or in cytoplasmic vesicles.

A very different distribution and type of particles was found in cells transfected with pNL43-ΔMA. The great majority of virus particles was observed within the cisternae of the ER (Fig. 5C). Budding structures at the plasma membrane (Fig. 5F) or extracellular particles (Fig. 5C [arrow] and Fig. 5G and H) were seen only rarely. The particles



	3d	6d	10d	15d
pNL4-3	>20%	>95%	n.d.	n.d.
Δ MA	0%	0%	0%	0%

FIG. 4. Infectivity of wild-type (pNL4-3) and mutant (ΔMA) virus. MT-4 cells were infected with equal amounts of virus particles. At the times indicated, cleared media were analyzed for newly synthesized Gag antigen by ELISA (upper panel) and cells were subjected to indirect immunofluorescence (lower panel). Antigen concentrations are given as relative absorbance units (RAU) per ml, an arbitrary unit derived from the absorbance readings, with 1,000 RAU/ml approximately equivalent to a CA protein concentration of 100 ng/ml. Abbreviations: p.i., postinfection; d, days, n.d., not done.

enclosed within the ER showed the typical morphology of immature retroviruses; a broad electron-dense region was seen to underlie the viral membrane and the central region remained electron lucent (Fig. 5E). All intracisternal particles contained a lipid envelope (Fig. 5E), indicating that they had budded into the lumen of the ER. Occasionally, typical retrovirus budding structures were seen at the ER membrane. In appropriate sections, it could be seen that the electron-dense ring structure was not always completely closed but showed a small gap (Fig. 5E). Depending on the plane of sectioning relative to the ER, one or more immature particles could be found in close proximity to each other, giving the impression of a dense packing within the ER (Fig. 5C). Virus particles were found mostly in sections of smooth ER or in sections locally devoid of ribosomes (Fig. 5D). An occasional particle was observed between the nuclear membranes (Fig. 5D), but none were found within the nucleus or other subcellular organelles. There was no indication of vesicular transport of viral particles, and no exocytotic release at the plasma membrane was seen. Aggregates of viral particles were observed, however, in large cytoplasmic vacuoles of unknown origin (data not shown), which were similar to the Golgi-derived vesicles seen in persistently HIV-1-infected cells (14) and to the virus-containing vesicles found in the case of a *vpu* mutant of HIV-1 (18). These particles were mostly aberrant in morphology with condensed core structures of various shapes and sizes but almost never typical of mature lentivirus cores.

In contrast to the intracisternal particles which had exclusively immature morphology, the few extracellular virions released from pNL43-ΔMA-transfected cells all exhibited the typical morphology of mature HIV (compare Fig. 5C to E and Fig. 5G and H). Similar to the wild-type virus (Fig. 5B), mutant virus cores were either cone-shaped (Fig. 5H) or round (Fig. 5G), depending on the plane of sectioning. In fact, extracellular mutant virions were morphologically almost indistinguishable from wild-type HIV, except for the lack of glycoprotein spikes which could be easily detected in wild-type particles (compare Fig. 5G and H and Fig. 5B).

DISCUSSION

Assembly of infectious retrovirus particles requires the migration of the structural proteins of the virion to a region within the cell where they interact with each other and with the genomic RNA to assemble an immature viral particle which is released by budding and converted to the mature virion (Fig. 6). The viral Env proteins are translated on membrane-bound ribosomes and are processed and transported via the secretory pathway (Fig. 6). Synthesis of the Gag and Gag-Pol polyproteins occurs on cytosolic polysomes and the mechanisms governing their transport to the plasma membrane are poorly understood. In contrast to other enveloped viruses, formation of noninfectious retrovirus-like particles does not require viral glycoproteins but depends solely on the expression of Gag proteins (for a review, see reference 41). The synthesis and transport of polyproteins, rather than individual components, can be viewed as a simple and efficient way of ensuring that several components of the virion are localized to the assembly site with a single targeting signal. Evidence for the presence of such a signal has been reported for the MA domain of several retrovirus Gag proteins and mutations within MA have been shown to arrest viral morphogenesis at defined stages (35, 37; for a review, see reference 41).

To analyze the contribution of the HIV-1 MA domain toward polyprotein targeting and particle assembly, we constructed a deletion mutant removing 84 of the 132 codons of MA. Since N-terminal myristoylation of the Gag polyprotein is essential for HIV-1 morphogenesis (4, 13), it was important to retain the signal for N-terminal myristoylation. For the Src protein, this signal is specified by the N-terminal amino acids (5) and, as expected, the presence of 15 authentic amino acids at the N terminus of the truncated polyprotein encoded by pNL43-ΔMA resulted in its efficient myristoylation. In addition, this deletion should not affect the MA-CA cleavage site, since the 33 C-terminal amino acids are retained in the mutant polyprotein. Interestingly, Papsidero et al. (28) reported that a monoclonal antibody against HIV-1 MA recognized two peptides corresponding to amino acids 12 to 19 and 100 to 105 of MA. These results indicate that amino acids flanking the deletion (amino acids 15 and 100) may be in close spatial proximity in the wild-type protein, which may be important for the observed stability of the protein and its capacity to assemble into particles. In contrast, expression of deletion mutants in the MA region of Mason-Pfizer monkey virus (7 to 84 amino acids long) yielded unstable Gag proteins which were defective in particle assembly and release (36). Similar results were reported by Bonefeld Jørgensen and coworkers (2) who constructed overlapping deletion mutants within the MA region of the Akv murine leukemia virus. All deletions affecting the N-terminal 100 amino acids of Gag yielded unstable proteins which were defective in particle assembly. A different phenotype was observed by Yu et al. (43, 44),

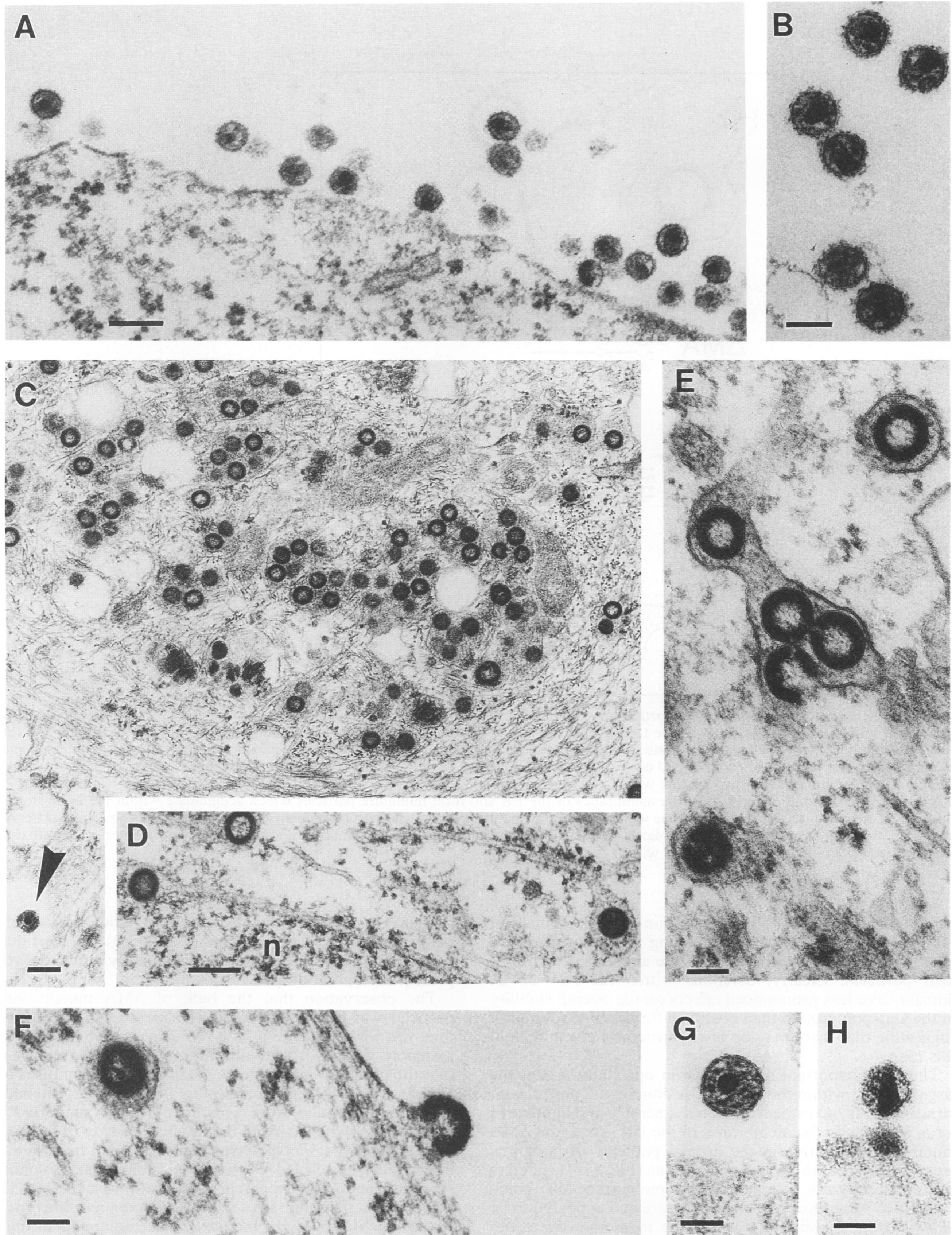


FIG. 5. Thin-section electron microscopy of COS 7 cells transfected with pNL4-3 (A and B) or pNL43-ΔMA (C to H) at 48 h after transfection. Panels A and B show large arrays of extracellular HIV-1 particles with the typical morphology of mature lentiviruses. No intracellular particles were observed, and immature particles were found very rarely. Panels C to F show intracellular immature particles in ΔMA-transfected cells, and panels G and H show extracellular mature particles. The arrowhead in panel C indicates a single extracellular mature virion. In panel D, the nucleus (n) is identified. Bars, 200 nm (panels A, C, and D) and 100 nm (panels B, E, F, G, and H).

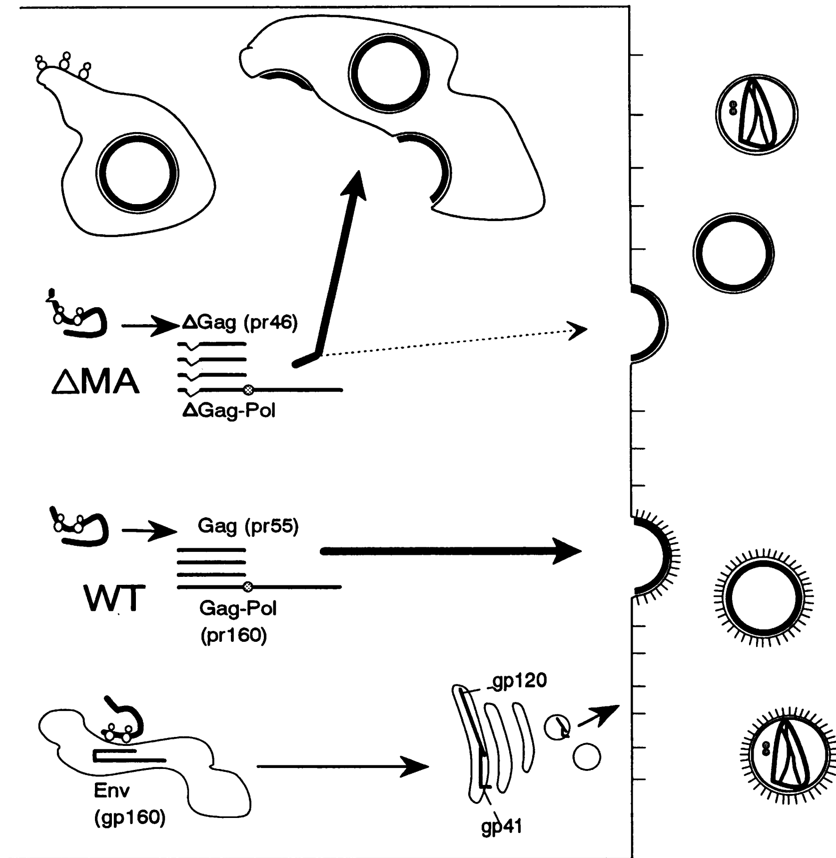


FIG. 6. Schematic representation of particle morphogenesis for wild-type (WT) and MA deletion mutant (ΔMA). Translation of the genomic length wild-type mRNA gives rise to Gag and Gag-Pol polyproteins that assemble and bud from the plasma membrane and form spherical immature virions which incorporate viral glycoproteins. Extracellular particles undergo rapid morphological conversion, and almost all particles exhibit the typical cone-shaped core of mature lentiviruses. The PR domain is shown as a circle on the Gag-Pol polyprotein, and the PR dimer is indicated as two circles within the mature particle. Translation of the ΔMA mRNA yields mutant Gag and Gag-Pol polyproteins that assemble and bud at the membranes of the ER and form immature particles which accumulate within the ER cisternae. Budding structures at the plasma membrane which give rise to mature extracellular particles lacking glycoprotein spikes are rarely observed. Viral glycoproteins are synthesized from spliced mRNA and are glycosylated, cleaved, and transported to the plasma membrane in both cases but are specifically incorporated only into wild-type particles.

who constructed serial deletions within the MA domain of HIV-1 and showed that the resulting Gag proteins were mostly stable and gave rise to variable amounts of extracellular particles. Clearly, deletions within the HIV-1 MA domain have less pronounced effects on the overall stability of the Gag polyprotein than observed for other retroviruses, suggesting that there may be fewer structural constraints in this case.

The most important observation in our study is that the large deletion within the HIV-1 MA domain did not prevent viral assembly but rather redirected assembly and budding of virus particles to the membranes of the ER. Thus, the ΔMA mutant was defective in the normal pathway of lentivirus morphogenesis at the plasma membrane (Fig. 6). This defect was apparently not complete, and some extracellular particles which were morphologically mature and appeared to be derived from budding at the plasma membrane were observed (Fig. 6). Despite the MA deletion, all extracellular particles appeared completely normal in core structure and were almost indistinguishable from wild-type HIV-1 but exhibited defective glycoprotein incorporation. These results indicate that the MA domain is required for the recruitment of viral Env proteins into virions and are in agreement with recent experiments showing that short deletions in HIV-1 MA abolished or significantly reduced glycoprotein incorporation (44).

The observation that the bulk of ΔMA mutant virus particles was found within the cisternae of the ER suggests that the viral structural polyproteins are competent for assembly whenever they are sufficiently concentrated and that the different morphogenic pathways of retroviruses are largely determined by the targeting of their Gag proteins to specific intracellular sites. This hypothesis is supported by experiments which showed that overexpression of unmyristoylated HIV Gag polyproteins in recombinant baculovirus-infected insect cells led to intracytoplasmic capsid assembly, independent of any apparent membrane association (11). Moreover, a point mutation in the MA domain of the type D oncovirus Mason-Pfizer monkey virus redirected its morphogenic pathway to that of a type C virus (35). Considering these results, it may be speculated that grafting a heterologous MA domain onto any retrovirus polyprotein, if structurally tolerated, should convert its pathway of morphogenesis to that of the donor virus.

Comparing the phenotype observed for pNL43-ΔMA-derived particles with those of other retroviruses, it is evident that the intracellular particles closely resemble murine IAP. IAP assemble and bud at the membranes of the ER and remain as immature particles within the ER cisternae (for a review, see reference 20). Sequence analysis of a number of IAP genomes revealed considerable homologies with each other and with other retroviruses but showed a conspicuous absence of homology in the N-terminal region of Gag, upstream of the MA-CA cleavage site (7, 24, 26, 33). Thus, IAP elements do not appear to contain a typical retroviral MA protein but may have acquired an unrelated N-terminal domain. It appears likely that the morphogenic pathway of IAPs is also determined by their N-terminal Gag domain and the ΔMA deletion may redirect mutant HIV particle assembly to a similar pathway. Assembly at the ER membrane may depend on specific targeting to the ER or may occur by random association of hydrophobic protein domains with intracellular membranes leading to a concentration at the most abundant membrane, namely, the ER. It has been suggested that the Gag polyprotein of the murine IAP element MIA14 is targeted to the ER by a N-terminal stretch of hydrophobic amino acids which may function as a signal sequence (20, 24). Other IAP elements, however, do not contain this hydrophobic N-terminal sequence, but similar to HIV, contain a consensus signal for N-terminal myristoylation (26, 33) which may have a similar function.

IAP contain only uncleaved polyproteins and always retain their immature morphology, which has led to the suggestion that they may be defective in viral PR function (for a review, see reference 20). Interestingly, all extracellular particles derived from pNL43-ΔMA showed mature-particle morphology, whereas the intracisternal particles remained exclusively immature, although they may contain at least partially cleaved core proteins. Clearly, viral PR is not defective in this case and it appears likely that particles in both compartments contain similar amounts of viral proteins, including PR. Therefore, additional factors, besides the presence of a wild-type PR sequence and the assembly and budding of virions, may be required to trigger maturation. Possible factors include the respective membrane acquired during envelopment of the viral core and the environment the particles are released into. If factors required for maturation are indeed absent in intracisternal particles, one can speculate that targeting of the IAP polyproteins to the plasma membrane by a heterologous signal may be sufficient to induce the release of mature virions. Experiments to address this question are currently under way in our laboratory.

Intracellular transport of proteins from their site of synthesis to their site of function is an important and highly regulated process. *cis*-acting signals that define the intracellular localization of a protein have been identified for proteins destined for the nucleus, mitochondria, the ER and other organelles (for reviews, see references 39 and 40). In each case, transport is facilitated by the interaction with specific cytosolic or membrane-associated signal receptors and subsequent transfer to the respective translocation apparatus (for a recent review see reference 12). Much less is known about the intracellular trafficking of cytoplasmic proteins, although it is likely to be a similarly complex process. Studies with the product of the *src* oncogene have shown that the myristoylated Src protein interacts with a specific receptor on the cytoplasmic face of the plasma membrane (32) and that this interaction is dependent on both protein acylation and N-terminal peptide sequence (31). The N-terminal peptide of Src can also act as a dominant targeting signal when grafted onto a heterologous protein (29) and can complement MA deletion mutants of Rous sarcoma virus which are defective in assembly (42). The acylated N-terminal sequence of the HIV Gag protein does not appear to contain a dominant targeting signal, since the ΔMA mutant polyproteins retain the authentic N-terminal sequence but exhibit defective assembly at the plasma membrane. This phenotype argues for the presence of a targeting signal in the internal part of MA, which is deleted in the mutant. Such a signal may function by direct interaction with a plasma membrane-associated Gag receptor, analogous to the Src receptor, or may permit association with a cytosolic or membrane-associated signal receptor with subsequent transport to the plasma membrane, similar to the described organelle targeting systems. The intracellular transport of the wild-type Gag polyprotein may also involve transient interaction with the cytoplasmic face of the ER membrane. Vesicular transport of retrovirus Gag proteins on the cytoplasmic side of the membrane has been suggested from immunofluorescence analyses (15, 17). Support for this hypothesis may be derived from experiments showing that viral glycoproteins, although not required for particle formation, can exert a dominant influence on the choice of budding site (27). In addition, monensin and brefeldin, two agents that interfere with the secretory pathway, have been shown to induce enhanced intracellular particle assembly with viruses budding into intracellular vesicles (3, 38). If targeting does involve transient association of the polyprotein with the ER, the phenotype of the ΔMA mutant may also be explained by an arrest of polyprotein transport at a specific stage of the normal pathway, leading to protein accumulation and budding at the ER membrane. Clearly, however, these experiments provide only circumstantial evidence and alternative explanations are also possible. Regardless of the precise path taken to the plasma membrane, the phenotype of the ΔMA deletion has allowed identification of a putative viral targeting domain which is likely to interact with a cellular signal receptor and may, therefore, be useful for the identification of a novel cellular targeting pathway.

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