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Mutations in the N-Terminal Region of Human Immunodeficiency Virus Type 1 Matrix Protein Block Intracellular Transport of the Gag Precursor

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The matrix domain of human immunodeficiency virus type 1 Gag polyprotein was studied for its role in virus assembly. Deletion and substitution mutations caused a dramatic reduction in virus production. Mutant Gag polyproteins were myristoylated and had a high affinity for membrane association. Immunofluorescence staining revealed a large accumulation of mutant Gag precursors in the cytoplasm, while wild-type Gag proteins were primarily associated with the cell surface membrane. These results suggest a defect in intracellular transport of the mutant Gag precursors. Thus, in addition to myristoylation, the N-terminal region of the matrix domain is involved in determining Gag protein transport to the plasma membrane. Wild-type Gag polyproteins interacted with and efficiently packaged mutant Gag into virions. This finding is consistent with the hypothesis that intermolecular interaction of Gag polyproteins might occur in the cytoplasm prior to being transported to the assembly site on the plasma membrane.

The Gag protein of human immunodeficiency virus type 1 (HIV-1), like those of other retroviruses, is initially synthesized as a polyprotein precursor. Gag precursors assemble at the cytoplasmic face of the plasma membrane (8). No other virion components are required for HIV-1 capsid formation (10, 19, 22, 40, 41). Concomitant with or soon after HIV-1 virions bud from infected cells, the virions undergo morphological maturation. The Gag precursor is then cleaved into smaller polypeptides by a virally encoded protease (5, 23). Mature Gag products include the matrix (MA) protein (p17), capsid (CA) protein (p24), nucleocapsid (NC) protein (P9), and a proline-rich protein (p6) (17, 18, 24, 44).

Capsid proteins form the conical core structure characteristic of HIV (9). Domains within the capsid protein are critical for Gag polyprotein interaction and retrovirus particle formation (15, 39, 48). Moreover, HIV-1 p24 can self-assemble in vitro (4). p24 is phosphorylated and constitutes a major antigenic determinant (43). HIV-1 NC protein contains two Cys-His boxes which are conserved among all retroviruses. Mutations which disrupt the Cys-His motif appeared to impair packaging of the viral RNA genome into mature virions (1). Recent evidence suggests that Cys-His boxes of murine leukemic virus NC proteins are required for NC protein binding to RNA, an interaction important for complete virion maturation, protection of the encapsidated RNA from degradation, and subsequent infectivity (2). Mutations in the p6 domain do not affect virus assembly, but they were reported to block the release of assembled particles from the cell surface (12).

Matrix proteins form a shell underneath the envelope in the mature virion. The HIV-1 MA protein is important for incorporation of viral envelope protein (50). It is widely believed that the MA protein provides a targeting signal for Gag polyprotein transport to the cell surface membrane. In most retroviruses, the MA protein is cotranslationally modified by N-terminal myristoylation (37). Mutations that block this modification completely inhibit HIV-1 and type C retrovirus assembly at the plasma membrane (3, 13, 32, 46). In type D viruses, capsid assembly can occur in the absence of myristoylation, but the assembled capsids are not transported to the cell surface (33). It appears that myristoylation is required for high-affinity association of Gag polyprotein with the cellular membrane (3) and is important for intracellular transport of the Gag polyprotein (15). Myristoylation-deficient Moloney murine leukemia virus as well as spleen necrosis virus Gag polyproteins are excluded from wild-type Gag particles (38, 46).

Myristoylation modification alone may not be sufficient for Gag polyprotein transport to the plasma membrane since not all myristoylated proteins are associated with the plasma membrane. They can be located in the nucleus, cytoplasm, or other membrane locations (37). Several retroviruses encode fully functional Gag polyproteins that are not myristoylated (37). Myristoylation-deficient Gag-Pol polyproteins of HIV-1 were reported to be packaged by Gag particles (26, 42). Thus, additional determinants in the matrix domain may also be involved in Gag polyprotein transport.

Signals for protein targeting to the plasma membrane can be provided by a polybasic domain (14). A similar polybasic region is found in the matrix domains of most primate lentiviral Gag polyproteins. We provide evidence that mutations within a polybasic region in the N terminus of the HIV-1 matrix sequence block Gag intracellular transport and inhibit virus production, suggesting that this region may provide an additional signal for Gag transport.

MATERIALS AND METHODS

Cells and sera. COS-7 and SupT1 cells were maintained in Dulbecco's modified Eagle's medium and RPMI 1640 medium, respectively, plus 10% fetal bovine serum. Sheep anti-gp120 and anti-p17 were obtained from the AIDS Research and

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Reference Reagent Program, National Institutes of Health, Bethesda, Md. Anti-p24 monoclonal antibody was purchased from Dupont, Boston, Mass. The fluorescein-conjugated anti-mouse immunoglobulin G (IgG) antibodies were obtained from AMAC Inc., Westbrook, Maine. HIV-1-positive sera were kindly provided by K. Mayer of the Fenway Community Health Center, Boston, Mass.

Plasmids. The HIV-1 proviral clone HXB2R3 has been previously described (50). It is a derivative of HXB2 (30) with full-length *vpr* and *nef* genes. The pGAG construct was a gift of C. D. Morrow. pGAG is also derived from HXB2 with the *Bcl*I (2007)-*Bal*I (4131) fragment deleted (31). This fragment contained most of the *pol* gene. As a result, pGAG does not express functional protease, reverse transcriptase (RT), or integrase activity (26). dBgldrev was constructed from pHXBΔenvCAT (kindly provided by J. S. Sodroski) (16). A frameshift and premature truncation of the *env* gene were created by deletion of the *Bgl*I (6618)-*Bgl*II (7198) fragment followed by self-ligation (31). The *rev* gene was truncated by inserting a stop codon containing a *Bam*HI adapter at the *Bam*HI (8053) site. Sequences of the adapter are as follows: sense, 5′gatcctctagac3′; and antisense, 5′gatcgtctagag3′. DNA oligonucleotides were synthesized on a model 391 DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.).

Sequence analysis. Protein sequence alignment was performed with the Gene Works program (version 2.01; IntelliGenetics, Inc., Mountain View, Calif.).

Oligonucleotide-directed mutagenesis of p17. Site-directed mutagenesis of p17 was performed as described before (50). Sequences of oligonucleotides for generating mutants dB5, MGA, B8, B5, and B3 were 5′catac tatat gtaac cgaat ttttt cccat cg3′, 5′gacgc tctcg tggcc atctc tctcc3′, 5′gccca tacta tatga tttaa gttat aatta ttatt tcccc ctggc cctaa cccaa tattt tccca tcgat ctaat tctc3′, 5′catac tatat gcttt aattt atact tcttc tttcc ccctg gcgtt aaccg3′, and 5′gtttt aattt atact tcttc tttcc ccctg3′, respectively. Mutant dB5 has a deletion of amino acids 22 to 32 of the matrix protein. In mutant MGA, glycine was replaced by alanine at position 2. This glycine is the target site of myristoylation modification. Mutant MGA also contains an amino acid substitution at position 3 (alanine to proline). B8, B5, and B3 contain amino acid substitutions of eight, five, and three basic amino acid residues, respectively. Mutant proviruses were generated by replacing the *Bss*HII-*Spe*I fragment in the provirus vector HXB2R3 with the respective fragments isolated from the mutagenesis vector pGEM7Zf(+)Sst I-Apa I (50). pGdB5, pGMGA, and pGB5 were generated by replacing the *Bss*HII-*Spe*I fragment of pGAG with fragments from mutants dB5, MGA, and B5, respectively. Mutations were verified by sequencing.

Transfection and RT assay. COS-7 cells were transfected by the DEAE-dextran method (50). Viruses produced from the transfected cells were collected by polyethylene glycol precipitation, and virion-associated RT activities were measured as described previously (36).

RIPA and immunoblot analysis. The radioimmunoprecipitation assay (RIPA) and immunoblot analysis were performed as previously described, with slight modification (50). During RIPA, the cells were metabolically labeled at 48 h rather than 60 h posttransfection. The specific activity of [^{3}H]myristic acid (NEN, Dupont) for labeling cells was 0.25 mCi/ml. Cells were labeled at 48 h posttransfection for 15 h.

Cell fractionation. At 48 h posttransfection, COS-7 cells were metabolically labeled with [^{35}S]cysteine at 0.1 mCi/ml for 3 h. Cells were rinsed once with cold phosphate-buffered saline (PBS), scraped, pelleted by centrifugation at 500 × *g* for 5 min, and resuspended in swelling solution (20 mM Tris-HCl [pH 7.5], 10 mM KCl, 1 mM EDTA, 100 μg of phenylmethylsulfonyl fluoride per ml). After resting on ice for 20 min, cells were broken with 60 strokes of a tight-fitting Dounce homogenizer (Wheaton, Millville, N.J.); the nuclei as well as unbroken cells were removed by centrifugation twice at 1,000 × *g* for 5 min each time. Supernatants were adjusted to 0.15 M NaCl and incubated on ice for 20 min. The soluble cytosol fraction (S−) was separated from the membrane fraction by centrifugation for 20 min at 100,000 × *g*. The membrane pellet was resuspended in swelling solution containing 0.15 M NaCl and divided into two halves. One half (P−) was saved for immunoprecipitation analysis. The other was adjusted to 1 M NaCl and centrifuged again at 100,000 × *g* for 20 min. The resulting supernatant and pellet fractions were designated S+ and P+, respectively; the + sign indicates high-salt washing. Equal portions from each of the above-mentioned fractions were adjusted to 1× RIPA lysis buffer (0.15 M NaCl, 0.05 M Tris-HCl [pH 7.5], 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) and subjected to RIPA with an HIV-1-positive serum.

Indirect immunofluorescence assay. COS-7 cells were transfected and grown in 4.26-cm^2 slide chambers (Nunc, Inc., Naperville, Ill.). At 48 h posttransfection, cells were rinsed twice with cold PBS and fixed in 2% paraformaldehyde–0.1% Nonidet P-40 for 30 min at 4°C. Cells were then rinsed with cold PBS and incubated with 5 μg of anti-p24 monoclonal antibody per ml for 1 h at 37°C. After being washed in PBS twice for 5 min each time, cells were incubated with fluorescein-conjugated anti-mouse IgG antibody (25 μg/ml) for 1 h at 37°C. Cells were washed with PBS twice and examined by laser scanning confocal microscopy (Sarastro 2000; Molecular Dynamics, Sunnyvale, Calif.) with a Nikon microscope. The argon ion laser was set at 10 mW and 10% transmittance, the excitation filter was set at 488 nm, the emission filter was set at 510 nm and above, and photo gain was set at 1×. The computer imaging analysis program was Image Space (Molecular Dynamics).

CAT assay. COS-7 cells were transfected with dBgldrev, either alone or with pGAG, pGdB5, and pGB5. After 72 h, virus-containing culture supernatants were precleared and filtered (0.2-μm-pore-size filter). Equivalent units of RT supernatants from each of the transfected cell cultures were added to SupT1 cells (5 × 10^6 cells in 1 ml of fresh medium) and incubated at 37°C for 2 h. The cells were washed and resuspended in 5 ml of fresh medium. At 48 h postinfection, cells were washed with PBS, pelleted, lysed by three cycles of freeze-thawing, and assayed for chloramphenicol acetyltransferase (CAT) activity as previously described (11).

RESULTS

Sequence analysis of the HIV-1 HXB2R3 matrix protein revealed a region in the N-terminal portion that is rich in basic amino acids (Fig. 1). Within this region (amino acids 20 to 32), seven residues are basic; none are acidic. This clustering of basic residues is conserved among HIV-1 isolates as well as HIV-2 and simian immunodeficiency virus isolates. Figure 1 compares the MA sequence of HXB2R3 with those of representative HIV-1, HIV-2, and simian immunodeficiency virus isolates. Most of the basic residues are preserved among all isolates, and there is less sequence variation within the polybasic region than in the surrounding regions. The HIV-1 isolates were selected to represent the phylogenetic grouping (groups a through d) compiled according to relatedness in the Gag sequence (25).

Since a polybasic domain in p21^{ras} can act in combination

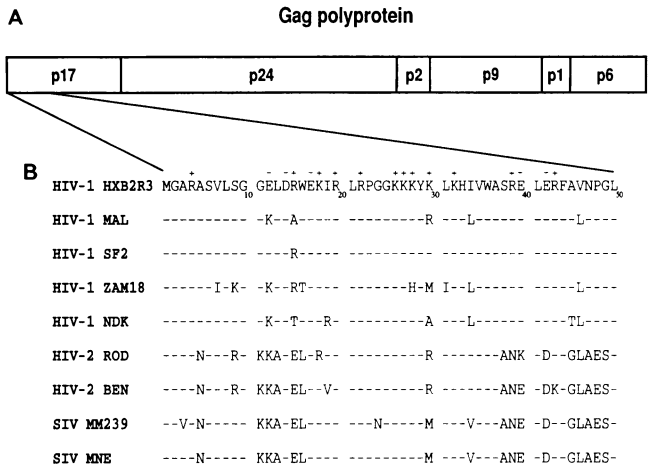

FIG. 1. Conserved polybasic region in the N-terminal portion of the matrix protein. (A) Gag precursor polyprotein and its processed products. (B) Sequence alignment of 50 amino acid residues in the amino terminus of several viral matrix proteins. + and − above the sequences indicate basic and acidic amino acid residues, respectively. Dashes within the sequence indicate amino acids identical to those in HXB2R3. SIV, simian immunodeficiency virus.

with the CAAX motif to target Ras protein to the cytoplasmic membrane (14), we hypothesized that the highly conserved polybasic region of the HIV-1 matrix protein might also function, in an analogous manner, to bring the Gag polyprotein to the cell surface membrane. Deletion mutant dB5 was constructed to test this hypothesis. dB5 has amino acids 22 to 32 removed, including six basic residues (see Fig. 7a).

Virus production. COS-7 cells were transfected with wild-type (WT) and mutant proviral DNAs. Virions were purified from the culture supernatant 72 h posttransfection and subjected to immunoblot analysis (Fig. 2b). Virus production from cells transfected with mutant dB5 was considerably less than

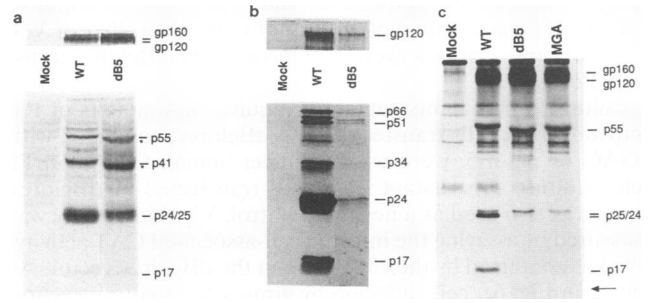

FIG. 2. Virus production of transfected cells. COS-7 cells were mock transfected or transfected with the WT and mutant dB5. At 72 h posttransfection, virus production and viral protein synthesis were analyzed. (a and b) Analysis of viral proteins by immunoblotting. Cell and virus proteins were separated by SDS–12.5% polyacrylamide gel electrophoresis and transferred onto two nitrocellulose filters. Cell (a) and purified virus (b) lysates were blotted with sheep anti-gp120 of HIV-1 (top) and with HIV-1-positive serum (bottom). Positions of the mutant Gag p55 precursor and p41 intermediate are indicated by asterisks. (c) Analysis of viral proteins by radioimmunoprecipitation. Transfected COS-7 cells were metabolically labeled with [^{35}S]cysteine. Viral proteins were precipitated with an HIV-1-positive serum and separated by SDS–12.5% polyacrylamide gel electrophoresis. Mutant p17 is marked by the arrow.

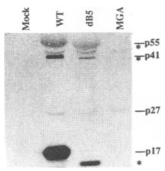

FIG. 3. Gag protein myristoylation. [^{3}H]myristic acid-labeled COS-7 cells were lysed with 1× RIPA lysis buffer and immunoprecipitated with an HIV-1-positive serum. Mutant p55, p41, and p17 are indicated by asterisks. The cells were mock transfected or transfected with the indicated proviruses.

that from WT-transfected cells, as indicated by the lower level of viral p24, p66/p51, and gp120 detected. Virion-associated RT activity in the mutant cell culture supernatant was more than 10-fold lower than that in the WT culture supernatant but detectably higher than in mock-transfected supernatant (data not shown).

When transfected cells were collected and analyzed by immunoblotting, no discernible difference in viral protein production between WT and dB5 was observed (Fig. 2a). Mutant p55 Gag precursor and p41 Gag intermediate (in Fig. 2a) were slightly smaller than their WT counterparts, presumably as a result of the internal deletion. Cell-associated viral proteins were also studied by RIPA (Fig. 2c). COS-7 cells were labeled with [^{35}S]cysteine for 15 h at 48 h posttransfection. Comparable amounts of gp160/120 and p55 were detected in WT- and dB5-transfected cells. With human serum, dB5 p17 was barely detectable as a faint band of about 15 kDa (indicated by an arrow in Fig. 2c). It was readily observed with hyperimmune antiserum to p17 (see Fig. 6). The amount of p24 capsid proteins was dramatically reduced in dB5 cells, while the amount of p25 capsid proteins was the same or slightly increased. A similar pattern was detected in myristoylation mutant cells, which served as a control for matrix protein mutants (Fig. 2c, lane MGA). The lack of p24 and reduced amount of p17 in dB5 mutant cells suggest that there was a defect in Gag polyprotein processing.

Membrane association of dB5 Gag polyprotein. For HIV-1 Gag polyproteins to assemble into virus particles, they must be transported to and associated with the cell plasma membrane. This is likely to be determined by the MA sequence within the precursor protein, which could in turn be partially dependent on myristoylation modification (3, 13). In dB5-transfected COS-7 cells labeled with [^{3}H]myristic acid, radiolabeled mutant p55, p41, and p17 proteins were clearly detected (Fig. 3, lane dB5). No myristoylated Gag proteins were detected in myristoylation mutant-transfected cells, despite the detection of myristoylated p27 Nef protein (Fig. 3, lane MGA). The determinant of protein myristoylation is reported to involve a short stretch of amino acids (21, 27). The first 10 N-terminal amino acids from Src as well as Mason-Pfizer monkey virus Gag proteins provided the myristoylation signal for Rous sarcoma virus Gag (49). The dB5 mutation is more than 20 amino acids downstream from the N terminus. To our knowledge, no similar mutations that are as far away from the N terminus have affected protein myristoylation. Lower amounts of labeled Gag proteins in mutant dB5-transfected cells might be due to a relatively reduced transfection efficiency, as the amount of p27 in the mutant cells was also lower. The ratio of mutant to WT p17 and p55 observed in [^{3}H]myristic acid-labeled cells was not discernibly different from the ratio in [^{35}S]cysteine-labeled cells (Fig. 2c, lane dB5). Thus, it appears that dB5 mutation did not grossly affect Gag myristoylation.

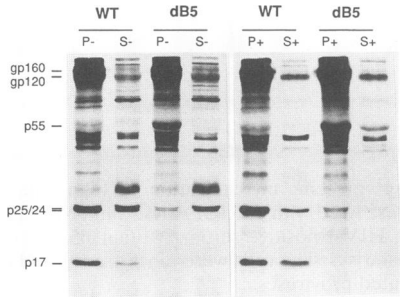

FIG. 4. Membrane association of the Gag polyproteins. [^{35}S]cysteine-radiolabeled transfected COS-7 cells were broken by Dounce homogenization, incubated with 0.15 M NaCl, and fractionated by centrifugation at 100,000 × *g* into soluble supernatant (S−) and membrane pellets. Pellets were resuspended in 0.15 M NaCl solution (P−), and a portion were adjusted to 1 M NaCl and separated into soluble (S+) and membrane (P+) fractions by similar centrifugation. Each fraction was adjusted to 1× RIPA lysis buffer, and viral proteins were immunoprecipitated by an HIV-1-positive serum. An autoradiogram of the SDS–12% polyacrylamide gel is presented.

However, because of the semiquantitative nature of the RIPA, a subtle reduction in myristoylation of mutant Gag proteins could not be totally excluded.

Membrane association of mutant Gag proteins was studied in a cell fractionation assay. At 48 h posttransfection, COS-7 cells were labeled with [^{35}S]cysteine for 3 h. Cells were swelled in hypotonic buffer and physically ruptured with a tight-fitting Dounce homogenizer. Cell membrane (P−) was separated from cytosol (S−) by centrifugation in the presence of 0.15 M NaCl. A portion of the membrane fraction (P−) was further washed with high-salt solution (1 M NaCl) and separated into membrane pellet (P+) and supernatant (S+). Each fraction was adjusted to 1× RIPA lysis buffer and subjected to RIPA with an HIV-1-positive serum. Proteins were separated on an SDS–12% polyacrylamide gel (Fig. 4).

Most of the dB5 Gag polyproteins (p55) were found in membrane fractions, at low (P−) or high (P+) salt concentrations (Fig. 4). This distribution is similar to that of the WT Gag polyproteins (WT, P−, and P+) except that WT p55 was reduced in quantity as it became processed more rapidly to p25/24 and p17. Thus, the association of dB5 Gag polyprotein with the membranes was not grossly affected. Similar assays have been widely used to define membrane association of proteins, e.g., Src (6), p21^{ras} (14), and HIV-1 Gag (3). However, this assay was not designed to differentiate between Gag polyprotein association with the plasma membrane or with intracellular membrane components. Alternatively, the possibility of Gag coprecipitation in the membrane fraction in some aggregate complex or precipitated with cytoskeleton could not be completely excluded.

dB5 Gag protein intracellular transport. The intracellular distribution of mutant Gag proteins was characterized by an indirect immunofluorescence assay. To characterize the Gag polyprotein, the pGAG construct (26) was adopted to avoid Gag intracellular processing, which would obscure the distribution of the Gag precursor. Intracellular processing of HIV-1 Gag proteins has previously been detected (20). Although Gag polyproteins from pGAG cannot be processed for lack of a viral protease, they still follow the Gag intracellular transport pathway and assemble into immature virus particles. Mutants pGdB5 and pGMGA were generated by replacing the *Bss*HII-*Spe*I fragment of pGAG with fragments from mutants dB5 and MGA, respectively.

At 48 h posttransfection, COS-7 cells were fixed with paraformaldehyde and permeated with mild detergent. Cells were probed with mouse anti-p24 monoclonal antibody and subsequently with fluorescein-conjugated anti-mouse IgG. The pGAG cells were predominantly labeled at the cell periphery, including the cell extension (Fig. 5b). This finding is consistent with the plasma membrane association of WT Gag precursors. Labeling of mutant pGdB5 cells significantly differed from that of the pGAG cells. The cells were labeled in discrete regions near the nucleus; the cell surface membranes were largely excluded (Fig. 5c). There was a noticeable punctate staining pattern in the mutant cells. For comparison, the fluorescence pattern observed with the myristoylation mutant pGMGA is shown in Fig. 5d.

Packaging of dB5 Gag polyproteins into WT virions. A complementation assay (16) was used to study the potential interaction between mutant and WT Gag proteins. Mutant Gag should be able to interact with WT Gag and be packaged into WT virions provided that it retains its ability to interact with WT Gag. This assay relies on the coexpression of mutant and WT Gag proteins in the same cells. dBgldrev produces WT Gag and Pol proteins and generates infectious viruses only when complemented by Rev and Env expression vectors, such as pGdB5. pGdB5 alone can produce only mutant Gag precursors that cannot be processed. When pGdB5 is cotransfected with dBgldrev, it provides the *rev* and *env* gene products for dBgldrev to form WT virus particles. The WT virus particles, in turn, package mutant Gag precursors and process them into their individual components, including the smaller p17. Thus, the presence of mutant 17 is a strong indicator of WT and mutant Gag precursor interaction and copackaging.

When dBgldrev was cotransfected with *pol*-deleted pGAG or pGdB5, RT activity significantly increased in the cotransfection cell culture supernatant compared with that in cells transfected by dBgldrev alone or by pGAG or pGdB5 alone (data not shown).

Viruses were collected and analyzed by immunoblotting (Fig. 6a and b). Env and Gag proteins were detected in viruses from cotransfected cells by an HIV-1-positive serum (Fig. 6a). When the proteins were analyzed with an anti-p17 antibody (Fig. 6b), a smaller MA protein was detected in viruses from cells cotransfected with dBgldrev and pGdB5. The amount of mutant p17 was comparable to, if not more than, that of WT p17. No viral proteins were produced from dBgldrev-transfected cells.

Culture supernatants containing equivalent amounts of RT activities from cells transfected with dBgldrev, combined with pGAG or pGdB5, were used to infect human CD4$^+$ SupT1 cells. Culture supernatant from cells transfected by dBgldrev alone was also used as a negative control. Virus infectivity was measured by assaying the infected cell-associated CAT activity, which is conferred by the CAT gene in the dBgldrev vector. As shown in Fig. 6c, cells infected by viruses from cotransfection with dBgldrev and pGdB5 produced significantly lower CAT activity than did cells infected by viruses from dBgldrev and pGAG cotransfection. This result confirmed the incorporation of mutant Gag proteins that transdominantly interfered with WT virus infectivity.

Amino acid substitution mutants. To further characterize the polybasic region of HIV-1 MA protein, mutations that replaced basic residues with acidic or neutral ones were introduced. The mutations in B8, B5, and B3 are indicated in Fig. 7a. Western blot (immunoblot) analysis revealed comparable amounts of viral proteins in cells transfected with these mutants or the WT sequence (Fig. 7b). When viruses collected from the culture supernatants were analyzed by immunoblot-

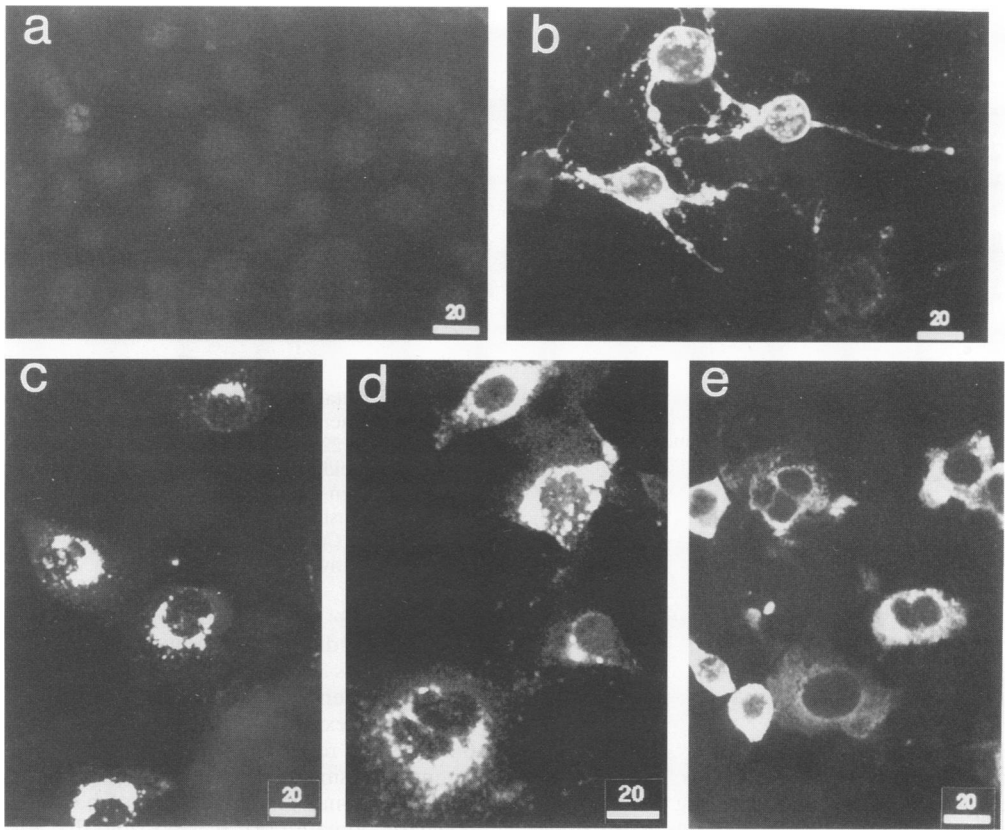

FIG. 5. Indirect immunofluorescence study of the intracellular distribution of Gag polyproteins. Cells were fixed in 2% paraformaldehyde and permeated with 0.1% Nonidet P-40 at 48 h posttransfection. The primary antibody was a mouse anti-p24 monoclonal antibody, and the secondary antibody was fluorescein-conjugated anti-mouse IgG. The stained cells were studied by confocal laser scanning microscopy. Cells were mock transfected (a) or transfected with pGAG (b), pGdB5 (c), pGB5 (d), or pGMGA (e). Bar: 20 μm.

ting, the amounts of virus produced by B8 and B5 were considerably reduced, while B3 produced an amount of virus comparable to that of the WT protein (Fig. 7c).

pGB5 was generated similarly to pGdB5. Indirect immunofluorescence staining of COS-7 cells revealed a punctated perinuclear pattern (Fig. 5d) that is like that observed with pGdB5-transfected cells (Fig. 5c). Viruses produced from COS-7 cells cotransfected with dBgldrev and pGB5 also contained a smaller mutant p17 (Fig. 6a and b) and produced considerably lower amounts of CAT activity in SupT1 cells (Fig. 6c). B5 and dB5 exhibited similar defects in virus production and intracellular transport of Gag protein. Together, they reveal the importance of N-terminal sequences of HIV-1 MA protein in virus assembly.

DISCUSSION

The molecular events of retrovirus particle formation are poorly understood. The Gag polyprotein contains functional domains that are sufficient for directing itself to the plasma membrane and for stable binding. The MA domain is believed to provide these functions, since it is localized at the periphery of the virion (9) and can be cross-linked to lipids and glycoproteins of the viral envelope (7, 28, 29).

Features of the MA protein that determine its specific interaction with the plasma membrane are still unclear. The myristic acid attached to the amino-terminal glycine of most Gag polyproteins is believed to play a role in targeting and binding to the site of assembly (37). Mutations that remove the glycine residue block myristoylation, resulting in a loss of membrane binding and virus particle formation (3, 13, 32, 46). Nevertheless, myristate alone is not sufficient to provide the specific signal for Gag polyprotein membrane targeting. A variety of myristoylated proteins are not localized on the plasma membrane (37). Gag polyproteins in some retroviruses are not myristoylated, but they can efficiently assemble virus particles (37).

The dB5 mutant Gag polyprotein remains myristoylated and has a high affinity for membranes, as indicated by its presence in comparable amounts in the membrane fraction under high-salt conditions. However, this Gag protein was severely compromised in its ability to be transported to the plasma membrane to assemble virus particles. Similar results were found with MA deletion mutants of Mason-Pfizer monkey virus (34) and Rous sarcoma virus (49). It appears that the MA domain contains some additional determinant(s) for Gag-plasma membrane interactions.

Whether the dB5 defect was due to a local alteration of the MA domain or due to a generalized conformational change in the Gag polyprotein remains unclear. Deletions in regions surrounding dB5 (D2 [deleting amino acids 21 to 31] and D3 [deleting amino acids 32 to 40]) did not affect virus production (50). Moreover, dB5 mutant Gag proteins were efficiently incorporated by WT virus. These results suggest that the

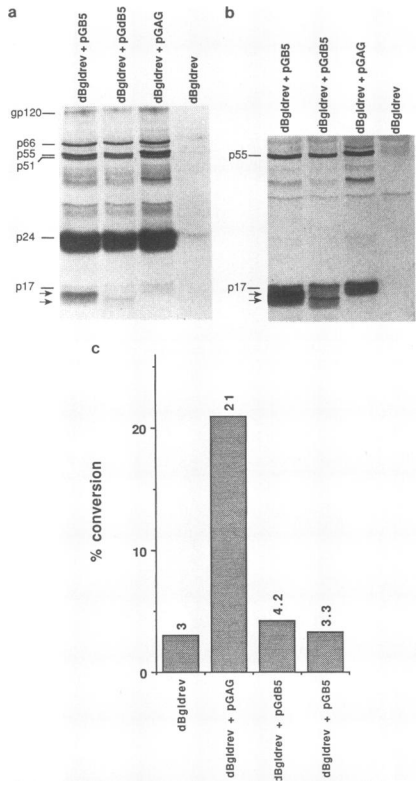

FIG. 6. Interactions between WT and mutant Gag proteins. (a and b) Packaging of dB5 and B5 mutant Gag polyproteins by WT virions. Culture supernatants from COS-7 cells cotransfected with dBgldrev and either pGB5 (lane dBgldrev + pGB5), pGdB5 (lane dBgldrev + pGdB5), or pGAG (lane dBgldrev + pGAG) or transfected with dBgldrev alone (lane dBgldrev) were collected and analyzed by immunoblotting. The blot was probed by an HIV-1-positive serum (a) and by an anti-p17 antiserum (b). The arrows indicate mutant p17. (c) Transdominant inhibition of WT virus infectivity by the mutants. Virus infectivity was measured by CAT activity in SupT1 cells at 48 h postinfection. CAT activity was measured by the percentage of conversion of [^{14}C]chloramphenicol into acetylated forms. Cells were infected with equal amounts of virus from COS-7 cells transfected with dBgldrev and pGB5 (dBgldrev + pGB5), dBgldrev and pGdB5 (Bgldrev + pGdB5), dBgldrev and pGAG (dBgldrev + pGAG), and dBgldrev alone (dBgldrev).

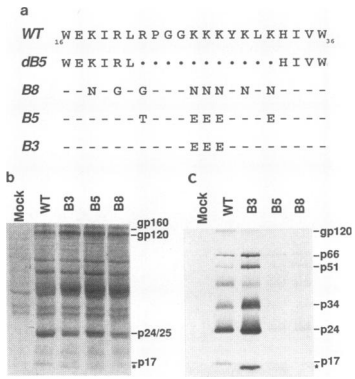

FIG. 7. Mutagenesis of the p17 polybasic region. (a) Comparison of mutant sequences with the WT sequence. dB5, mutant with deletion of amino acid 22 to 32; B8, B5, and B3, mutants with eight, five, and three amino acid residues substituted; •, amino acid deletion; -, amino acid that is identical to the WT sequence. (b and c) Virus production from COS-transfected cells. Methods are as described in the legend to Fig. 2a and b. Cell (b) and virus (c) lysates were immunoblotted with an HIV-1-positive serum. *, mutant p17.

overall structure of the Gag protein was not drastically perturbed. The dB5 region may serve as a part of the Gag transport signal.

Many primate lentiviruses contain a polybasic region in the N-terminal regions of their MA proteins. dB5 deleted most of the basic amino acid residues of the polybasic region of the HIV-1 matrix protein (Fig. 7a). Consistent with the deletion mutation, mutations that simultaneously substituted eight and five basic residues in the dB5 region with neutral and acidic residues (B8 and B5) also significantly blocked virus production. Mutant B5 Gag polyprotein displayed a limited intracellular distribution similar to that of the dB5 Gag (Fig. 5d). However, when the number of amino acids substituted was reduced to 3 (B3), virus production was not affected.

A minimal number of basic amino acid residues in the N-terminal region of MA protein may be required for its functions. This may explain differences between D2 (with a deletion of amino acids 21 to 31) (50) and dB5 (with residues 22 to 32 deleted). Although largely overlapping, they had dramatically different phenotypes. The D2 deletion brought basic residues 32 and 39 close to basic residues 18 and 20 (Fig. 7a), thus regenerating a local basic cluster. The dB5 deletion removed an extra basic residue, 32, and may not have fulfilled the minimal requirement. In support of this hypothesis, the extent of plasma membrane binding of p21^{ras} was also found to be proportional to the number of basic residues within the polybasic domain (14). It is possible that the basic domain of HIV-1 MA protein plays a role in Gag protein transport to the plasma membrane.

After synthesis and folding, nascent Gag polyproteins must interact with each other to form virus particles. When and where Gag molecules of HIV-1 first interact is currently unknown. In type B and D viruses, Gag proteins interact and assemble at the cytoplasm. The assembly sites are not random, for mutations in Mason-Pfizer monkey virus MA protein lead to an accumulation of cores deep in the cells and close to the nucleus (33). A dominant targeting signal within the Mason-Pfizer monkey virus Gag protein has been hypothesized to specifically direct Gag to an intracellular assembly site. After Gag proteins interact and condense to form an immature core, another targeting signal is generated to subsequently direct the core to the plasma membrane. A single amino acid substitution in the MA protein could alleviate the first signal and allow the second signal to be recognized without intracellular particle formation, thereby allowing the Gag polyproteins to proceed to the plasma membrane and assemble in the manner of a C-type virus (35).

Although the morphological data from electron microscopy suggest that the assembly of type C retroviruses and lentiviruses first occurs at the cell membrane, there is very little information about where the Gag proteins first interact and how they are transported to the assembly site. It is possible that lentiviral Gag polyproteins first interact in the cytoplasm to form regular but electron-lucent structures which condense only after transport to and interaction with the cytoplasmic membrane. Immunofluorescence staining revealed that the dB5 mutant Gag protein accumulates in the cytoplasm close to the nucleus (Fig. 5c). The staining pattern seems similar to that of the Golgi apparatus (45). The punctuated staining suggests the proteins may be associated with intracellular membrane vesicles (47). This is consistent with the considerable associa-

tion of mutant Gag proteins with the membrane fraction in cell fraction analysis. However, further studies are needed to define the precise structure where the mutants reside. The WT Gag proteins were clearly associated with the cell surface membrane. The difference in intracellular distribution suggests that the dB5 mutant Gag is blocked at the step of Gag protein transport from the cytoplasm to the surface membrane. This blockage can be relieved through interaction with the WT Gag protein, for WT Gag can efficiently package mutant Gag into virions. A considerable amount of mutant Gag was packaged by the WT virion, as indicated by the comparable amounts of mutant and WT p17 in the virions (Fig. 6a and b). It is likely that interaction between WT and mutant Gag proteins occurs in the cytoplasm, where a majority of mutant Gag polyproteins was found (Fig. 5c).

The dB5 Gag protein distribution also differed from that of the myristoylation mutant Gag protein, which is homogeneous throughout the cytoplasm. A similar distribution was observed with myristoylation mutant spleen necrosis virus Gag (46). Myristoylation mutant HIV-1 Gag polyproteins are reported to have a significantly reduced affinity for the plasma membrane (3). Myristoylation-deficient Gag proteins are likely to be defective in steps of the transport process that require stable association with membranes. Our results suggest that HIV-1 Gag protein transport is a multistep process. The Gag polyproteins might be transported to an intracellular site, possibly the intracellular membrane compartment, where they interact and subsequently be transported to the cell membrane assembly site. These steps could be potential targets for antiviral intervention.

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