Characterization of human immunodeficiency virus type 1 Pr55\textsuperscript{gag} membrane association in a cell-free system: Requirement for a C-terminal domain

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ABSTRACT  Association of the human immunodeficiency virus type 1 (HIV-1) gag polyprotein precursor with cellular membranes is necessary for assembly of virions. We used \textit{in vitro} synthesized HIV-1 gag to study its association with isolated cellular membranes. Rabbit reticulocyte lysates programmed with HIV-1 gag mRNA incorporated \textsuperscript{[35S]}methionine and \textsuperscript{[3H]}myristate into two predominant species of 55 kDa and 40 kDa. Radioimmunoprecipitation with HIV-1-specific antibodies suggested that the 55-kDa protein represented the polyprotein precursor (Pr55\textsuperscript{gag}), while the 40-kDa protein was a mixture of N- or C-terminal truncations of the gag precursor. The Pr55\textsuperscript{gag} protein bound to cellular membranes, while the 40-kDa mixed protein species did not. Membrane binding studies with C terminus-truncated and point mutants revealed that the seven-amino acid sequence located between the two Cys-His arrays in the nucleocapsid region was necessary for stable association to occur. Therefore, we propose that signals in addition to myristate are required for the membrane association of HIV-1 gag proteins and that these signals include a domain in the nucleocapsid protein.

Human immunodeficiency virus type 1 (HIV-1) is a member of the type C family of retroviruses, which undergo capsid assembly at the plasma membrane of the host cell (1–3). As with other retroviruses, RNA binding and budding activities are properties of the HIV-1 gag protein, as has been shown by its ability to form particles in the absence of other viral products (4–6). The HIV-1 capsid protein Pr55\textsuperscript{gag} is synthesized as a cytosolic 55-kDa gag polyprotein precursor possessing discrete structural and functional domains in common with other retroviral gag proteins. Cleavage of HIV-1 Pr55\textsuperscript{gag} by the viral protease occurs during virion formation and yields the major core proteins—namely, matrix (MA), capsid (CA), and nucleocapsid (NC) proteins with molecular masses of 17 (p17), 24 (p24), and 7 (p7) kDa, respectively (7). Additionally, a 6-kDa proline-rich C-terminal protein, p6, is generated (8). While cleavage of HIV-1 Pr55\textsuperscript{gag} is necessary for the production of infectious virions, it is not required for budding to occur, as shown by viral protease inhibitor (9) and mutagenesis studies (10).

Mutagenesis and biochemical studies have elucidated the critical role that MA plays in membrane association (11, 12) and in virus assembly and budding (13–16). A role for the C terminus of HIV-1 gag in virion assembly is suggested by mutagenesis studies in which C-terminal truncations of Pr55\textsuperscript{gag} prevent particle formation (8, 17–19). We theorized that these truncations may inhibit particle formation through abrogation of gag membrane association. To address this question, we evaluated the cellular membrane association of full-length and truncated HIV-1 gag proteins by assaying the ability of \textit{in vitro} translated proteins to associate with isolated membranes derived from CEM cells. Our results show that the \textit{in vitro} generated gag proteins are myristoylated and that the Pr55\textsuperscript{gag} will associate with membranes when added post-translationally. Characterization of myristoylated, truncated HIV-1 gag proteins revealed that deletions extending into the N terminus of the NC region abolished membrane binding completely.

MATERIALS AND METHODS

Construction of Plasmids for Use in the \textit{in Vitro} Transcription/Translation System. A fragment containing the HIV-1 gag and truncated pol (5) open reading frames was ligated into the \textit{in vitro} expression plasmid pSP65 (Promega) to generate the plasmid pSP65\textsuperscript{gag} (see Fig. 1A).

Truncated and point mutants of HIV-1 gag gene were generated as described below and introduced into the pSP65\textsuperscript{gag} plasmid to replace the wild-type (WT) gag sequences. All nucleic acid manipulations were performed by standard protocols (20).

\textit{Minus myristate mutant}. The mutagenic primer pair 5'-CGGCCGATTGACGATGATGCTGACGATGCTGATTAAGC-3' (sense) and 5'-CGGCCGCTGAGCCTCCTCATTTAT-3' (antisense) was used to generate a PCR fragment in which the codon for the penultimate glycine residue in MA (GCT) was changed to a codon for alanine (GCT; underlined region in sense primer).

\textit{TC363}. A termination codon (for subsequent truncation) was inserted at the C terminus of p24 by using the following primer pair: 5'-CGGAAAGTACGAGGAGATC-3' and the reverse primer: 5'-CGGAGATCGACATGATTTAAGCCTTCTC-3' (antisense). The termination codon within the reverse primer is underlined and is read as UAA during translation.

\textit{TC410}. A premature termination codon was substituted for codon 411 to generate a truncated gag protein of 410 amino acids possessing the first Cys-His array and the 7-amino acid basic region distal to the array. The mutagenic primer pair used was the sense primer described for TC363 (see above) and the following antisense primer: 5'-CGGAGATC-3'.

\textit{TC403}. A premature termination codon was substituted for codon 404, generating a truncated gag protein of 403 amino acids possessing the first Cys-His array and the 7-amino acid basic region distal to the array. The mutagenic primer pair used was the sense primer described for TC363 (see above) and the following antisense primer: 5'-CGGAGATC-3'.

Abbreviations: HIV-1, human immunodeficiency virus type 1; RRL, rabbit reticulocyte lysate; Pr55\textsuperscript{gag}, 55-kDa gag polyprotein precursor; MA, CA, and NC, major core matrix, capsid, and nucleocapsid proteins; WT, wild type; RIPA, radioimmunoprecipitation assay; mAb, monoclonal antibody.

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acids possessing sequences up to and including the first Cys-His array. Thus, this truncated gag protein is 7 amino acids shorter than TC410. Mutagenic primer pair used was the sense primer described for TC363 and the following antisense primer: 5'-CGGGATCTTTAGCAATTTCTG-GCTATGTCGCCTTCTT3'. The underlined bases are read as the termination codon UAA during translation.

WT<sup>TE</sup>. Codons encoding Pro-406 and Arg-407 were changed to codons for leucine and glutamic acid, respectively. This was accomplished by excising a 90-bp fragment of HIV-1 gag open reading frame with an Apa I/Bgl II double digest of pSP65gag. The mutation was then introduced by ligating hybridized synthetic oligonucleotides designed to have the appropriate base changes and contain Apa I and Bgl II ends as follows: 5'-CTCTGAGAAAAAGGCTGTTG-GAAAAGGAAAGACAACAAAATGAAAGAT-TGTACTGAGACAGGGCTTCTTCTTCTTCTC-3' (sense) and 5'-GATCTTTCCCTAAAAATTAGCCTGTC-TCTCAGTTCAATCTTCTATTGGGTCTTCTTCTTTC-CACATTCCAACAGGCCTTCTTCTCGAGGGCC-3' (antisense). The presence of the codons for leucine and glutamic acid (CTC and GAG, respectively) was confirmed by the creation of a new Xho I site (CTCGAG) (see underlined sequences). All constructs were confirmed by restriction digestion analysis and sequencing.

In Vitro Transcriptions/Translations. The linearized pSP65gag plasmid (at EcoRV; see Fig. 1A) was transcribed in <i>vivo</i> according to the manufacturer's recommendations (Promega) except that the reaction volumes were halved (21). After incubation for 2 hr at 37°C, 2 μl of the transcription reaction mixture was used to program 35 μl of nuclease-treated rabbit reticulocyte lysate (RRL) containing 20 μM amino acids, 20 μM myristate, and 1 μCi (37 kBq) of [<sup>35</sup>S]methionine or [<sup>3</sup>H]myristate (Amersham) per μl in a final volume of 50 μl. The translation mix was incubated for 1 hr at 30°C.

Isolation of Cellular Membranes. Cellular membranes were prepared from CEM cells by the method of Lin and Wharton (22). Briefly, 10<sup>6</sup> cells were resuspended in homogenization buffer (50 mM mannitol/10 mM Hepes, pH 7.4) supplemented with 10 mM CaCl<sub>2</sub> and aspirated through a blunt 25-gauge needle. Nuclei and cell debris were removed by centrifugation at 13,000 rpm in an Eppendorf Microfuge. The membranes were sedimented from the resulting supernatant by ultracentrifugation at 32,000 rpm in an SW 55 Ti rotor (Beckman). The resulting membrane pellet was resuspended in NTE buffer (100 mM NaCl/10 mM Tris chloride, pH 7.4/1 mM EDTA) to a final concentration of 0.5–1 mg/ml, and aliquots were taken and stored at −70°C.

Membrane Binding Assay. Fifteen microliters of <i>vivo</i> translated HIV-1 gag was incubated with 30 μl of isolated CEM membranes (0.5–1 mg/ml) or with NTE buffer alone at room temperature for 10 min. Membranes were sedimented by ultracentrifugation in either a SW 55 Ti rotor at 32,000 rpm for 1 hr or in an Airfuge (Beckman) at 20 psi (137.8 KPa) for 10 min. The resulting pellet and supernatant were solubilized respectively in phosphate-buffered saline containing 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS and in 10× radioimmunoprecipitation assay (RIPA) lysis buffer (0.15 mM NaCl/0.05 mM Tris-HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS) (see Fig. 2A). Radioimmunoprecipitated material was analyzed by SDS/PAGE as described below. Relative distribution of gag proteins in the pellet and supernatant fractions was quantitated by densitometric analysis of the autoradiograms.

**Fig. 1.** In <i>vivo</i> translation of HIV-1 gag proteins. (A) Schematic of the HIV-1 gag gene engineered downstream of the SP6 promoter, indicating the location of the EcoRV site used to linearize the pSP6gag plasmid for <i>vivo</i> transcription. (B) <i>In vitro</i> translated gag proteins radiolabeled with <sup>[35</sup>S]methionine and immunoprecipitated with human anti-HIV serum (lane B), rabbit anti-HIV serum (lane C), monoclonal antibody (mAb) specific to p24 (lane D), or mAb specific for p17 (lane E). The immunoprecipitated proteins are compared to proteins from unimmunoprecipitated translation mixtures (lane A). (C) Immunoprecipitation of <i>vivo</i> translated gag protein mutantized to replace the penultimate glycine with alanine. Incorporation of <sup>[35</sup>S]methionine (lanes B–D) is contrasted with incorporation of <sup>[3</sup>H]myristate (lanes F–H). Lanes A and E present the WT gag protein. Proteins were fractionated in 10% acrylamide gels.
RIPA, SDS/PAGE, and Fluororadiography. RIPA were performed as described (23) except that the SDS elution and a second antibody incubation step were omitted. The RIPAs were conducted with rabbit polyclonal sera generated against inactivated HIV-1 virions (sera 243; ref. 24) or, where indicated, with human anti-HIV serum and mAbs specific for the MA protein (mAb 18-1) or the CA protein (mAb 25-3) (Genetic Systems, Seattle; ref. 5). The immunoprecipitated proteins were fractionated by SDS/PAGE in 10% acrylamide gels and were visualized by autoradiography.

RESULTS

Characterization of in Vitro Synthesized HIV-1 gag. RRLs programmed with in vitro synthesized HIV-1 gag mRNA incorporated [35S]methionine into two prominent proteins of 55 kDa and 40 kDa (Fig. 1B, lane A). Occasionally, higher molecular mass species were detected that most likely arose from ribosomal frameshifiting (25). Incubating programmed lysates with HIV-1-specific human and rabbit anti-HIV sera (24) and mAbs specific for either p17 (mAb 18-1) or p24 (mAb 25-3) (5) resulted in the immune precipitation of both the 55-kDa and 40-kDa proteins (Fig. 1B, lanes B, C, D, and E). Antibodies generated against the carboxyl-terminal p6 region also immunoprecipitated both proteins (data not shown). These antibody surveys suggest that the 55-kDa protein most likely represents the HIV-1 gag precursor, Pr55gag, while the 40-kDa species represents a mixture of proteins truncated at either the C terminus (possessing p17 and p24; see Fig. 1A) or N terminus (possessing p24, p7, and p6); similar processing intermediates have been observed in mammalian cells expressing HIV-1 gag proteins (5). The mixture of HIV-1 Pr55gag-derived truncated proteins whose approximate molecular mass was 40 kDa was designated p40 (Fig. 1B and C).

Since myristoylation of the HIV-1 gag polyprotein is necessary for particle formation and membrane association (16), it was important to establish that myristoylation of gag occurred in the RRL system. Therefore, RRLs were programmed with wild-type gag mRNA or with gag mRNA generated from a mutant gag gene in which the normal myristoylation site, the penultimate glycine, was changed to an alanine. Immunoprecipitation and SDS/PAGE analysis of these lysates revealed that both Pr55gag and p40 incorporated [3H]myristate, further supporting the notion that Pr55gag and at least a fraction of p40 possess intact N termini (Fig. 1C, lane E). The myristate modification was specific for the penultimate glycine because substitution with alanine abolished incorporation of [3H]myristate (Fig. 1C, lanes F-H) without altering [35S]methionine incorporation (Fig. 1C, lanes B-D).

Membrane Binding Studies of in Vitro Translated HIV-1 gag. To investigate the membrane binding properties of HIV-1 gag, we developed an assay, described in Fig. 2A, in which in vitro translated proteins were incubated with isolated cellular membranes and the membrane-bound proteins were sedimented by ultracentrifugation. A similar approach was used by Resh to characterize the cellular membrane association of the oncogene product pp60src (26). Analysis of the pellet and supernatant fractions by RIPA revealed the membrane-binding capacity of the HIV-1 gag proteins tested. Pr55gag appeared predominantly in the particulate fraction (P) in the presence of membranes (Fig. 2B, lanes C vs. D compared with lanes A vs. B), while p40 remained in the supernatant (S), suggesting that the latter protein lacked a domain required for stable membrane association. Additionally, Pr55gag exhibited some pelleting in the absence of membranes suggestive of aggregation, while the p40 species showed no such pelleting. Association of Pr55gag with cellular membranes occurred within 1 min of incubation at all temperatures tested: 4°C, 22°C, and 37°C (data not shown).

Membrane Binding of HIV-1 gag Truncation Mutants. Since the 40-kDa species generated in the in vitro translation reaction was a mixture of C- or N-terminal truncated proteins, a mutant HIV-1 gag gene was constructed in which a termination codon was substituted for the codon encoding the first amino acid of the connecting peptide between p24 and p7. This truncated gene encodes an HIV-1 gag protein of 40
kDa designated TC363 for which the actual terminating amino acid was known (Fig. 3A). Incubation of TC363 with membranes followed by ultracentrifugation, revealed that, although myristoylated (Fig. 3B, lane H), TC363 remained in the supernatant both in the presence and absence of membranes (Fig. 3C, lanes N and P), suggesting that a domain located C-terminally to p24 is required for membrane binding. This result was consistent with that derived from the spontaneous truncated protein (p40, see Fig. 2A) generated in the in vitro translation reaction. To further delineate domains within the C terminus that may play a role in membrane binding, other truncated HIV-1 gag proteins were generated by introducing termination codons within the NC coding region. Presented in Fig. 3A are two mutant genes that encode gag proteins that were truncated upstream of the second Cys-His arrays at amino acids 404 (TC403) or 411 (TC410). TC403 possesses the first Cys-His array but lacks subsequent amino acids, while TC410 possesses an additional seven-amino acid basic region distal to the first Cys-His array. RRLs programmed with mRNA synthesized from these mutant genes produced truncated, myristoylated (Fig. 3B, lanes F and G) HIV-1 gag proteins of approximately 41.5 kDa (TC403) and 42 kDa (TC410) (Fig. 3B, lanes C and B, respectively). Membrane binding studies showed that, in addition to TC363, TC403 did not sediment with membranes (Fig. 3C, lanes I-L). In contrast, the presence of seven additional amino acid residues, Arg-Ala-Pro-Arg-Lys-Lys-Gly, in the mutant TC410 completely restored binding to membranes (Fig. 3C, lanes E-H) at levels similar to full-length Pr55<sup>WT</sup> (Fig. 3C, compare lanes A-D with lanes E-H). The seven additional amino acids restored aggregation as well (Fig. 3C, compare TC403 lane 1 with TC410 lane E).

Since it is possible that the above truncations may grossly affect HIV-1 gag conformation and thus its ability to associate with membranes, it was important to assess the role of Arg-Ala-Pro-Arg-Lys-Lys-Gly in the full-length Pr55<sup>WT</sup>. Toward this end, point mutations were introduced into this region. Pro-406 and Arg-407 were selected for mutagenesis because of their conserved nature among strains of HIV-1. Pro-406 was changed to leucine (L) and Arg-407 changed to glutamic acid (E) to generate the construct WT<sup>LE</sup> (Fig. 4A). Expression of WT<sup>LE</sup> in vitro yielded a protein of 55 kDa (Pr55<sup>WT<sup>LE</sup></sup> Fig. 4B, lanes E-H) migrating identically with Pr55<sup>WT</sup>. Incubation of this protein with membranes revealed that, in contrast to TC363 and TC403 (Fig. 3C), WT<sup>LE</sup> exhibited some aggregation detected by pelleting in the absence of membranes (Fig. 4B, lane E). However, like TC363 and TC403 and unlike TC410 (Fig. 3C), WT<sup>LE</sup> did not show increased sedimentation upon incubation with membranes (Fig. 4B, lanes E vs. F compared with lanes G vs. H; also compare with WT protein in Fig. 4B, lanes A-D). These data further support the notion that the Arg-Ala-Pro-Arg-Lys-Lys-Gly sequence may play an important role in HIV-1 gag membrane binding in vitro.

**DISCUSSION**

Using a cell-free system, we found that myristoylated C-terminal truncated mutants of HIV-1 gag, TC363 and TC403, did not bind to isolated cellular membranes, whereas the myristoylated truncated mutant TC410 did. These results suggest that the presence of a fatty acid moiety such as myristate does not irrevocably dictate membrane association and that characteristics other than fatty acid modification can determine membrane association. The data further suggest that one region allowing efficient and stable membrane binding resides

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**Fig. 3.** Membrane binding analysis of truncated HIV-1 gag mutants. (A) Schematic presentation of the truncated HIV-1 gag genes, delineating the structural regions of Pr55<sup>WT</sup>. Broken vertical lines represent the Cys-His arrays. The amino acid positions immediately preceding the inserted termination codons are indicated by the designations TC410, TC403, and TC363. (B) Radioimmunoprecipitation of WT and truncated gag proteins (TC410, TC403, and TC363) radiolabeled with either <sup>35</sup>S-methionine (lanes A-D) or <sup>3</sup>H-myristate (lanes E-H). (C) Binding of the truncated mutants to membranes. The truncated gag proteins, TC410 (lanes E-H), TC403 (lanes I-L), and TC363 (lanes M-P), and WT protein (lanes A-D) were incubated with and without membranes for 10 min at 22°C and then separated into P and S fractions as described. The proteins were then immunoprecipitated and fractionated by SDS/PAGE. The percentage distribution of gag proteins in the P fractions was as follows: WT, 41% (lane A) and 78% (lane C); TC410, 41% (lane E) and 74% (lane G); TC403, 16% (lane I) and 15% (lane K); and TC363, 12% (lane M) and 12% (lane O).
within the NC region of HIV-1 gag, encompassing the seven-amino acid sequence Arg-Ala-Pro-Arg-Lys-Lys-Gly located between the two Cys-His arrays (TC403 versus TC410). The lack of enhanced sedimentation with membranes exhibited by TC403 is most likely due to the absence of the seven amino acids and not to misfolding of the truncated protein, since full-length Pr55Gag containing two point mutations within this region does not show increased pelleting in the presence of membranes either. Thus, changing the above sequence to Arg-Ala-Leu-Glu-Lys-Lys-Gly is sufficient to greatly reduce membrane association of the HIV-1 gag polyprotein. At present it is not known whether the seven-amino acid basic region of HIV-1 gag enables membrane binding by direct contact with lipids or by maintaining a membrane binding-competent conformation of N-terminal regions. A role for basic amino acid sequences for targeting cytosolic proteins to membranes has been uncovered by Hancock et al. (27), who noted that a six-amino acid stretch of lysines at the carboxyl terminus of p21K-ras(B) could act as a plasma membrane localization signal in conjunction with prenoid addition at the consensus sequence Cys-Ala-Ala-Xaa.

While it is conceivable that regions within NC exert their effects by binding to membranes directly, such an interaction would most likely be a transient occurrence during virion assembly, since crosslinking of mature viral particles from other systems (11, 12) did not demonstrate an interaction of gag C-terminal sequences with lipids. Alternatively, the seven-amino acid basic stretch may be necessary for formation of an assembly domain (2) that facilitates the multimerization of HIV-1 gag proteins and thus their stable association with membranes. The existence of a multimerization domain residing in the C terminus of HIV-1 gag is suggested by the work of Trono et al. (19), who demonstrated that mutant HIV-1 gag proteins unable to generate particles themselves but possessing intact C termini inhibit particle formation of WT gag upon cotransfection. Nonfunctional gag mutants lacking C-terminal sequences were unable to inhibit particle production when coexpressed with WT gag.

Since it has been demonstrated that RNA packaging is not necessary for particle formation (28, 29), we feel that the inability of TC403 and TC363 to bind membranes is not due to loss of RNA binding. It is interesting to note that the domain (Arg-Ala-Pro-Arg-Lys-Lys-Gly) of these mutants lacks, which we have found to be important for membrane binding, has also been shown to bind RNA independently of the Cys-His arrays (30). Taken together, these data suggest that the NC protein may be important for both RNA binding and membrane binding.

Recent evidence from a number of researchers suggests that HIV-1 particle formation requires the full-length gag protein (17, 18). For example, Hoshikawa et al. (18) found that constructs expressing either C terminus truncations of HIV-1 gag proteins <48 kDa as well as p17 or p24 alone did not lead to particle formation. Our work suggests that this failure to form particles results from the inability of C-terminal truncation mutants to bind membranes.

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