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Structural Interactions between Retroviral Gag Proteins Examined by Cysteine Cross-Linking

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We have examined structural interactions between Gag proteins within Moloney murine leukemia virus (M-MuLV) particles by making use of the cysteine-specific cross-linking agents iodine and *bis*-maleimido hexane. Virion-associated wild-type M-MuLV $Pr65^{Gag}$ proteins in immature particles were intermolecularly cross-linked at cysteines to form $Pr65^{Gag}$ oligomers, from dimers to pentamers or hexamers. Following a systematic approach of cysteine-to-serine mutagenesis, we have shown that cross-linking of $Pr65^{Gag}$ occurred at cysteines of the nucleocapsid (NC) Cys-His motif, suggesting that the Cys-His motifs within virus particles are packed in close proximity. The M-MuLV $Pr65^{Gag}$ protein did not cross-link to the human immunodeficiency virus $Pr55^{Gag}$ protein when the two molecules were coexpressed, indicating either that they did not coassemble or that heterologous Gag proteins were not in close enough proximity to be cross-linked. Using an assembly-competent, protease-minus, cysteine-minus $Pr65^{Gag}$ protein as a template, novel cysteine residues were generated in the M-MuLV capsid domain major homology region (MHR). Cross-linking of proteins containing MHR cysteines showed above-background levels of Gag-Gag dimers but also identified a novel cellular factor, present in virions, that cross-linked to MHR residues. Although the NC cysteine mutation was compatible with M-MuLV particle assembly, deletions of the NC domain were not tolerated. These results suggest that the Cys-His motif is held in close proximity within immature M-MuLV particles by interactions between CA domains and/or non-Cys-His motif domains of the NC.

Successful completion of the retrovirus life cycle requires that viral components in an infected cell colocalize and assemble particles that bud through the plasma membrane, mature properly, enter a target cell, and integrate their genomes into the genome of the host. During assembly of Moloney murine leukemia virus (M-MuLV), core (Gag) proteins form a protein capsid including the viral genomic RNA and enzymatic (Pol) proteins, which are synthesized by a termination suppression mechanism in approximately 5% of Gag translations that produces the fusion protein GagPol. Expression of the Gag protein is necessary and sufficient for the formation of enveloped, virus-like particles (13, 22, 36, 43), and assembly of type C retroviruses such as M-MuLV usually occurs at the plasma membrane (44) but also can occur on intracellular membranes (14), which may or may not be a productive route of assembly.

The Gag protein of M-MuLV is synthesized as a myristylated precursor polypeptide, $Pr65^{Gag}$ (16), which is processed by the viral protease (PR) during or after budding into four mature Gag proteins: matrix (MA), p12, capsid (CA), and nucleocapsid (NC) (2, 5, 29, 38, 46). The MA protein is myristylated (34), associates with membranes, and may contact the carboxy-terminal region of the viral transmembrane (Env) protein (44, 48). The M-MuLV p12 protein has no avian or primate retroviral homologs, and a large portion of the p12 protein has been shown to be dispensable for virus particle assembly (7). In contrast, the Gag capsid domain has been shown to facilitate interactions between $Pr65^{Gag}$ proteins and appears to be essential for particle assembly (15, 17, 18, 41, 45). Within the CA protein sequence lies the determinant of murine C-type NIH-BALB/c (N/B) cell tropism (44), as well as the major homology region (MHR), a stretch of 20 to 30 residues which represents the only highly conserved sequence among retroviral CA proteins (24, 40, 45). At the C terminus of CA is the NC protein, which specifically binds viral genomic RNA and is required for RNA encapsidation (4, 12); point mutations in the nucleocapsid Cys-His finger eliminate this function (11).

Although general functions have been attributed to the mature M-MuLV Gag protein products, little is known of specific protein-protein contacts in immature or mature virions. Some genetic analyses have implicated CA as a major region of intermolecular contacts (17, 18, 42), and Gag-Gag binding in a yeast two-hybrid system has been attributed to the CA domain (23). The pioneering use of cross-linking agents to examine virus structure (10, 32, 33) demonstrated that chemical treatment of avian retroviruses produced homotypic dimers of all mature Gag proteins but did not yield any heterotypic dimers (33). Similar results were shown for murine C-type retroviruses, in which matrix and nucleocapsid domains also cross-linked in $Pr65^{Gag}$ (32). A limitation of these experiments is that the cross-linkers employed were amine reactive and had multiple reaction target sites, making it difficult to identify specific regions of protein-protein contact. To extend previous results, we have adapted the method of Pakula and Simon (31) for specific cross-linking at cysteine residues, in which proteins or protein complexes are treated with membrane-permeable cysteine-specific cross-linking agents, such as molecular iodine or *bis*-maleimido hexane (BMH), and cysteines on neighboring proteins can form a covalent bond, creating an intermolecular cross-link. Using these reagents, we have found that neither mature CA nor NC showed significant homodimerization but that $Pr65^{Gag}$ in immature particles cross-linked via NC cysteines to form oligomers up to pentamers or hexamers. $Pr65^{Gag}$ cross-linking was resistant to nonionic but sensitive to ionic detergent treatments, and $Pr65^{Gag}$ did not cross-link with

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coexpressed human immunodeficiency virus (HIV) $Pr55^{Gag}$. Cysteines created in the MHR of the M-MuLV capsid domain showed increased levels of $Pr65^{Gag}$ intermolecular cross-linking and also cross-linked to a nonviral protein with a molecular size of 140 to 160 kDa. Interestingly, while cysteine-minus Gag proteins formed virus-like particles of wild-type (wt) density, implying that the NC Cys-His motif is unnecessary for assembly, deletion of the NC domain greatly reduced the assembly efficiency and stability of particles.

MATERIALS AND METHODS

Recombinant constructs. The M-MuLV stable expression vectors 2051T *dl* NC and 2189T are nucleocapsid (NC) truncation mutants derived from previously described Gag–β-galactosidase fusion proteins (18) by replacing the β-galactosidase moiety with a termination signal. The vector 3229T C504S/C507S (numbers indicate Gag cysteine codons mutated to serines) has the same plasmid backbone and was derived from C26S/C29S (numbers indicate NC codons; the mutations are identical), the kind gift of Alan Rein, and has been described before (11). The sequences at gag termination junctions, in which the underlined nucleotide (nt) marks the M-MuLV nt position (37) designated in the construct name and TAA is the termination codon, are as follows: 2051T *dl* NC, 5′ CTA TTG GCG GAT CCC CCT **TAA** GTT AAC TTA AGG GCT GCA GGA ATT 3′; 2189T, 5′ GGA CCT CGG CGG GAT CCC CCT **TAA** GTT AAC TTA AGG GCT GCA GGA ATT 3′; and 3229T C504S/C507S, 5′ CGG ATC CCC CTT AAG TTA ACT **TAA** GGG CTG CAG GAA TTC 3′. Note that, in addition to creating deletions, the 2051T and 2189T constructs alter C-terminal residues from LLATVV to LLADPP for 2051T and from GPRGPRP to GPRRDPP for 2189T. M-MuLV transient-expression vectors were derived from pXM (47), which contains a simian virus 40 (SV40) origin of replication and the adenovirus major late promoter. M-MuLV was modified at the Psi2 position 563 *Hin*dIII site (26) by addition of 5′ *Eco*RI and *Eco*RV sites (GAATTCGATATCAAGCTT) and used to create pXMGPE, which expresses gag, pol, and env genes and spans M-MuLV nt 566 to 7846; pXM2453 is protease deficient (PR^-), deleted in pol and env genes, and encodes M-MuLV nt 566 to 2453, where a termination signal was placed downstream of nt 2453. Junction sequences are as follows: pXM2453, 5′ GGT AAG GTC ACC GCG GAT CCC CCT **TAA** GTT AAC TTA AGG G CTG CAG GAA TTC 3′, in which the M-MuLV nt indicated in the name is underlined and the termination codon is TAA; pXMGPE, 5′ TTTGGCAAGCT AGA 3′, in which M-MuLV nt 7840 from 3′ untranslated sequence is underlined. The parental vectors, pXMGPE and pXM2453, were used as templates to introduce cysteine-to-serine point mutations at each of the M-MuLV cysteine codons. All five cysteine-to-serine point mutations, including two from the previously described 3229T C504S/C507S (11), were then cloned singularly or in combination into the parental vectors. (In particular, note that all five mutations were cloned into two vectors to yield pXMGPE cysteine-minus [C^-] and pXM2453 cysteine-minus.) Using the C^- constructs as templates, we then introduced novel cysteine codons into the M-MuLV MHR. The sequences of mutant pXM constructs, in which the altered M-MuLV codon designated in the construct name appears in boldface and point mutations, conservative and nonconservative, are underlined, are as follows: C39S, 5′ TGG GTT ACG TTC TCC **TCT** GCA 3′; C270S, 5′ TGG GAC GAT **AGT** CAA CAG 3′; C517S, 5′ AAA GAT **TCT** CCC AAG AAG CCT CGA 3′; L369C, 5′ TCT CCC AGC GCC TTC **TGT** GAG AGA C 3′; E370C, 5′ TCT CCC TCG GCC TTC CTA **TGTC**GA C 3′; and E370C/K373C, 5′ TTC CTA **TGTC**GA CTT **TGC** GAA GCC TAT CGC ACG TAC ACT CCA TAT 3′. Two nucleocapsid truncation mutants, pXM2051 *dl* NC and pXM2189, were derived from their stable expression counterparts, described above, and have the same junction/termination sequences. pXM2453 *dl* p12 has a deletion of p12 coding sequence from nt 1035 to nt 1265, and the junction sequence, in which nt 1034 and nt 1266 are underlined and the first codon of CA is in boldface, is 5′ CT CTA GGC GAT ACC GTC GAT **CCC** CTC CGC 3′. The vector HIV gpt Bcl was used to express HIV-1 $Pr55^{Gag}$ and HIV-1 $Pr160^{GagPol}$ and has been described previously (30). All mutants were constructed by standard cloning techniques (1, 25, 27) and verified by dideoxy sequencing (35).

Cells and viruses. NIH 3T3, Psi2 (26), and PA317 (28) cells were grown as described previously (18). The TR291F cell line, expressing an M-MuLV protease-deficient (PR^-) mutant, was the kind gift of Alan Rein (20). Stable cell lines expressing retroviral constructs were established in NIH 3T3 cells by the transfection-infection protocol of Jones et al. (18), where retroviral vectors are transfected into packaging cell lines (26, 28), and viruses produced by transfected cells are used to deliver recombinant proviruses to NIH 3T3 fibroblasts. Cos7 cells were grown and transfected with transient-expression vectors as described previously (42). Briefly, confluent 10-cm-diameter dishes of Cos7 cells were split 1:5 the day prior to transfection. Fifteen micrograms of plasmid DNA per dish was mixed with 1 ml of HEPES-buffered saline (HBS [pH 7.05]; 21 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; CalBiochem], 137 mM NaCl, 5 mM KCl, 0.7 mM sodium phosphate, 5 mM dextrose), and 40 μl of 2 M $CaCl_2$ was added while vortexing. The DNA solution was incubated at room temperature for 45 min, added dropwise to the monolayer, and then incubated at room temperature for 20 min. After addition of 10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Sigma), 500 U of penicillin and streptomycin per ml, 10 mM HEPES buffer solution (GIBCO BRL), and 25 μl of 50 mM chloraquine, plates were incubated for 4 to 6 h at 37°C. Following incubations, cells were washed with 5 ml of DMEM, incubated in 3 ml of 15% glycerol in HBS (pH 7.05) for 3 min at 37°C, washed twice, and fed with 10 ml of DMEM with fetal calf serum and 50 μg of gentamicin (GIBCO BRL) per ml. Supernatants and cells were collected at 72 h and either filtered through a 0.45-μm filter (Gelman) or spun at 560 × *g* (2,000 rpm on an MSE centrifuge) for 5 min to clear cells.

Protein preparation. Cell-free supernatants from transfected Cos7 cells were centrifuged through a 4-ml sucrose cushion consisting of 20% sucrose in TSE (10 mM Tris-hydrochloride [pH 7.4], 100 mM NaCl, 1 mM EDTA) at 83,000 × *g* (25,000 rpm on an SW28 rotor) for 2 h at 4°C. Pellets were suspended in TSE or electrophoresis sample buffer (12.5 mM Tris-hydrochloride [pH 6.8], 2% sodium dodecyl sulfate [SDS], 20% glycerol, 0.25% bromphenol blue) with 5% β-mercaptoethanol (β-me), depending on the experiment. In experiments comparing cellular and extracellular Gag proteins, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS; 9.5 mM sodium potassium phosphate [pH 7.4], 137 mM NaCl, 2.7 mM KCl), collected in IPB (20 mM Tris-hydrochloride [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 0.02% sodium azide), vortexed for 1 min, and microcentrifuged (13,700 × *g*, 4°C, 10 min) to remove insoluble material. Solubilized cellular material was mixed with an equal volume of 2× electrophoresis sample buffer with 5% β-me unless otherwise indicated. In most experiments, supernatant and cell samples, 50% and 10% of the total samples, respectively, were prepared for electrophoresis and immunoblot analysis.

Sucrose gradients. Extracellular virions were pelleted through a 4-ml sucrose cushion of 20% sucrose in TSE at 83,000 × *g* for 2 h at 4°C. The pellets were resuspended in 0.5 ml of TSE and applied to sucrose gradients consisting of 1.1-ml steps of 20, 30, 40, and 50% sucrose in TSE that had been prepared at least 60 min in advance and placed at 4°C to permit mixing. Gradients were centrifuged at 300,000 × *g* (50,000 rpm on an SW50.1 rotor) overnight at 4°C, and 400-μl fractions were collected from top to bottom. Fractions were mixed and aliquoted for measurement of sucrose densities and of Gag protein levels by densitometric quantitation of immunoblot bands (Bio-Rad model 620). Gag protein levels were normalized to the densest band from each gradient.

Detergent treatments. In cross-linking experiments utilizing detergents, 10% Triton X-100 (Bio-Rad) was added to a final concentration of 0.5% and 10% SDS was added to a final concentration of 0.1%. Samples were vortexed gently and incubated at room temperature for 5 min prior to further treatment.

Subcellular fractionation. Typically, three 10-cm-diameter plates of confluent cells were washed twice, pelleted in PBS, and resuspended in 1 ml of Dounce buffer (20 mM HEPES [pH 7.4], 100 mM KCl, 85 mM sucrose, 100 μM EGTA [ethylene glycol tetraacetic acid]). The cells were lysed by Dounce homogenization 200 times in a 2-ml Wheaton Dounce homogenizer, using the type A (tight) pestle. Whole-cell lysates were spun for 5 min at 5,000 × *g* (8,500 rpm on an Eppendorf microfuge) at 4°C. Postnuclear supernatant was collected and either treated or not treated with Triton X-100 to a final concentration of 0.5%. Treated or untreated postnuclear supernatant was spun at 201,000 × *g* (55,000 rpm on a Beckman TLS-55 rotor) for 15 min at 4°C. Second supernatants (S2) and second pellets (P2) were resuspended in electrophoresis sample buffer with β-me, or untreated second pellets were suspended in 200 μl of TSE for intracellular particle cross-linking experiments.

Immunoblotting. Samples for immunoblot analysis were prepared in electrophoresis sample buffer (without dithiothreitol and, where indicated, without β-me) and applied to Laemmli SDS-polyacrylamide gels (21). After polyacrylamide gel electrophoresis (PAGE), proteins were electroblotted to nitrocellulose membranes (0.45-μm pore size; Schleicher and Schuell). For immunoblotting, nonspecific binding sites were blocked in TBST (20 mM Tris-hydrochloride [pH 7.6], 150 mM NaCl, 0.05% Tween 20 [Bio-Rad]) with 3% gelatin (Bio-Rad) for 30 min at 25°C on a shaker platform. Blocking solution was replaced with a primary antibody solution of 1% gelatin in TBST for 30 min and then washed three times for 5 min each with TBST. After the washes, filters were incubated in a solution of alkaline phosphatase-conjugated secondary antibody in TBST with 1% gelatin for 30 min and then washed three times for 5 min each with TBST. After the final washes, filters were incubated in 20 ml of color reaction solution containing 0.33 mg of nitro blue tetrazolium, 0.17 mg of 5-bromo-4-chloro-3-indolyl phosphate, 100 mM Tris-hydrochloride (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$ until bands appeared. Primary antibodies were as follows: rat monoclonal antibody hybridoma cell supernatants anti-M-MuLV $p12^{Gag}$ and anti-M-MuLV $p30^{Gag}$ (6, 14) used at 1:10 dilutions; mouse anti-HIV $p24^{Gag}$ hybridoma 183 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institutes of Health; hybridoma 183, clone H12-5C from Bruce Chesebro) supernatant used at a 1:10 dilution; purified mouse monoclonal antivinculin used at a 1:1,500 dilution, a gift from P. Stenberg; polyclonal goat anti-M-MuLV $p30^{Gag}$ (National Cancer Institute) used at a 1:4,000 dilution; and rabbit anti-M-MuLV Gag-interacting protein, the kind gift of K. Alin and S. Goff, used at a 1:4,000 dilution. Secondary alkaline phosphatase-conjugated antibodies were goat anti-mouse immunoglobulin G (IgG; Promega) used at a 1:20,000 dilution; rabbit anti-goat IgG (Boehringer Mann-

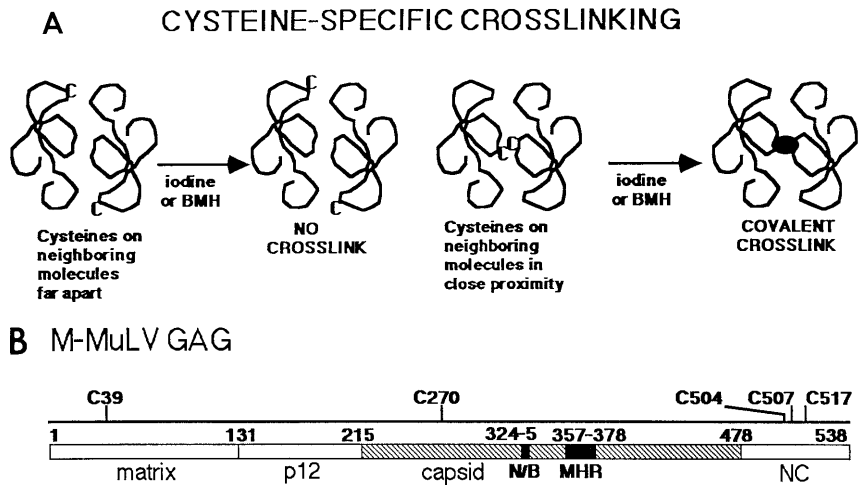

FIG. 1. Cysteine cross-linking and location of cysteine residues in $Pr65^{Gag}$. (A) Schematic illustration of the consequences of molecular iodine or BMH cysteine-specific cross-linking. Distant cysteine residues from neighboring molecules do not cross-link covalently when exposed to membrane-permeable iodine or BMH, but neighboring cysteine residues in close proximity can form a covalent intermolecular cross-link. The iodine cross-link is a reversible oxidative formation of cystines, while BMH produces an irreversible, maleimide-bridged cross-link. The maximum cross-link distance between cysteine residues is approximately 5 and 15 Å (0.5 and 1.5 nm) for iodine and BMH, respectively. (B) The M-MuLV $Pr65^{Gag}$ protein consists of four domains; matrix, p12, capsid (stippled lines), and nucleocapsid, that are cleaved to maturation by viral protease (PR) during the budding process. The precursor polypeptide is 538 codons in length, and the juncture codons are indicated. $Pr65^{Gag}$ contains five cysteine residues that are indicated on the top bar with codon numbers. Within the capsid domain, the N/B tropism determination site is localized to codons 324 and 325, and the MHR spans residues 357 to 378. The nucleocapsid domain contains a Cys-His zinc finger motif with three cysteines, C504, C507, and C517.

heim), used at a 1:10,000 dilution; and goat anti-rabbit IgG (Boehringer Mannheim), used at a 1:10,000 dilution.

Cross-linking techniques. For BMH (Pierce) treatments, virus particles or proteins were prepared as described above. BMH was prepared in dimethyl sulfoxide (DMSO) as a 100 mM solution. Samples were prepared in 200 μl of TSE and halved into equivalent 100-μl fractions that were mock treated (1 μl of DMSO) or BMH treated (1 μl of 100 mM BMH in DMSO). Reaction mixes were vortexed gently and incubated for 60 min at room temperature, reactions were terminated by the addition of electrophoresis sample buffer with 5% β-me, and the mixtures were boiled for 5 min. Iodine treatments were performed in a fume hood following the protocol of Pakula and Simon (31). Briefly, 500 mg of iodine crystals (Aldrich) was placed in a flask containing 100 ml of water that was parafilmed twice and vortexed for 5 min. The saturated solution turned orange and was used immediately by mixing 25 μl with 25 μl of sample in parallel with distilled-water mock treatments. Reactions progressed for 1 min and were quenched by pipetting 50 μl of 80 mM iodoacetic acid (Aldrich) directly into the reaction and quickly adding 100 μl of electrophoresis sample buffer without dithiothreitol or β-me. Iodoacetic acid was prepared 10 min prior to use by dissolving 185 mg in 10 ml of water, adjusting the pH to 6.8 with 5 M and 100 mM NaOH solutions, and bringing the volume to 12.33 ml with water. For some control samples, β-me (10 μl) was added directly to sample tubes, and all samples were immediately heated at 90°C for 4 min prior to electrophoresis. Note that excessive boiling of iodine-treated samples appeared to cause oxidative protein degradation.

RESULTS

Cysteine cross-linking of the M-MuLV $Pr65^{Gag}$ protein. To identify specific regions of contact between $Pr65^{Gag}$ molecules in immature virus particles, we followed the experimental design of Pakula and Simon (31), which is illustrated in Fig. 1A. Briefly, unique cysteines on adjacent molecules will not cross-link with membrane-permeable, cysteine-specific cross-linking agents such as molecular iodine (cross-link distance, approximately 5 Å [0.5 nm]) and BMH (cross-link distance, approximately 15 Å [1.5 nm]) if the cysteine residues are far apart. However, adjacent molecules with cysteine residues in close proximity should cross-link, and oligomers can be detected after gel electrophoresis by immunoblotting. The M-MuLV Gag protein is particularly amenable to this type of analysis, since it possesses only five cysteine residues (Fig. 1B), three of which occur in the Cys-His box of the nucleocapsid (NC) domain. As an initial test of this strategy, we subjected $Pr65^{Gag}$ proteins in immature, protease-minus (PR^-) virions to iodine-catalyzed cysteine cross-linking. As shown in Fig. 2A (lane B), iodine treatment of PR^- particles yielded a novel Gag antibody-reactive band at 130 kDa, the predicted size of a $Pr65^{Gag}$ dimer. This band was not present in the mock-treated sample (lane A) or after reduction with β-me (lane C), suggesting that iodine-catalyzed cystine formation between $Pr65^{Gag}$ proteins was responsible for the 130-kDa protein in lane B. Similar results were observed when BMH was employed as a cross-linker. As shown in Fig. 2B, although the mock-treated lane showed a faint Gag-reactive band at 130 kDa (lane A), BMH treatment yielded a greatly enhanced dimer band (lane B). Additionally, we observed putative oligomers to tetramers and pentamers (lane B), with a relative reduction in putative trimers. The mobility of each oligomer, as calculated in semilog plots, was slightly less than predicted, presumably because cross-linking reduced the Stokes' radius, permitting the molecules to traverse the acrylamide gel matrix faster than predicted. Interestingly, the reduction in putative trimer band intensity is reminiscent of results reporting the in vitro oligomerization of purified HIV capsid protein (8).

The protein at 130 kDa in Fig. 2 is anti-$p30^{Gag}$ immunoreactive and contains at least one $Pr65^{Gag}$ moiety. However, the mobility shift of monomeric $Pr65^{Gag}$ could be due to a cross-link formed with an unidentified protein of approximately 65 kDa, such as bovine serum albumin. In order to prove that cross-linked molecules were composed of $Pr65^{Gag}$ dimers, we deleted a portion of the $p12^{Gag}$ coding sequence to create a smaller Gag protein; the p12 domain contains no cysteines, and similar deletions have been shown to be assembly competent (7). To examine the composition of Gag-reactive cross-linked molecules, protease-minus (PR^-) wt and *dl* p12 proteins in virus particles were subjected to BMH cross-linking (Fig. 3). As expected, extracellular wt virions showed Gag-reactive bands at 65 and 130 kDa (lane A). Cross-linking of extracellular virions composed of *dl* p12 PR^- proteins produced bands at 57 and 115 kDa (lane D), consistent with the notion that the *dl* p12 protein assembles and cross-links as

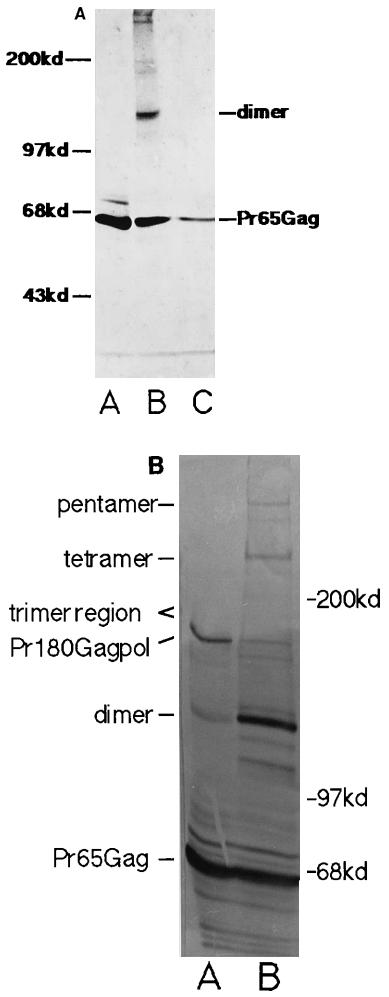

FIG. 2. M-MuLV Gag protein cross-linking. M-MuLV protease-deficient (PR^-) TR291F (20) extracellular virions were treated with cysteine-specific cross-linking agents, molecular iodine or BMH, to examine the effect of cross-linking on M-MuLV Gag proteins expressed in stably infected cells. (A) Parallel 50-μl samples of TR291F virions in TSE were mock treated (lane A) or iodine treated (lanes B and C). Reactions were terminated after 1 min by addition of iodoacetic acid and 2× sample buffer. β-me was added to 5% to one sample (lane C). Samples were immediately boiled, subjected to SDS-PAGE on a 7.5% gel, and electroblotted, and Gag proteins were detected by the sequential addition of anti-p30 antibody, goat anti-mouse IgG alkaline phosphatase-conjugated antibody, and color detection solution. Lanes: A, untreated; B, iodine treated; C, iodine and β-me treated. Note that although the monomer band in lane C appears fainter than that in lane B, longer exposures of iodine-plus β-me-treated samples have not shown dimer bands. The positions of size standard proteins, $Pr65^{Gag}$, and dimers are indicated. (B) Parallel samples of TR291F virions were mock treated (lane A) or BMH treated (lane B) at 25°C for 1 h. Reactions were terminated by the addition of electrophoresis sample buffer with 5% β-me; samples were subjected to SDS-PAGE on a 7.5% gel and transferred to a nitrocellulose filter; and M-MuLV Gag proteins were detected with an anti-$p30^{Gag}$ antibody. The positions of marker proteins, $Pr65^{Gag}$, $Pr180^{GagPol}$, and oligomers are indicated.

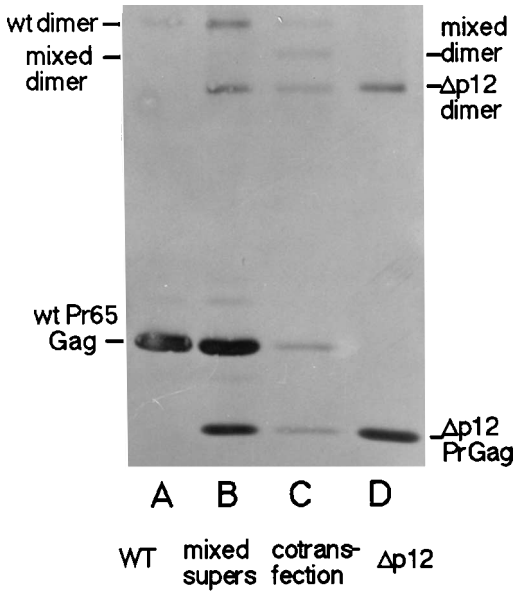

FIG. 3. Composition of cross-linked species. Homogeneous and heterogeneous virus particles released from transiently transfected cells and composed of protease-minus (PR^-) wt and/or *dl* p12 M-MuLV Gag proteins were treated with BMH to examine the protein composition of cross-linked species. Virus particle preparation and BMH cross-linking reactions were done as described in Materials and Methods. Proteins were subjected to SDS-PAGE on a 6% gel and electroblotted, and M-MuLV Gag proteins were detected by immunoblotting. Lanes derive from BMH cross-linking of the following PR^- virus samples: A, pXM2453 wt virus; B, mixed supernatants of wt and pXM2453 *dl* p12 virus; C, cotransfected wt and pXM2453 *dl* p12 virus; D, pXM2453 *dl* p12 virus alone. The positions of marker proteins, wt and *dl* p12 monomers and dimers, and mixed dimers are indicated. Note that dimers did not appear with mock-treated samples (data not shown).

dimers. When virions composed of homogenous wt protein and virions composed of homogeneous *dl* p12 protein were mixed and treated with BMH, bands at 115 and 130 kDa were observed (lane B), but the absence of an intermediate cross-linking product suggests that cross-linking of free protein in the supernatant did not occur and that proteins in separate particles did not cross-link. However, when mixed virus particles were produced by coexpression of wt and *dl* p12 proteins, BMH treatment yielded homogeneous dimers as well as a novel intermediate band at 122 kDa. The appearance of the 122-kDa band is indicative of wt-*dl* p12 heterodimers and shows that cysteine cross-linking produces Gag-Gag dimers. Larger oligomers, tetramers and pentamers, also appeared to be composed of the Gag protein, as seen in the same experimental system (data not shown).

From the above results, our assumption was that cross-linking of Gag proteins takes place primarily between Gag proteins within an individual particle. As an independent test of this, we BMH-treated particles that had been incubated in our standard buffer in buffer supplemented with 0.5% Triton X-100, which should not disrupt immature particles (14, 39), and in buffer plus 0.1% SDS, which dissociates particles (14, 39). As shown in Fig. 4, dimer formation occurred after BMH treatment under standard conditions (lane B) or with Triton (lane C), but the dimer band was reduced to near control levels after SDS treatment (lane D). Taken together, these results suggest that oligomers are formed only by particle-associated Gag proteins.

We used cysteine cross-linking to examine whether M-MuLV Gag and HIV Gag proteins could coassemble into particles and cross-link. To do so, M-MuLV and HIV Pr^{Gag} molecules were coexpressed in Cos7 cells, and extracellular virions were collected and treated with BMH (Fig. 5). Duplicate control and experimental samples were electrophoresed and immunoblotted for detection of HIV $Pr55^{Gag}$ and M-MuLV $Pr65^{Gag}$ proteins. In lanes A and B, detection of HIV Gag shows that HIV Gag proteins were released from cells (lane A) and that HIV $Pr55^{Gag}$ dimers were formed (lane B). Similarly, lanes C and D show M-MuLV Gag protein release (lane D) and BMH

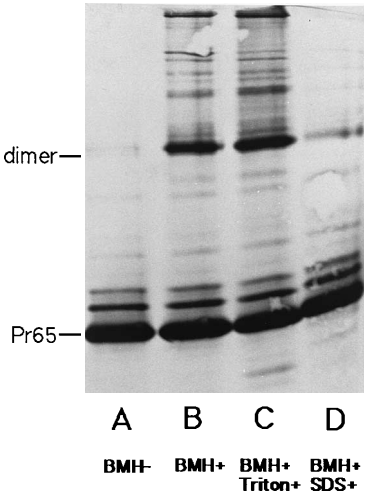

FIG. 4. Detergent effects on Gag protein cross-linking. M-MuLV PR-deficient virus particles from transiently transfected Cos7 cell supernatants were mock treated (lane A) or BMH cross-linked in the absence (lane B) or presence of either 0.5% Triton X-100 (lane C) or 0.1% SDS (lane D). Samples were processed and proteins were detected as described in the legend to Fig. 2B. The positions of $Pr65^{Gag}$ and dimers are indicated.

dimers (lane C). However, neither of the treated lanes (lanes B and C) showed a mixed dimer band at a predicted size of 120 kDa. The absence of a heterodimer indicates either that the HIV and M-MuLV Pr^{Gag} proteins did not coassemble or that they coassembled but did not cross-link. That M-MuLV $Pr65^{Gag}$ and HIV $Pr55^{Gag}$ proteins do not bind to each other in the yeast two-hybrid system (23) supports the interpretation that these heterologous Pr^{Gag} proteins do not coassemble.

The above results indicate that $Pr65^{Gag}$ proteins were cross-linked in immature particles. In contrast, treatment of mature M-MuLV particles showed no evidence of cysteine-specific cross-linking of p12 (which contains no cysteines), capsid, or nucleocapsid proteins (data not shown), and we were unable to examine cross-linking of mature matrix domains because we have not found antibodies suitable for our purposes. These results might imply that matrix domains mediate cysteine cross-linking of immature $Pr65^{Gag}$ and possibly mature MA proteins. Alternatively, it is conceivable that cross-linking of $Pr65^{Gag}$ proteins could occur via CA or NC cysteines and that structural differences between immature and mature particles account for differences in cross-linking results. In order to identify residues directly involved in $Pr65^{Gag}$ cysteine cross-linking, we removed gag cysteine codons, singly or in combination, changing them to serine residues via point mutations. In all cases, we verified that particles produced by mutant proteins were of wt density (1.14 to 1.18 g/ml) by sucrose density gradient centrifugation (data not shown). Analysis of our $Pr65^{Gag}$ variant with the MA and CA cysteines mutated to serines (C39S and C270S) showed that $Pr65^{Gag}$ proteins in mutant particles were cross-linked above background oxidation levels by iodine treatment (Fig. 6A, compare lane C with lanes A and B). The covalent bond was reduced with β-me (lane B), indicating that the iodine treatment was responsible for cystine bond formation. Dimer formation, by a protein lacking the MA and CA cysteines, indicates that the remaining cysteines in the NC domain were responsible for $Pr65^{Gag}$ cysteine cross-linking. To support this assessment, $Pr65^{Gag}$ variants with single, double, and triple cysteine-to-serine NC point mutations were BMH treated (Fig. 6B). The wt (lane A), single mutation (C504S, lane B), and double mutation (C504S/C507S, lane C) all cross-linked to form dimers, while the triple

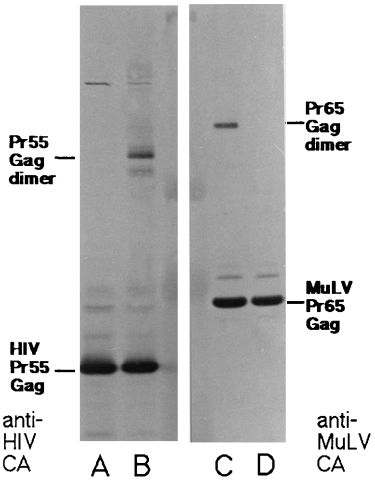

FIG. 5. M-MuLV and HIV Gag proteins do not cross-link. Cos7 cells were transiently cotransfected with 5 μg of pXM2453 plasmid DNA, encoding wt PR^- M-MuLV $Pr65^{Gag}$ protein, and 10 μg of HIV gpt Bcl plasmid DNA, encoding wt PR^- HIV-1 $Pr55^{Gag}$ protein. Supernatants were collected, pooled, and prepared as described in the legend to Fig. 3. Supernatant pellets were suspended in 200 μl of TSE, halved, and mock treated (lanes A and D) or BMH treated (lanes B and C). Reactions were terminated, and duplicate samples were electrophoresed, electroblotted, and immunoblotted for detection of HIV Gag proteins (lanes A and B) or M-MuLV Gag proteins (lanes C and D). The positions of HIV $Pr55^{Gag}$ and M-MuLV $Pr65^{Gag}$ monomers and dimers are indicated; no mixed dimers were observed. A size standard between lanes B and C was used to align the left- and right-hand panels.

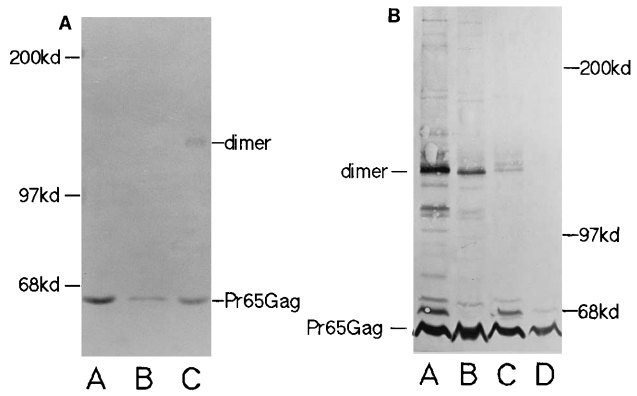

FIG. 6. Gag proteins cross-link at cysteines in the nucleocapsid domain. The five cysteine residues of $Pr65^{Gag}$ were changed to serine residues by point mutation either singly or in combination to determine the site of cysteine cross-linking. (A) Cos7 cells were transiently transfected with pXM2453 C39S/C270S plasmid DNA, encoding the M-MuLV gag gene containing cysteine-to-serine point mutations of the cysteines in the matrix (MA) and capsid (CA) domains but retaining NC cysteines. Supernatants were collected, pooled, and processed as described in the legend to Fig. 3. The supernatant pellet was suspended in 150 μl, divided into three equivalent samples of 50 μl each, mock treated (lane A) or treated with molecular iodine (lanes B and C), and subjected to reducing (lane B) or nonreducing (lanes A and C) conditions. Electrophoresed and electroblotted proteins were immunoblotted, and the positions of $Pr65^{Gag}$ and dimers are shown. (B) Supernatants from Cos7 cells transiently transfected with protease-minus M-MuLV $Pr65^{Gag}$ expression constructs encoding pXM2453 wt (lane A) and nucleocapsid single (lane B, C517S), double (lane C, C504S/C507S), and triple (lane D, C504S/C507S/C517S) cysteine-to-serine mutants were collected and BMH cross-linked as described in the legend to Fig. 2B. Samples were electrophoresed, electroblotted, and immunoblotted with an anti-p30 capsid antibody as described in Materials and Methods. The positions of $Pr65^{Gag}$ monomer and dimer bands are indicated.

RETROVIRAL CAPSID MAJOR HOMOLOGY REGIONS

HIV-1	IRQGPKEPFRDYVDRFYKTLRA
HIV-2	IHQGPKEPFQSYVDRFYKSLRA
SIV	IRQGPKEPFKDYVDRFYKAIRA
HTLV-1	ILQGLEEPYHAFVERLNIALDN
HTLV-2	ILQGLEEPYCAFVERLNVALDN
BLV	IVQGPAESSVEFVHRLQISLAD
MPMV	VKQGPDEPFADFVHRLITTAGR
RSV	IMQGPSESFVDFANRLIKAVEG
FLV	VVQGKEETPAAFLERLKEAYRM
MMTV	LKQGNEESYETFISRLEEAVYR
M-MULV	ITQGPNESPSAFLERLKEAYRR

357 378

FIG. 7. Alignment of the MHR. The MHR (24, 40) of several *Retroviridae* family members has been aligned to demonstrate the residue conservation. Virus sequences are as follows: HIV-1 and HIV-2, human immunodeficiency virus types 1 and 2; SIV, simian immunodeficiency virus; HTLV-1 and HTLV-2, human T-cell leukemia virus types 1 and 2; BLV, bovine leukemia virus; MPMV, Mason-Pfizer monkey virus; RSV, Rous sarcoma virus; FLV, feline leukemia virus; MMTV, mouse mammary tumor virus; M-MuLV, Moloney murine leukemia virus. The M-MuLV $Pr65^{Gag}$ residue numbers from 357 to 378 are indicated at the bottom, and molecular modeling with the FRODO software package (19) suggests that an amphipathic helix can be formed between residues 366 and 378. Sites of point mutations creating one or two novel cysteine residues (L369C, E370C, and E370C/K373C) are indicated by solid circles.

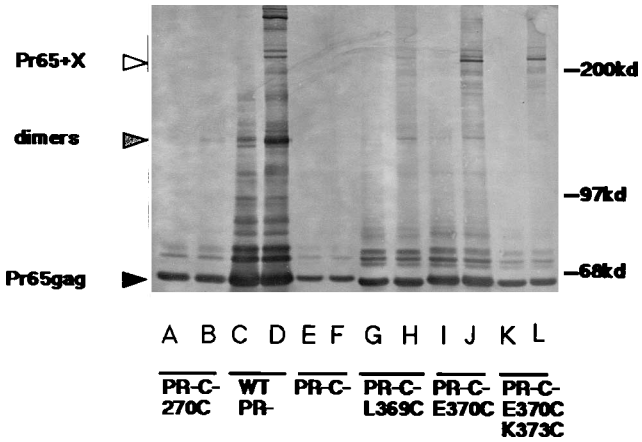

FIG. 8. Cross-linking M-MuLV Gag proteins with capsid mutations. Cos7 cells were transiently transfected with the designated pXM2453-derived PR^- $Pr65^{Gag}$ expression plasmids, and after 72 h, supernatants were collected and prepared for cross-linking treatment as described in the legend to Fig. 3. Samples were halved, mock treated (lanes A, C, E, G, I, and K) or BMH treated (lanes B, D, F, H, J, and L), and processed for Gag protein detection as described in Materials and Methods. Plasmids used in transfections were as follows: lanes C and D, wt; lanes E and F, all cysteines to serines (C^-); lanes A and B, C^- plus 270C; lanes G and H, C^- plus L369C; lanes I and J, C^- plus E370C; lanes K and L, C^- plus E370C/K373C. The positions of size markers are as indicated, as are $Pr65^{Gag}$ monomers (solid arrowhead), dimers (shaded arrowhead), and cross-links to a cellular protein of 140 to 160 kDa ($Pr65^+$ X; open arrowhead).

point mutation (C504S/C507S/C517S, lane D) did not cross-link detectably. The intensity of the dimers decreased as the number of NC cysteines decreased, and larger oligomers were present only when there were at least two cysteines in the NC domain. Thus, we conclude that most of the cysteine-specific cross-linking of $Pr65^{Gag}$ occurred between cysteine residues in the NC domain and that an intact Cys-His motif does not appear to be required, as its structure presumably is disrupted in the single and double point mutants.

Cysteine cross-linking of the $Pr65^{Gag}$ capsid domain. Throughout our studies, we found that it was possible to eliminate all cysteine codons of $Pr65^{Gag}$ without apparent impairment of assembly (Fig. 6, and see below). Consequently, the M-MuLV Gag protein appeared to be suitable for analysis of intermolecular associations by cross-linking of mutant Gag proteins possessing unique cysteines at novel locations. To do so, we constructed a PR^- cysteine-minus (C^-) $Pr65^{Gag}$ mutant that assembled particles and introduced cysteine residues into the MHR. We examined this region because it maintains a high degree of homology throughout the *Retroviridae* family, as shown in Fig. 7. Retroviral MHRs have been shown to be sensitive to mutagenesis at conserved residues (24, 40), but the region has not been characterized structurally. From its putative potential to form an amphipathic alpha helix, we modeled the MHR using the FRODO software (19) and, for mutagenesis, selected MHR positions with some degree of evolutionary variability on either the putative hydrophobic helical face (L369C) or the modeled hydrophilic face (E370C and E370C/K373C; see Fig. 7). All of these mutants produced virus particles which were of wt density and, in a PR^+ background, proteolytically processed mutant $Pr65^{Gag}$ proteins at wt levels (data not shown).

Having created Gag proteins with cysteine residues in the MHR domain, we wished to determine if these residues could facilitate the cross-linking of $Pr65^{Gag}$ molecules. Figure 8 shows the results of such an experiment after electrophoresis and immunoblotting to detect Gag proteins of untreated or BMH-treated PR^- virions. As shown (lanes C and D), positive-control wt virions cross-linked to form $Pr65^{Gag}$ dimers (lane D) above background levels, while cysteine-minus (C^-) Gag proteins did not cross-link to detectable levels when treated with BMH (lane F). As illustrated in lanes A and B, particles composed of C^-/270C Gag proteins, which contain a single cysteine, the natural CA cysteine at position 270, appeared to cross-link above background levels (lane B versus lane F), but not nearly to the extent of wt $Pr65^{Gag}$ (lane B versus lane D). These results are consistent with those shown in Fig. 6B. Proteins with cysteines introduced at the MHR, L369C (lanes G and H) and E370C (lanes I and J), showed low levels of dimer formation. Interestingly, proteins with two cysteine residues in the MHR domain, E370C and K373C, did not produce detectable dimers (Fig. 8, lane L). The structure of these non-cross-linking proteins may be slightly different from that of E370C proteins; the two cysteine residues could preferentially form an intramolecular cross-link, or the reduced levels of Gag protein in this lane may have resulted in our failure to detect a dimer band; we have not delineated between these two possibilities. However, in addition to Gag dimers, a novel band was detected when proteins with cysteine residues in the MHR were chemically cross-linked (Fig. 8, lanes H, J, and L; designated Pr65 + X). The novel band was evident with L369C proteins, pronounced with E370C, and the major cross-link product with E370C/K373C proteins. This band was not seen with 270C proteins (lane B), but may correspond to a wt cross-link band (lane D). Evidence suggests that the Pr65 + X band is the product of a cross-link formed between the $Pr65^{Gag}$ mutant protein and a nonviral protein, since viral particles were generated by transfection of plasmids lacking env and pol sequences. Although the novel band, approximately 215 kDa, is similar in size to $Pr65^{Gag}$ trimers (approximately 195 kDa), the presence of only one cysteine residue for L369C or E370C proteins should preclude the formation of $Pr65^{Gag}$ trimers. Thus, the X factor appears to be a cellular protein of 140 to 160 kDa present in virions, with a cysteine in close proximity to the MHR. A similar cross-link product has been seen after cross-linking of HIV Gag proteins possessing introduced MHR cysteines (8a) and when particles were derived from different cells (data not shown).

Role of NC in virus assembly. The formation of $Pr65^{Gag}$ oligomers by cysteine-specific cross-linking of residues in the Cys-His motif (Fig. 2 and 6) suggests a close association of

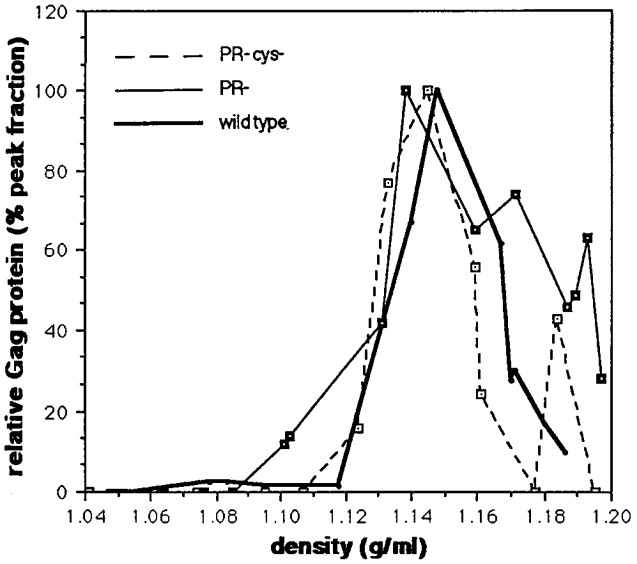

FIG. 9. Sucrose density gradient fractionation of M-MuLV particles. Wild-type (thick black line), protease-minus (PR^-; thin black line), and PR^- cysteine-minus (PR^- C^-; dashed line) extracellular particles were fractionated by sucrose density centrifugation on a 20 to 50% gradient as described in Materials and Methods. Fractions were collected, and aliquots were assayed for density and for total Gag protein content by immunoblot detection. Sucrose density is plotted on the *x* axis, while Gag protein levels are plotted as a percentage of the densest band from each immunoblot on the *y* axis.

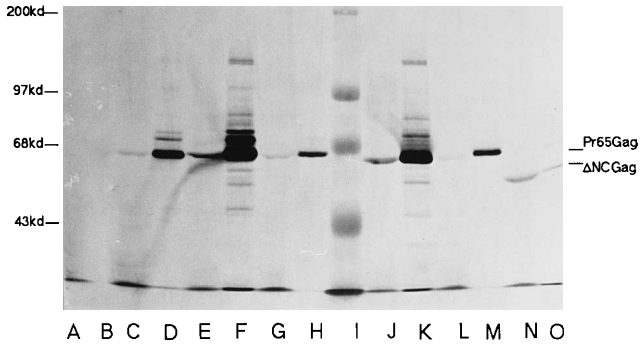

FIG. 10. Expression and release of M-MuLV nucleocapsid domain mutant proteins. Cos7 cells were transiently transfected with the designated PR^- pXM2453-, pXM2189-, or pXM2051-derived expression plasmid, and at 72 h, cell lysates and supernatants were collected and processed as described in Materials and Methods. Matching supernatant (lanes B, D, F, H, K, M, and O; corresponding to 50% of the total sample) and cell samples (lanes A, C, E, G, J, L, and N; corresponding to 10% of the total sample) were fractionated by SDS-PAGE on a 10% gel and transferred to a nitrocellulose filter, and Gag proteins were detected with an anti-p30 antibody as described in the legend to Fig. 3. Samples derive from transfections as follows: lanes A and B, mock; lanes C and D, pXM2453 wt; lanes E and F, pXM2453 C504S/C507S; lanes G and H, pXM2453 C504S/C507S/C517S; lanes J and K, pXM2189; lanes L and M, pXM2453 C517S; lanes N and O, pXM2051 *dl* NC. Molecular size markers were run in lane I; their positions are indicated at the left, and those of wt and *dl* NC $Pr65^{Gag}$ proteins are indicated on the right.

these motifs, corroborating previous results (32). The arrangement of Cys-His motifs may facilitate a functional interaction, although several observations have seemed to point against such a role in immature particle structure. $Pr65^{Gag}$ Cys-His motif point mutations that presumably disrupt the structure of the motif cross-linked at remaining Cys-His motif cysteines, and mature NC domains apparently did not cysteine cross-link. We also found no apparent interaction in the yeast two-hybrid system (9) when M-MuLV NC domains were expressed on two complementary constructs (data not shown). Furthermore, we previously found that a fusion protein comprised of β-galactosidase fused at the carboxy terminus of the CA domain, replacing the NC domain (GBG2051 [18]), incorporated into assembling wt particles, and in the absence of wt proteins, the fusion protein assembled to form low-density particles.

Because of the seemingly contradictory NC results, further analysis was necessary to explore the effect of NC on assembly and structure. As noted above, all of our cysteine substitution mutants formed particles of wt density, an example of which is shown for the protease-minus cysteine-minus (PR^- C^-) mutant in Fig. 9. As illustrated, mature wt virus particles, PR^- but otherwise wt virus particles, and PR^- C^- particles sedimented at 1.14 to 1.18 g/ml, the expected density of M-MuLV in sucrose (44). We further examined the role of NC in particle formation by expressing truncation mutants: pXM2051 *dl* NC encodes a complete deletion of the nucleocapsid domain and has three foreign residues (DPP) at the Gag C terminus, while pXM2189 encodes a carboxy-terminal deletion of the final six NC residues and has four foreign C-terminal residues (RDPP). To obtain a rough measure of wt and mutant particle assembly, the amounts of Gag proteins in matching cellular and particle-associated supernatant samples were compared (Fig. 10). As shown, protease-minus but otherwise wt $Pr65^{Gag}$ proteins were efficiently released from cells (compare lanes D and C). Similarly, pXM2189 (lanes J and K) and single (C504S; lanes L and M), double (C504S/C507S; lanes E and F), and triple (C504S/C/507S/C517S; lanes G and H) NC cysteine point mutant proteins were efficiently released, although there appeared to be a slight reduction in the case of the triple point mutant (Fig. 10, lanes H and G). However, we found that release of the complete NC-deleted protein (pXM2051 *dl* NC) was reduced in transient-expression assays (Fig. 10, lanes O and N) as well as stable-expression assays (data not shown). These data suggest that some non-Cys-His motif component of the NC domain is needed for efficient assembly or that the exposed carboxy terminus of *dl* NC (containing the three foreign residues DPP) is detrimental to Gag protein stability, transport, or assembly.

The implication from the data in Fig. 10 is that the presence of NC affects virus assembly, structure, and/or release. However, examination of virus structure with extracellular virions composed of *dl* NC proteins was not possible because of a lack of sufficient extracellular protein for either gradient or stability analysis. As an alternative, we examined intracellular proteins, characterizing their subcellular fractionation patterns in the presence and absence of Triton X-100, which helps dissociate Gag protein complexes (14). To do so, postnuclear cell supernatants were either untreated or treated with Triton X-100 and centrifuged to generate second supernatant (S2) and pellet (P2) fractions. As expected, all Gag proteins associated with intracellular membranes in the absence of detergent (Fig. 11A, lanes F, J, N, and R versus E, I, M, and Q). However, wt, C504S/C507S, and 2189T intracellular proteins remained largely associated with the pellet fraction after Triton treatment (Fig. 11A, lanes H, L, and P versus G, K, and O), while the NC-deleted intracellular Gag protein (2051T *dl* NC) was solubilized almost completely by Triton (lane S versus T), suggesting that *dl* NC proteins did not form stable Gag-Gag contacts. As a further test, P2 fractions from cells expressing wt and 2051T *dl* NC proteins were treated with iodine (Fig. 11B) to examine the cross-linking profile of intracellular particles. Wild-type Gag proteins were cross-linked to form β-me-sensitive dimers (lanes A to D). Not surprisingly, *dl* NC proteins showed no dimer formation (lanes E to H), and no *dl* NC

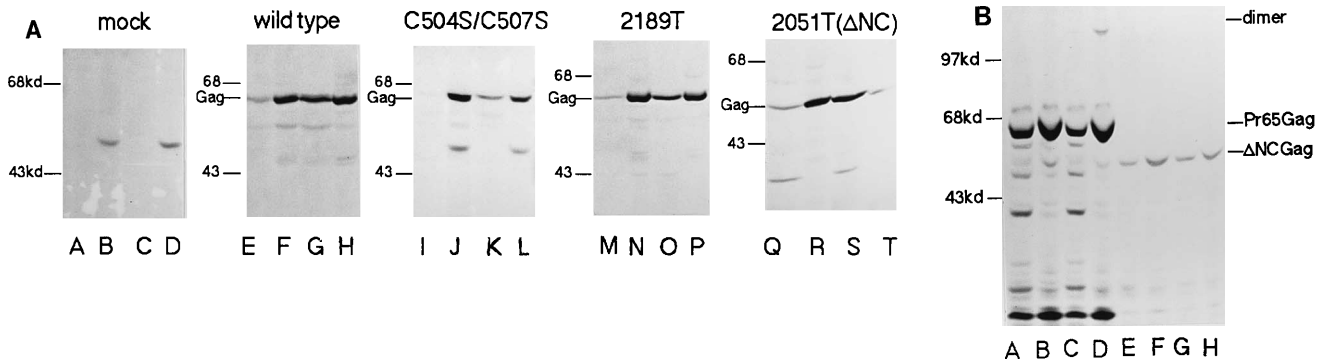

FIG. 11. Intracellular Gag protein fractionation and cross-linking. (A) Stable cell lines expressing the designated protease-minus proteins were established as described in Materials and Methods. NIH 3T3 mock-treated cells (lanes A to D) and NIH 3T3 cells expressing wt (lanes E to H), C504S/C507S (lanes I to L), 2189T (lanes M to P), and 2051T (lanes Q to T) proteins were washed in ice-cold PBS, scraped into 1 ml of PBS, and pelleted at 630 × *g*. Cellular pellets were dounced and centrifuged at 5,000 × *g* at 4°C for 5 min to obtain a postnuclear supernatant (PNS) fraction. PNS fractions were either untreated (lanes A and B, E and F, I and J, M and N, and Q and R) or treated with 0.5% Triton X-100 (lanes C and D, G and H, K and L, O and P, and S and T) for 5 min at 25°C prior to centrifugation at 201,000 × *g*. PNS second supernatants (S2, corresponding to 10% of the total sample; lanes A, C, E, G, I, K, M, O, Q, and S) and second pellets (P2, corresponding to 33% of the total sample; lanes B, D, F, H, J, L, N, P, R, and T) were collected, subjected to SDS-PAGE on a 10% gel, and transferred to a nitrocellulose filter, and Gag proteins were detected with an anti-p12 antibody. The positions of molecular size markers and Gag proteins are indicated. (B) Intracellular wt (lanes A to D) and NC-deleted 2051T (lanes E to H) Gag proteins were iodine treated to detect Gag-Gag dimers. Second pellets (P2) from NIH 3T3 cells stably expressing viral constructs were prepared as described for panel A, resuspended in 100 μl of PBS, separated into four equivalent 25-μl samples, and treated with 25 μl of water (lanes A, B, E, and F) or 25 μl of iodine solution for 1 min (lanes C, D, G, and H). Reactions were quenched by the addition of 50 μl of 80 mM iodoacetic acid (pH 6.8) and 100 μl of sample buffer with (lanes A, C, E, and G) or without (lanes B, D, F, and H) β-me. Samples were immediately heated at 90°C for 4 min, electrophoresed, electroblotted, and immunoblotted with an anti-p12 antibody. The positions of molecular size markers and *dl* NC and $Pr65^{Gag}$ proteins and dimers are indicated.

protein dimers were observed on a parallel immunoblot that was subjected to a 5- to 10-fold-longer exposure. From these data, we conclude that M-MuLV CA and non-Cys-His motif regions of NC are necessary for particle assembly and that interactions between these regions hold zinc finger motifs in close enough proximity to cross-link.

DISCUSSION

The results of this study demonstrate that particle-associated M-MuLV $Pr65^{Gag}$ monomers can be cross-linked via cysteines into oligomers, from dimers to at least pentamers or hexamers, upon exposure to cysteine-specific cross-linkers (Fig. 2, 3, 4, and 6). Assignment of cross-link residues to NC cysteines agrees with previous reports which identified NC domain cross-links in $Pr65^{Gag}$ but did not identify specific bridging residues (32, 33). In our cross-linking studies, the relative reduction in trimer formation, in comparison with tetramers (Fig. 2B and 4), was reminiscent of results reporting HIV CA in vitro oligomerization (8). The lack of observed M-MuLV $Pr65^{Gag}$ trimers could be due to an unknown cross-linking preference or could indicate that trimers are an unlikely subunit structure in the formation of immature M-MuLV particles. Interestingly, M-MuLV $Pr65^{Gag}$ proteins did not cross-link to HIV $Pr55^{Gag}$ proteins when the molecules were coexpressed (Fig. 5), suggesting either that the two Pr^{Gag} proteins did not coassemble or that they coassembled but did not cross-link via cysteines. We favor the former interpretation, since M-MuLV and HIV Gag proteins do not interact in the yeast two-hybrid system (23).

The fact that BMH can cross-link NC domains, which presumably are located inside of immature virions (5), suggests that small membrane-permeable molecules can penetrate immature virus cores. This result could occur if immature particles are not completed prior to budding, thus maintaining a complete lipid bilayer but an incomplete protein shell. Alternatively, immature cores may not be close-packed shells, but rather a cage or lattice with pores or holes through which BMH can gain access. A more trivial alternative is that our virus preparations may have a high percentage of broken particles. We currently are trying to distinguish between these possibilities.

We interpret the cysteine cross-linking results to indicate that nucleocapsid cysteines in particle-associated $Pr65^{Gag}$ proteins are held in close proximity by domains in CA and non-Cys-His motif regions in NC. Proteins possessing NC cysteine mutations retained their ability to cross-link (Fig. 6B, lanes B and C), implying that residues of these motifs are maintained in close proximity to one another, but their structure is not required for cross-linking. What interactions keep Cys-His motifs in close proximity? In the yeast two-hybrid system, Gag-Gag interactions were localized to CA (23), an important region for particle assembly (17, 18, 41). $Pr65^{Gag}$ protein with a deleted NC domain (*dl* NC) was assembly impaired (Fig. 10, lanes N and O) and Triton sensitive (Fig. 11). Thus, it appears that both CA and a subregion of NC are important to the M-MuLV core structure. These results are consistent with those of Bennet et al. (3), which implicate the HIV NC domain as necessary for tight packing of particle-associated Gag proteins.

To extend our observations on wt $Pr65^{Gag}$ proteins, we used cysteine mutagenesis to create novel cysteines in the MHR (24, 40) of a cysteine-minus template (Fig. 8). The natural CA cysteine residue, 270C, contributed little to cross-linking of adjacent $Pr65^{Gag}$ proteins (Fig. 6 and 8), while cysteine residues created in the M-MuLV MHR cross-linked at slightly above control levels (Fig. 8). We also observed an apparent reduction of dimer formation when two cysteines were created in the MHR, which could have resulted as a consequence of preferential intramolecular cross-link. In interpreting these results, it is important to note that mutant protein structures may deviate from that of the wt and that cross-linkers may not have had complete access to cysteine residues. However, with protease-competent variants of our MHR mutants, we observed no obvious processing defects (data not shown), arguing against global conformational disparities. Also, an indication that the MHR cysteines were accessible to reagents was the formation of a novel cross-link band (Fig. 8, Pr65 + X). The

unknown moiety appeared to be 140 to 160 kDa, and while this band may correspond to a band present when wt proteins are cross-linked, it appeared to be pronounced with hydrophilic MHR substitutions. A similar or identical factor has been seen when parallel HIV MHR cysteine substitutions were chemically cross-linked (8b). Evidence suggests that the X factor is nonviral. The expression constructs used in these experiments were env^- pol^-, and although the mobility of the novel band is similar to the expected mobility of Gag trimers, the factor was present after cross-linking of proteins that contained single cysteine residues, which should preclude $Pr65^{Gag}$ oligomers larger than dimers. At this point, evidence suggests that the X factor is not a membrane-anchored protein, as cross-linking was stable in 0.5% Triton (data not shown). Also, immunoblot analysis suggests that the protein is neither vinculin nor one of the recently identified cellular Gag-interacting proteins identified by K. Alin and S. Goff that appear to associate with the M-MuLV Gag protein in the yeast two-hybrid system (9). We currently are examining the identity and significance of the X factor, as well as the role of the amino-terminal region of NC in particle assembly, transport, and/or release.

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