Identification of Human Immunodeficiency Virus Type 1 Gag Protein Domains Essential to Membrane Binding and Particle Assembly

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Assembly of human immunodeficiency virus type 1 (HIV-1) particles occurs at the plasma membrane of infected cells. Myristylation of HIV-1 Gag precursor polyprotein Pr55^{Gag} is required for stable membrane binding and for assembly of viral particles. We expressed a series of proteins representing major regions of the HIV-1 Gag protein both with and without an intact myristyl acceptor glycine and performed subcellular fractionation studies to identify additional regions critical for membrane binding. Myristylation-dependent binding of Pr55^{Gag} was demonstrated by using the vaccinia virus/T7 hybrid system for protein expression. Domains within the matrix protein (MA) region downstream of the initial 15 amino acids were required for membrane binding which was resistant to a high salt concentration (1 M NaCl). A myristylated construct lacking most of the matrix protein did not associate with the plasma membrane but formed intracellular retrovirus-like particles. A nonmyristylated construct lacking most of the MA region also was demonstrated by electron microscopy to form intracellular particles. Retrovirus-like extracellular particles were produced with a Gag protein construct lacking all of p6 and most of the nucleocapsid region. These studies suggest that a domain within the MA region downstream from the myristylation site is required for transport of Gag polyprotein to the plasma membrane and that stable plasma membrane binding requires both myristic acid and a downstream MA domain. The carboxyl-terminal p6 region and most of the nucleocapsid region are not required for retrovirus-like particle formation.

Assembly of human immunodeficiency virus (HIV) particles occurs at the plasma membrane of infected cells. The major structural proteins of HIV originate as Pr55^{Gag}, the Gag polyprotein precursor, and Pr160^{Gag-Pol}, the product of a ribosomal frameshift near the carboxyl terminus of Pr55^{Gag}. These polyprotein precursors are produced from the unspliced genomic RNA on free ribosomes within the cytoplasm (29). Pr55^{Gag} and Pr160^{Gag-Pol} then move to the plasma membrane, where particles are initially seen as crescent-shaped buds on the plasma membrane. Complete budding leads to formation of immature particles with spherical cores, followed by HIV protease-mediated cleavage of the Gag and Gag-Pol precursor polyproteins and formation of mature HIV particles. The entire process of membrane binding, particle assembly, and maturation may occur in the absence of the viral envelope protein (12, 25), although the envelope protein may help direct Gag polyproteins to specific membrane assembly sites (15). Many details of Gag protein transport, membrane binding, and assembly of HIV particles remain to be elucidated. One important requirement which has been identified is myristylation of Pr55^{Gag}.

Cotranslational addition of the 14-carbon fatty acid myristic acid to the N-terminal glycine residue of a number of retroviral Gag proteins is essential for assembly of infectious viral particles. Myristic acid appears to play an important role in plasma membrane targeting and binding of these retroviral Gag polyproteins. Myristylation of Pr65^{Gag} of Moloney murine leukemia virus is required for membrane association and

budding of viral particles (3, 18). Myristylation of the Gag polypeptide of Mason-Pfizer monkey virus, a type D oncovirus, is not required for intracellular capsid assembly but is essential for transport of capsids to the plasma membrane for budding (20). The Gag and Gag-Pol precursors of HIV type 1 (HIV-1) are similarly modified with myristic acid, and this modification has also been shown to be essential for infectious particle production (1, 10). Moreover, subcellular fractionation studies comparing myristylated (Myr⁺) and nonmyristylated (Myr⁻) HIV-1 Pr55^{Gag} revealed that Myr⁺ Pr55^{Gag} binds to cellular membranes in a manner resistant to a high-ionic-strength solution (1 M NaCl), while the Myr⁻ Gag protein is dissociated from the membrane pellet by this treatment. This phenomenon has been designated "tight" membrane binding of myristylated Pr55^{Gag} (1). Some researchers have suggested that Myr⁺ Pr55^{Gag} may bind to a specific, cytoplasmic-face plasma membrane receptor, analogous to p60^{v-src} of Rous sarcoma virus (19, 29). While there is no direct evidence for such a myristyl-Gag receptor, the importance of myristylation of Pr55^{Gag} in membrane binding and subsequent assembly steps is clear.

Myristylation of Pr55^{Gag} may not be sufficient for its transport to the plasma membrane and stable membrane association, as evidenced by studies with other myristylated proteins. A single point mutation within the matrix protein (MA) of Mason-Pfizer monkey virus eliminated core transport to the plasma membrane, despite having no effect on the myristylation site (21). Similarly, a mutant form of p60^{v-src} which is myristylated but not membrane bound has been identified (7). Domains within Pr55^{Gag} other than the myristylation site may therefore be important for stable membrane binding. In this report, we describe membrane-binding studies with Pr55^{Gag}

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and a series of Myr⁺ and Myr⁻ Gag protein constructs with deletions of large regions of the polyprotein in order to identify the major regions involved in stable membrane binding. On the basis of these data, we propose that stable membrane binding of Pr55^{Gag} requires not only myristylation but also domains within MA which lie outside the myristylation site. In addition, we describe two Gag protein constructs lacking most of MA which form particles at an intracellular site, suggesting an important plasma membrane targeting role for this region. Furthermore, we describe a construct which lacks the carboxyl-terminal portion of Gag (p6 and most of the nucleocapsid [NC] region) but is still competent for transport, binding, and particle production at the plasma membrane.

MATERIALS AND METHODS

Construction of Gag and Gag-Pol expression plasmids. All HIV sequences were initially derived from HIV-1 proviral clone pHXB2gpt2 (17). Site-directed mutagenesis by the method of Kunkel (13) was used to introduce an *Nco*I site at the Gag initiation codon (primer 1) and to change the N-terminal glycine codon to an alanine codon (primer 2) (1):

*Nco*I primer 1, GGAGAGCCATGGGTGCGAGAGCGTCAGTATTAAGC;
*Nco*I primer 2, GGAGAGCCATGGCTGCGAGAGCGTCAGTATTAAGC.

The resulting products were sequenced to confirm the presence of the mutation. An *Nco*I-to-*Nco*I fragment containing the entire Gag and Pol coding regions was then cloned into the *Nco*I site of pTM3 to create plasmids pGPG1 (glycine-1) and pGPA1 (alanine-1). The correct orientation of the *gag* reading frame with respect to the T7 promoter was verified by restriction enzyme digestion analysis.

Additional expression constructs were made from pGPG1 and pGPA1. p55G1 and p55A1 were constructed by digestion of the relevant plasmids (pGPG1 and pGPA1, respectively) with *Bcl*I and filling in with T4 DNA polymerase and deoxynucleoside triphosphates. This created a frameshift mutation in the Pol reading frame beyond the Gag stop codon and introduced a stop signal at codon 126 of Pol. p41G1 and p41A1 were constructed by digestion of pGPG1 and pGPA1, respectively, with *Apa*I and frameshifting with T4 DNA polymerase. This mutation results in a frameshift at codon 407 of the Gag open reading frame and a termination codon 16 codons downstream. This construct was designated to express the p41 protein (MA plus CA), as well as the amino-terminal portion of the NC protein. p17G1 and p17A1 were constructed by digestion of pGPG1 and pGPA1, respectively, with *Nsi*I and religation. This resulted in deletion of the Gag reading frame after codon 154 and deletion of all of Pol.

The remaining constructs were produced via PCR to introduce a new *Cla*I site at the 5' end and a new *Bcl*I site at the 3' end of the p24 or p15 reading frame. To facilitate subcloning, a shuttle vector (pHXE) lacking *Cla*I or *Bcl*I sites and containing a single *Nco*I site was employed. pHXE had previously been constructed as follows. An *Eco*RI-to-*Eco*RI fragment (nucleotides 4647 to 5742) of HXB2 was cloned into the *Eco*RI site of SP65. The *Nco*I-to-*Nco*I fragment of pGPG1 was ligated into pHXE to form intermediate cloning vector pHXE/GPG1; an identical strategy used with pGPA1 created cloning vector pHXE/GPA1. The following oligonucleotide primers were used to amplify the p24 (CA) region:

Cla-24; TTGATCGATACCTATAGTGCAGAACATC;
Bcl-24; TTGTGATCACTAACTCTTGCCCTATGGCCGGGTCC.

The resulting PCR product was digested with *Cla*I and *Bcl*I and ligated into *Cla*I- and *Bcl*I-digested plasmids pHXE/

GPG1 and pHXE/GPA1. An *Nco*I-to-*Nco*I fragment was then subcloned from these products into the *Nco*I site of pTM3 to create p24G1 and p24A1. Thus, p24G1 and p24A1 express fusion proteins with the first 15 amino acids of MA (containing the myristylation site) attached to the CA protein. An identical strategy was used to create p15G1 and p15A1 by use of the following oligonucleotide primers:

Cla-15, TTGATCGATTAATGATGCAGAGAGGGCAATTTTAGG;
Bcl-15, TTGTGATCACTACTGTCTTACTTTGATAAAACCTCCAA.

p39G1 and p39A1 were constructed by using the *Cla*-24 and *Bcl*-15 primers described above, and they express the initial 15 amino acids of MA fused to CA plus p15. All constructs were checked by restriction digest analysis by using standard methods.

Cell culture and infection-transfection. BSC-40 (African green monkey kidney) cells were kindly provided by T. Braciale and maintained in Dulbecco's modified Eagle medium (supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 1 mM sodium pyruvate) at 37°C in 5% CO₂. Recombinant vaccinia virus VTF 7-3 (6) was supplied by C. Rice.

BSC-40 cells (1.5×10^6) were inoculated onto 100-mm² dishes on the evening prior to infection-transfection. Infection with VTF 7-3 was performed with an inoculum of 10 PFU per cell at room temperature, followed in 30 min by transfection with 10 µg of cesium chloride-purified plasmid DNA with lipofectin (GIBCO BRL, Gaithersburg, Md.) as described by the manufacturer.

Fractionation studies. Cells were harvested 7 to 8 h following transfection by being scraped into NTE buffer (0.10 M NaCl, 10 mM Tris [pH 7.4], 1 mM EDTA), gently pelleted at $500 \times g$, resuspended in ice-cold NTE, and pelleted again. Cells were then suspended in hypotonic lysis buffer (10 mM Tris [pH 7.4], 1 mM MgCl₂, pepstatin A at 1 µg/ml, antipain at 1 µg/ml, leupeptin at 1 µg/ml, aprotinin at 5 µg/ml, and phenylmethylsulfonyl fluoride at 1 mM). The cells were allowed to swell in this buffer for 15 min on ice and then were lysed with 15 to 25 strokes of a Dounce tissue homogenizer. Lysis was monitored by phase-contrast microscopy and terminated when 80 to 90% of the cells had broken and the nuclei remained intact. The solution was then adjusted to 0.15 M NaCl and centrifuged at $1,000 \times g$ for 10 min at 4°C to pellet the unbroken cells and nuclei. The resulting supernatant (S1) was used for further studies; the nuclear-cellular pellet was discarded. The S1 sample was divided into equal portions, and one half was adjusted to 1 M NaCl. Both the 0.15 and 1 M NaCl samples were incubated on ice for 10 min with intermittent gentle agitation. Samples were next centrifuged at $100,000 \times g$ in a 70.1 Ti rotor (Beckman Instruments, Fullerton, Calif.) at 4°C for 30 min. The supernatants (S100) were collected, and the pellets (P100) were collected in ice-cold NTE buffer with protease inhibitors. Total protein in each sample was assayed by using the BCA Protein Assay (Pierce, Rockford, Ill.). 5'-Nucleotidase activity was assayed in the S100 and P100 fractions of several experiments by using a commercial kit (Sigma Chemical, St. Louis, Mo.). Samples were concentrated prior to further analysis by precipitation with 10% trichloroacetic acid or via filtration in Centricon-10 microconcentrators (Amicon, Beverly, Mass.).

Western immunoblot analysis of fractionated samples. Proteins present in the soluble (S) and pellet (P) fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroblotting to nitrocellulose membranes. The total amount of protein per lane of the gel was equalized prior to electrophoresis; approximately

150 μ g of membrane or soluble protein was loaded per lane. Primary antibodies employed for probing of the membranes included anti-p24 mouse monoclonal antibody C246 (16) (provided by T. W. Chang, Tanox Biosystems, Houston, Tex.) for analysis of 55G1, 55A1, 41G1, 41A1, 24G1, and 24A1 products; anti-p17 rat monoclonal antibody G11G1 (24) (provided by A. Pinter, Public Health Research Institute, New York, N.Y.) for analysis of 17G1 and 17A1 products; and an anti-p6 rat monoclonal antibody (28) (provided by M. Sarngadharan and F. Veronese, Advanced Bioscience Laboratories, Kensington, Md.) for analysis of 15G1 and 15A1 products. Secondary antibodies conjugated with horseradish peroxidase were employed, and the immunoblots were developed by using enhanced-chemiluminescence techniques as recommended by the manufacturer (Amersham, Arlington Heights, Ill.). To establish the response linearity of this system for quantitation of detected protein in the supernatant and pellet fractions, serial dilutions of these fractions were prepared and analyzed by Western blotting as described above. Laser densitometry was performed on the blots with an Ultrascan XL densitometer (LKB, Bromma, Sweden). The sample amount loaded on each diagnostic blot was kept within the linear response range.

[³H]myristic acid labelling and radioimmunoprecipitation. BSC-40 cells were infected and transfected as described above. Following 4 h of incubation in complete medium, the medium was removed and replaced with Dulbecco's modified Eagle medium containing 5% delipidated fetal calf serum and 50 μ Ci of [³H]myristic acid per ml (55 Ci/mmol; Amersham). Following an additional 8 h of incubation, immunoprecipitation was performed with either pooled HIV-positive patient antiserum or a rabbit anti-p24 polyclonal antiserum. The rabbit anti-p24 serum was produced by inoculation of a New Zealand White rabbit with 100 μ g of recombinant soluble p24 (provided by American Biotechnology through the National Institutes of Health AIDS Research and Reference Reagent Program) followed by 100- μ g booster doses given 2 and 4 weeks later.

Fractionation of postnuclear supernatants. BSC-40 cells were used to produce HIV Gag protein constructs with the vaccinia virus/T7 hybrid system as described above. Following 3 h of incubation in complete medium, the cells were labelled for 4 h with [³H]leucine in leucine-deficient medium. The cells were then harvested and lysed, and a postnuclear supernatant (S1 fraction) was prepared as described above. This sample was then layered over a linear 15 to 50% sucrose gradient and centrifuged for 16 h at 4°C in an SW28.1 rotor (Beckman). Ten equal fractions were collected; an aliquot was used for quantitation of alkaline phosphodiesterase 1 activity (26). The density of sucrose in each fraction was determined by refractometry, and the remainder of the sample was immunoprecipitated by using anti-p24 rabbit serum (for the p55G1 and p39G1 products) or anti-p17 rat monoclonal antibody G11G1 (for p17G1). The resulting immunoprecipitates were analyzed by SDS-PAGE and fluorography with salicylic acid as the scintillant.

Particle isolation and sucrose density gradient analysis. Supernatants from BSC-40 cells infected with VTF 7-3 and transfected with each plasmid construct were labelled with 100 μ Ci of Tran³⁵S-label per ml (1,065 Ci/mmol; ICN, Irvine, Calif.) in Dulbecco's modified Eagle medium deficient in cysteine and methionine following 4 h of incubation in unlabelled medium. After an additional 12 h, supernatants were collected and debris was pelleted at 1,000 $\times$ g and discarded. The supernatants were then layered over a 20% sucrose cushion and centrifuged at 28,000 rpm in an SW28.1 rotor. The resultant pellet was carefully resuspended in cold phosphate-buffered saline, and an aliquot was analyzed by radioimmuno-

precipitation with pooled HIV-positive patient antiserum to detect specific viral proteins. Only samples positive by this analysis were subjected to further testing. For these samples, the remainder of the resuspended pellet was loaded over a 20 to 60% linear sucrose gradient and centrifuged at 20,000 rpm for 16 h at 4°C. Fifteen equal fractions were collected and analyzed by loading an aliquot of each fraction on an SDS-PAGE gel. The density of sucrose in each fraction was assessed by refractometry. Laser densitometry was performed on resulting fluorographs with an Ultrascan XL densitometer (LKB).

Electron microscopy. BSC-40 or CV-1 cells were infected with VTF 7-3 and transfected with plasmid DNA as described above. Following 16 h of incubation, the cells were harvested, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer containing 8% sucrose, washed in the same buffer overnight, and then postfixed with 1% osmium tetroxide at 4°C for 1 h. Dehydration with graded ethanol was performed, and the cell samples were embedded in Eponate-12 resin or Spurr's resin (Ted Pella, Redding, Calif.). Sections 70 nm thick were made, stained with uranyl acetate and lead citrate, and observed under a Zeiss EM 900 electron microscope. Additional sections were observed under a Zeiss 10A electron microscope.

RESULTS

Construction and characterization of Gag expression plasmids. Covalent attachment of myristic acid to proteins requires an intact N-terminal glycine residue, as well as a myristylation consensus sequence (27). To study the role of myristic acid and to decipher the possible role of other domains within Pr55^{Gag} in membrane binding, a series of Gag expression plasmids were constructed with either an intact myristyl acceptor glycine or a mutation converting the codon for glycine to a codon for alanine. This glycine-to-alanine mutation has been extensively characterized in a full-length proviral clone and found to prevent myristylation, eliminate particle production, and lead to less stable membrane binding of Pr55^{Gag} compared with myristylated Pr55^{Gag} (1).

The regions expressed in each construct are shown in Fig. 1. The parent plasmids pGPG1 and pGPA1 contain the entire HXB2 Gag-Pol open reading frames within expression plasmid pTM3 (Fig. 1A). The p55G1 and p55A1 constructs were designed with a frameshift mutation within the protease gene to study membrane binding and particle formation of Pr55^{Gag} in the absence of proteolytic cleavage products (Fig. 1B). p41G1 and p41A1 express regions of Pr55^{Gag} similar to the intermediate cleavage product p41, although our constructs extend into the amino terminus of NC (frameshifted at codon 407, just after the first cysteine-histidine box coding region). p17G1 and p17A1 express myristylated and nonmyristylated MA protein, respectively. p24G1 and p24A1 were designed to express the p24 (CA) region of Gag but with the N-terminal myristylation acceptor sequence (the first 15 amino acids of p17) attached. The reasons for this experimental design were twofold. (i) It is possible that the myristate moiety functions as a membrane-targeting signal in addition to its role in membrane binding. If p24 itself contains any domains important to membrane binding, it may require a myristate-mediated transport mechanism for this to be apparent in our membrane-binding assay. (ii) If the myristylation site is the only requirement for stable membrane binding of Pr55^{Gag}, then attachment of this site to the p24 region or to other regions should confer upon the resultant protein the binding characteristics of the intact, myristylated polyprotein. The p15G1 and p15A1 constructs were designed in a similar manner to study the possible membrane interactions of this region, and p39G1 and

FIG. 1. Schematic diagram of HIV-1 Gag and Gag-Pol constructs. (A) pGPG1 and pGPA1 express the entire Gag and Gag-Pol reading frames of HIV-1 proviral clone HXB2. pGPG1 contains an intact myristylation acceptor glycine; pGPA1 is identical except for a single nucleotide change resulting in an N-terminal alanine. The indicated coding regions were expressed by using the T7 promoter in plasmid pTM3. P_{T7}, T7 promoter; EMC, encephalomyocarditis virus leader sequence; T_{T7}, T7 transcriptional terminator. (B) Gag protein constructs expressing the shaded regions of the Gag gene. Constructs derived from pGPG1 and expressing the wild-type myristylation acceptor glycine are labelled M. On the right is a summary of the results of the membrane-binding and particle formation studies discussed in the text.

p39A1 were designed to express p24 (CA) together with p15 (NC and p6).

To establish the applicability of the vaccinia virus/T7 expression system for binding experiments with HIV Gag proteins, we first examined whether Gag products were produced and myristylated appropriately. The expression of Gag proteins by pGPG1 and pGPA1 is shown in Fig. 2A. Pr55^{Gag} and cleavage products p41 and p17 are demonstrated in this immunoblot. In separate experiments, cleavage product p24 was also demonstrated (Fig. 2C). No difference between the products of pGPG1 and pGPA1 was apparent in this experiment. In contrast, upon labelling with [³H]myristic acid and immunoprecipitation with anti-p24 serum, bands were seen at 55 and 41 kDa for the pGPG1 product while no label was seen for pGPA1 (Fig. 2B). This confirms that Pr55^{Gag} and cleavage product p41 originating from pGPG1 are appropriately myristylated, while the proteins with the alanine mutation are not. It should be noted that the processing defect previously reported for nonmyristylated Pr55^{Gag} in transfected HeLa cells (1) was not observed in this more highly efficient expression system.

Membrane fractionation and particle formation studies

with pGPG1 and pGPA1. Previous studies have shown that Pr55^{Gag}, when expressed with a full-length proviral clone, binds to membranes in a manner resistant to a high salt concentration (1 M NaCl), while nonmyristylated Pr55^{Gag} binds under physiologic salt conditions (0.15 M NaCl) but dissociates from membranes at 1 M NaCl (1). We next sought to demonstrate that the membrane binding seen with Pr55^{Gag} produced in our vaccinia virus-based expression system would reflect this characteristic of myristylation-dependent binding. A subcellular fractionation study was performed on cells expressing proteins from pGPG1 or pGPA1. The distribution of proteins in the soluble fraction (S) or membrane pellet (P) was compared by immunoblotting (Fig. 2C). Control lanes included lysates from cells transfected with expression plasmid pTM3 alone and processed identically; no protein bands were seen in these lanes. With a low-salt (0.15 M NaCl) buffer, Pr55^{Gag} was found primarily in the pellet fraction for both myristylated and nonmyristylated samples. At 1 M NaCl, although some of the myristylated Pr55^{Gag} was found in the soluble fraction, most remained in the pellet. The nonmyristylated protein, on the other hand, was found entirely in the soluble fraction when 1 M NaCl was employed. This result is similar to those obtained with fractionations performed with cells expressing products of integrated proviruses (1). Myristylation-dependent binding of p41, which is resistant to 1 M NaCl, is also evident in Fig. 2. In contrast, p24 was present primarily in the soluble (cytosolic) fraction.

To demonstrate further the usefulness of this expression system for examination of the membrane binding and assembly of HIV Gag proteins, the supernatants of [³⁵S]cysteine-methionine-labelled cells expressing pGPG1 and pGPA1 protein products were collected and screened for particle production. The pGPG1 construct was demonstrated to produce retrovirus-like particles which were released into the supernatant and banded upon density equilibrium centrifugation at 1.16 to 1.17 g/ml (Fig. 2D). This result was confirmed by electron microscopy (data not shown). The supernatants from pGPA1-transfected cells contained no retrovirus-like particles as determined by pelleting through a 20% sucrose cushion and analysis on SDS-PAGE (data not shown). Although the production of retrovirus-like particles in vaccinia virus systems has previously been reported by several investigators (4, 12, 25), it serves to validate the ability of the myristylated constructs used in these experiments to produce HIV-1 Gag proteins which form particles which bud from the plasma membrane.

Expression and characterization of Gag protein constructs. The 12 Gag constructs shown schematically in Fig. 1B were expressed in BSC-40 cells by using the vaccinia virus/T7 hybrid system. Immunoblot analysis of cell lysates is shown in Fig. 3A. Proteins of the predicted size were produced from all of the expression constructs, and no differences in mobility were seen between myristylated and nonmyristylated proteins. Figure 3B demonstrates [³H]myristic acid-labelled proteins detected by immunoprecipitation and fluorography. Only those constructs with glycine-1 intact are labeled.

Subcellular localization of mutant Gag proteins. Fractionation studies were performed with both low (0.15 M) and high (1 M) NaCl concentrations to map the regions important for myristylation-dependent, salt-resistant binding. A 5'-nucleotidase assay confirmed that greater than 85% of this plasma membrane marker was present in the pelleted material in repeated experiments. Results from experiments with the protein products of p55G1 and p55A1, p41G1 and p41A1, and p17G1 and p17A1 are shown in Fig. 4A. The p55G1 product was found primarily in the pellet at low and high salt concentrations, while the 55-kDa product lacking myristic acid was

FIG. 2. Expression and characterization of Gag-Pol constructs with the vaccinia virus/T7 RNA polymerase hybrid system. (A) Western blot of lysates of BSC-40 cells expressing protein products of pGPG1 and pGPA1; pTM3-transfected cells served as a negative control. The primary antibody used was an anti-p17 rat monoclonal antibody. The positions of molecular size markers are indicated on the left in kilodaltons. (B) Results of $[^3\text{H}]$ myristic acid labelling and immunoprecipitation with rabbit polyclonal anti-p24 serum. Molecular size marker positions are indicated on the left in kilodaltons. (C) Western blots of membrane fractionation studies expressing protein products of pGPG1 and pGPA1. Cells expressing pTM3, pGPG1, and pGPA1 were lysed by hypotonic lysis and Dounce homogenization, and nuclei and unbroken cells were removed by low-speed centrifugation. The samples were divided equally into low-salt (0.15 M NaCl) and high-salt (1 M NaCl) portions and subjected to centrifugation at $100,000 \times g$ for 30 min. Supernatant (S) and membrane pellet (P) fractions were collected and analyzed by SDS-PAGE and immunoblotting with mouse anti-p24 monoclonal antibody and enhanced-chemiluminescence detection. Molecular size marker positions are indicated on the left in kilodaltons. (D) Particle formation analysis of pGPG1-transfected cellular supernatant. The pGPG1 protein product was expressed in BSC-40 cells and labelled with $[^{35}\text{S}]$ cysteine-methionine as

FIG. 3. Expression and characterization of Gag protein constructs. (A) Western blotting of cell lysates. Proteins were expressed in BSC-40 cells, and cell lysates were examined by immunoblotting with a mouse anti-p24 monoclonal antibody (pTM3, 55G1, 55A1, 41G1, 41A1, 24G1, 24A1, 39G1, or 39A1), a rat anti-p17 monoclonal antibody (17G1 or 17A1), or a rat anti-p6 monoclonal antibody (15G1 or 15A1). Blots were developed by using enhanced-chemiluminescence techniques. (B) $[^3\text{H}]$ myristic acid labelling and immunoprecipitation. Proteins expressed in BSC-40 cells were labelled with $[^3\text{H}]$ myristic acid and immunoprecipitated with rabbit anti-p24 serum (pTM3, 55G1, 55A1, 41G1, 41A1, 24G1, 24A1, 39G1, or 39A1) or pooled HIV-positive patient antiserum (17G1, 17A1, 15G1, or 15A1). Analysis was via SDS-PAGE and fluorography. Molecular mass marker positions are indicated on the left in kilodaltons.

located entirely in the soluble fraction when 1 M NaCl was employed. This result is consistent with previous observations both in full-length proviral expression (1) and with the vaccinia virus/T7 system expression of pGPG1 and pGPA1 discussed above. The phenotype of myristylation-dependent, salt-resistant binding was also demonstrated for the protein product of p41G1 (Fig. 4A, middle) and for the p17G1 product (Fig. 4A, bottom). The ability of the p17 (MA) region to bind to membranes in this manner suggests that this region alone is sufficient to confer upon the Pr55^{Gag} polyprotein its membrane-binding properties.

To determine whether myristic acid itself is sufficient for membrane binding or if a domain within MA or another region of Pr55^{Gag} in conjunction with myristic acid is required, a series of six Gag protein constructs lacking most of MA but retaining the N-terminal 15 codons were constructed, expressed, and tested by subcellular fractionation. The results are shown in Fig. 4B. The p24G1 product was seen in the pellet under low-salt conditions (41% in the pellet by laser densitometry of an enhanced-chemiluminescence immunoblot), but the

described in the text. Supernatants were centrifuged through a 20% sucrose cushion, and the pellet was loaded on a 20 to 60% linear sucrose gradient. An aliquot of each fraction was run on an SDS-12% PAGE gel. The results of laser densitometry versus sucrose density are shown above the corresponding fluorograph. The bottom of the gradient is at the left. Symbols: ●, sucrose density (in grams per cubic centimeter); □, absorbance.

FIG. 4. Membrane fractionation studies with Gag protein constructs. Proteins were expressed in BSC-40 cells as described in the text. Cells were lysed by hypotonic lysis and Dounce homogenization followed by centrifugation at $1,000 \times g$ to remove nuclei and unbroken cells. Samples were then divided into low-salt (0.15 M NaCl) and high-salt (1 M NaCl) portions and subjected to centrifugation at $100,000 \times g$ for 30 min. Supernatant (S) and membrane pellet (P) fractions were collected and examined by SDS-PAGE and immunoblotting with the appropriate monoclonal antibody (see Materials and Methods). Molecular mass marker positions are indicated on the left in kilodaltons.

amount present in the pellet when 1 M NaCl was employed decreased to 20%. The proportion of the p24G1 product in the membrane pellet under high-salt conditions (20%) was much lower than that seen for the protein products containing the MA region (70% for p55G1). This result was consistent in three separate experiments. The change in distribution from a low to a high salt concentration with this protein was also much more dramatic than that seen with the products of p55G1, p41G1, and p17G1, suggesting a weaker or more easily dissociable interaction. The difference in soluble versus pellet fraction distribution was not due to inefficient myristylation of the p24G1 product (data not shown). The p15G1 product, in contrast, was found entirely in the membrane pellet when a low salt concentration was used and entirely in the soluble fraction when a high salt concentration was employed. Thus, this construct failed to demonstrate any salt-resistant membrane binding. Taken together, these data suggest that domains within the p17 region of Pr55^{Gag}, but not the myristylation site alone, are necessary for the phenotype of high salt concentration-resistant membrane binding. The possibility of a lesser contribution to the myristylation-dependent binding of the intact Pr55^{Gag} molecule from the CA region cannot be ruled out.

Fractionation studies with the p39G1 and p39A1 products are shown in the top panels of Fig. 4B. These constructs, which express p24 (CA) and p15 (NC and p6) but lack all of MA downstream of the N-terminal 15 amino acids, would be predicted by the results discussed above to lack high salt concentration-resistant binding. However, both constructs are present in the pellet to a significant degree, even with a high salt concentration. Remarkably, the 39G1 product was found entirely in the membrane pellet. This result led to the hypothesis that these protein products demonstrate pelleting on

another basis, not related to plasma membrane binding, and led to further experiments (discussed below). A summary of the results of fractionation studies with all 12 of the Gag protein constructs is presented in Fig. 1B, for which densitometry results were designated + for significant membrane binding (presence of at least 40% of the total soluble and pellet fractions in the membrane pellet), +/- for lesser degrees of binding (15 to 39%), and - for minimal presence in the pellet (less than 15%). Each construct was tested in a minimum of three separate experiments to generate these results. The fractionation studies with Gag products containing the alanine-1 mutation revealed that these proteins all were present principally in the soluble fraction at the high salt concentration (with the single exception of p39A1). However, the Myr⁻ constructs containing the p15 region of Pr55^{Gag} (p55A1 and p15A1) were found in the membrane pellet at the low salt concentration, while those lacking this region (p41A1, p17A1, and p24A1) were not. This suggests that there is a domain within p15 which mediates myristylation-independent membrane binding. This type of binding is dissociated by a high salt concentration.

Subcellular fractionation on sucrose density gradients. The fractionation scheme utilized for the experiments discussed above may not distinguish pelleting of a membrane-bound protein from other explanations for pelleting, such as the formation of large aggregates or the pelleting of Gag proteins in the form of virus-like particles. To address this concern, radiolabelled postnuclear supernatants from BSC-40 cells expressing the p55G1, p17G1, and p39G1 protein products were further examined by centrifugation on linear sucrose gradients. The results are shown in Fig. 5. For this series of experiments, a marker for the presence of the plasma membrane, alkaline

FIG. 5. Sucrose gradient analysis of intracellular Gag protein production. (A) Postnuclear (S1) supernatants prepared from BSC-40 cells labelled with [3 H]leucine and expressing the indicated protein products were layered over 15 to 50% sucrose gradients. Centrifugation was performed at 20,000 rpm for 16 h in an SW28.1 rotor. Equal fractions were collected and analyzed by immunoprecipitation with rabbit anti-p24 sera (55G1 and 39G1) or a rat anti-p17 monoclonal antibody (17G1) followed by SDS-PAGE and fluorography. The resulting fluorographs are shown; molecular mass markers (lanes M) are indicated at the left in kilodaltons; the bottoms of the gradients are on the left. Faint bands were present in fractions 2 and 3 from the sucrose gradient of 39G1. Triton X-100 was added to the S1 fraction expressing the p39G1 product prior to centrifugation to a concentration of 0.5% (bottom fluorograph; B, pellet resuspended from bottom of tube). (B) Alkaline phosphodiesterase 1 activity was assayed in each fraction of the gradients; a representative result from the experiment with p55G1 is shown in the top graph plotted versus sucrose density. The left ordinate scale is relative enzyme activity as determined by a spectrophotometric assay. The results of laser densitometric analysis of the fluorographs of the 55G1 product (left ordinate scale, in relative absorbance units) and the 39G1 product (right ordinate scale) are plotted on the bottom graph. The sucrose density plot from the 55G1 experiment (shown in the top graph) was nearly identical to that of the 39G1 experiment (data not shown).

phosphodiesterase 1, was measured in each fraction. As shown in the top graph of Fig. 5B, this enzyme was found to have a wide distribution on the 15 to 50% gradient employed but was concentrated primarily in the upper fractions of the gradient (fractions 6 to 10). The myristylated p55G1 product, which should bind to the plasma membrane, was found primarily in fractions 7 to 10, thus overlapping the plasma membrane-containing fractions (Fig. 5A, top, and 5B, bottom, open squares). The p17G1 product was also found after fractionation in these upper fractions of the gradient, although the distribution was not identical (Fig. 5A). In contrast, the p39G1 protein was seen primarily in the denser fractions (primarily fractions 2 and 3), at a sucrose density of 1.15 to 1.17 g/ml. Triton X-100 has been used to remove the lipid envelope of retroviral particles and results in a shift in density to approximately 1.25 g/cm³ (31). Addition of 0.5% Triton X-100 to the postnuclear supernatant prior to centrifugation completely eliminated the p39G1 product from the gradient fractions, but a pellet of protein was found at the bottom of the tube (Fig. 5A, bottom gel, B), corresponding to a density of greater than

1.175 g/cm³. These results are consistent with the formation of intracellular retrovirus-like particles by this construct. Addition of 0.5% Triton X-100 to the postnuclear supernatants of cells expressing p55G1 and p17G1 did not result in this marked shift of protein to the bottom of the tube; a small shift to the upper two gradient fractions was noted (data not shown). The marked difference in distribution between the p55G1 and p39G1 proteins is illustrated by the bottom graph of Fig. 5B (open squares, p55G1; dark squares, p39G1).

Particle formation studies with mutant Gag constructs. One indirect indicator of plasma membrane binding for HIV subviral constructs is extracellular particle formation, since budding must occur through this membrane. For this reason, we examined all 12 Gag protein constructs for extracellular particle formation by radiolabelling and centrifugation of cell-free supernatants through a 20% sucrose cushion. Three constructs consistently were found to produce proteins in the supernatant which pelleted through 20% sucrose: p55G1, p41G1, and p24G1. One construct (p39G1) was inconsistently positive in this analysis, possibly secondary to cell lysis or low-level

FIG. 6. Retrovirus-like particle detection. Supernatants from cells expressing the indicated Gag protein construct and labelled with [³⁵S]methionine-cysteine were clarified by low-speed centrifugation followed by centrifugation through 20% sucrose cushions. The resulting pellets were then loaded on 20 to 60% linear sucrose gradients and subjected to density equilibrium centrifugation. Densitometry of the fluorograph of fractions (□) and sucrose density (●) are shown. The corresponding fluorograph is shown below. Panels: A, 55G1 particle analysis; B, 41G1 particle analysis.

extracellular particle formation (see electron microscopy results below). None of the nonmyristylated proteins were found in the supernatants of infected-transfected cells. The results of density equilibrium centrifugation on sucrose gradients for cells expressing p55G1 and p41G1 products are presented in Fig. 6. Both experiments show evidence of retrovirus-like particles banding at 1.16 to 1.17 g/ml. The 24G1 protein, although it did pellet through 20% sucrose, did not band at this density but instead travelled into the top three or four fractions of the gradient (data not shown). Thus, only two constructs, both containing the matrix protein and an intact myristylation site, were able to produce retrovirus-like particles which were released into the supernatant. These two constructs are indi-

cated in the table portion of Fig. 1B by a plus sign for extracellular particle formation.

Electron microscopic examination of retrovirus-like particles. To confirm the results of the particle studies described above and to examine the site of intracellular particle formation by the p39G1 product, we performed transmission electron microscopy on BSC-40 cells expressing the p55G1, p55A1, p41G1, p39G1, p39A1, and p17G1 protein products. No retrovirus-like particles were seen in cells expressing the full-length Gag protein in its nonmyristylated form (p55A1) or from cells expressing the p17G1 product (data not shown). Retrovirus-like particles with immature morphology were seen budding from the plasma membrane of cells transfected with p55G1 (Fig. 7A). These particles were approximately 100 nm in diameter and were easily distinguished from the intracellular vaccinia viruses. As predicted by sucrose gradient analyses, the p41G1 product also formed particles on the plasma membrane which were released into the supernatant (Fig. 7B). Most of the particles produced by this construct were seen as pictured outside of cells; rarely, a few incomplete ring forms were seen in the cytoplasm of a transfected cell (data not shown). The particles produced at the plasma membrane by this construct were indistinguishable from those produced by p55G1.

The formation of particle-like structures from construct p39G1 within the cytoplasm of cells was confirmed by electron microscopy. As shown in Fig. 7C, perinuclear collections of particles were frequently found. Both complete ring-shaped particles and more bizarre appearing, incomplete rings were seen in these discrete locations. Collections of particles were also found in some cells at locations farther from the nucleus (data not shown). We were unable to find particles budding from the plasma membrane with this construct. Complete lysis of some cells with release of numerous particles was also noted. A specific membrane compartment in which these particles formed was not identified; it appeared that fine cellular morphology had been disrupted by either vaccinia virus infection, processing of cells, or perhaps production of large numbers of these retrovirus-like particles. A somewhat unexpected finding was the production of particle-like forms in the cytoplasm with the nonmyristylated p39A1 construct (Fig. 7D). These particles had an intracellular distribution and morphology identical to those of the particles produced by p39G1.

DISCUSSION

The importance of myristylation of Pr55^{Gag} to plasma membrane binding has been well established. When the myristylation acceptor glycine is mutated, membrane binding is abrogated and particle assembly is prevented. However, the precise mechanism by which myristic acid promotes HIV particle assembly is not clear. Some myristylated proteins, exemplified by the myristylated, alanine-rich C kinase substrate (MARCKS) protein, appear to interact with the plasma membrane by a simple hydrophobic interaction between myristate and the lipid bilayer (8). Poliovirus protein VP0, on the other hand, is a myristylated protein which plays an important role in the stability of viral capsids but does not appear to interact directly with cellular membranes (14). Another myristylated viral protein, p60^{v-src} of Rous sarcoma virus, has been reported to interact with a cytoplasmic-face-plasma membrane receptor (19). In this last example, both myristic acid and amino acid sequences unique to the Src protein are required for interaction with the putative receptor.

We now present evidence that domains within the MA protein of Pr55^{Gag} but distinct from the myristylation site itself

FIG. 7. Electron microscopic analysis of particle formation. BSC-40 cells expressing Gag protein constructs were analyzed by transmission electron microscopy. (A) 55G1 particle analysis (magnification, $\times 24,400$; bar, 400 nm). (B) 41G1 particle analysis (magnification, $\times 21,500$; bar, 500 nm). (C) 39G1 intracellular particle analysis (magnification, $\times 40,000$; bar, 250 nm). Note the nucleus in the upper left-hand corner. (D) 39A1 intracellular particle analysis (magnification, $\times 53,100$; bar, 200 nm).

are essential for stable binding of Pr55^{Gag} to the plasma membrane. In addition, we present evidence that a domain(s) downstream from the myristylation site in MA is required for targeting to the plasma membrane; myristic acid and the N-terminal 15 amino acids are not sufficient for this transport. The current study used a vaccinia virus-based protein expression system which has previously been shown to be capable of producing HIV-like particles which bud from the plasma membrane in a manner analogous to particle assembly in HIV-infected cells (4, 12). The phenotype of myristylation-dependent tight membrane binding (resistant to 1 M NaCl) which was previously described for Pr55^{Gag} produced by an integrated provirus (1) was reproduced in this system by using a series of subviral constructs. Only those protein constructs containing the intact myristylation site and MA demonstrated this stable form of binding. A myristylated Gag protein lacking most of MA was found to pellet in a simple fractionation assay even after treatment with 1 M NaCl, but this pelleting was demonstrated to be due to intracellular particle formation and not to plasma membrane association. In addition, the expression of the first 15 amino acids of MA was not sufficient to confer the salt resistance phenotype on other regions of Pr55^{Gag} downstream of MA. This strongly suggests that domains within p17 (MA) but outside of the myristylation site are required for plasma membrane binding in concert with myristate.

Important to the interpretation of the data in this report is the potential for Gag proteins to multimerize and possibly to form insoluble aggregates. The presence of Gag proteins in the pellet after ultracentrifugation of cell lysates could be due to association with membranes or sedimentation of insoluble Gag multimers. If the results of this study reflect pelleting of multimers or aggregates not associated with membranes, then myristic acid and domains within p17 must be essential to the stability of these multimers or aggregates in the presence of 1 M NaCl. Although we have not totally ruled out this possibility, the sucrose gradient analysis of the p55G1 and p17G1 products showing these proteins in the upper, plasma membrane-rich fractions is presumptive evidence that we examined plasma membrane binding.

The importance of domains within MA for membrane binding and particle assembly is not unique to HIV-1 among the retroviruses. Wills et al. studied particle formation and membrane binding of Rous sarcoma virus Pr76^{Gag}, a nonmyristylated Gag protein, by utilizing a number of proviral constructs with deletions in MA (30). They found that these mutant Gag proteins were defective in particle formation, although they could be rescued into particles by coexpression of constructs with an intact MA. Deletions or substitutions within the MA protein of the myristylated Gag proteins of Mason-Pfizer monkey virus (22) and Akv murine leukemia virus (11) also interfere with virus particle assembly. A previous study of deletions in the MA protein of HIV-1 reported a decrease in particle production for some mutants, as well as a defect in incorporation of the HIV envelope protein (32). A proviral construct with a large internal deletion of the MA protein but an intact myristylation site produced particles which budded into the endoplasmic reticulum (5), a result which is consistent with our sucrose gradient and electron microscopic data on the p39G1 product. This result suggests an important plasma membrane targeting role for domains within the MA protein of HIV-1.

The ability of a nonmyristylated Gag protein lacking most of MA (the p39A1 product) to form intracellular particles in the cytoplasm of cells is intriguing. This provides evidence that myristic acid is not necessary for multimerization of the Pr55^{Gag} molecule or for the formation of a recognizable

particle structure. However, the many abnormally formed ring structures seen by electron microscopy of both the p39G1 and p39A1 products suggests that the MA region plays an important part in the ordered formation of the immature particle. Whether these particles form in association with an intracellular membrane has not been established.

While the importance of domains within p17 for membrane binding and particle assembly seems clear, the role of the carboxyl-terminal p15 region is less evident. We found that a myristylated protein construct lacking p6 and two-thirds of NC produced retrovirus-like particles which were released into the medium. Gottlinger et al. have shown that deletion of p6 sequences in proviral constructs leads to a defect in particle maturation and a marked reduction in particles release from the cell (9). The fact that our 41G1 particles were released into the supernatant may reflect differences in our construct from that of Gottlinger et al. (our construct also deleted the distal two-thirds of the NC protein and contained a short stretch of heterologous amino acids at the carboxyl end) or may be due to differences in the expression system employed. The current findings are also consistent with a report of particle production from an HIV Gag protein construct lacking all of p15 expressed in baculovirus-infected cells (23). Taken together, these studies indicate that p6 is not absolutely required for retrovirus-like particle formation, although it may still play an important role in the HIV life cycle. The finding that a membrane-association domain also exists in the p15 region of Pr55^{Gag} which is independent of myristylation is a novel finding but of unclear significance. We have also demonstrated the membrane-binding characteristics of this region in a reticulocyte lysate-based *in vitro* membrane-binding assay (data not shown).

Several models of HIV particle assembly may be postulated to explain the results presented in this report. In one model, the Pr55^{Gag} molecules produced on free ribosomes first travel to an intracellular location, where these molecules interact to form an undefined assembly intermediate which is invisible by electron microscopy. An assembly domain within MA but downstream from the N-terminal 15 amino acids then mediates transport of assembly intermediates to the plasma membrane. Myristylation-dependent tight plasma membrane binding then occurs, forming a nucleation site for addition of further Gag protein and ordered particle formation. Loss of the transport function of MA (as with p39G1 or p39A1) would lead to accumulation of Gag proteins at the intracellular site, which multimerize and are able to form particles at this site. Nonmyristylated Gag proteins with an otherwise intact MA (such as the p55A1 and p41A1 constructs) may be transported away from the intracellular assembly site but fail to bind to the plasma membrane in the necessary fashion. Alternatively, the same transport domain in MA may facilitate movement of Gag polyproteins as monomers to the plasma membrane, where myristylation-dependent membrane binding and multimerization occur. Additional studies to define the specific steps in intracellular Gag protein targeting, multimerization, and plasma membrane binding are under way.

What do the additional domains within p17 required for transport and stable binding of Pr55^{Gag} represent? A recent report of phosphorylation of HIV-1 MA protein at serine 111 by protein kinase C raises one intriguing possibility (2). The researchers report that phosphorylation at this site may enhance membrane binding. It is possible that both myristylation and phosphorylation are important for steps in membrane targeting, binding, and subsequent assembly. Further studies are needed to decipher these crucial steps in HIV particle assembly.

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