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Compensatory Substitutions Restore Normal Core Assembly in Simian Immunodeficiency Virus Isolates with Gag Epitope Cytotoxic T-Lymphocyte Escape Mutations

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The evolution of human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) as they replicate in infected individuals reflects a balance between the pressure on the virus to mutate away from recognition by dominant epitope-specific cytotoxic T lymphocytes (CTL) and the structural constraints on the virus' ability to mutate. To gain a further understanding of the strategies employed by these viruses to maintain replication competency in the face of the intense selection pressure exerted by CTL, we have examined the replication fitness and morphological ramifications of a dominant epitope mutation and associated flanking amino acid substitutions on the capsid protein (CA) of SIV/simian-human immunodeficiency virus (SHIV). We show that a residue 2 mutation in the immunodominant p11C, C-M epitope (T47I) of SIV/SHIV not only decreased CA protein expression and viral replication, but it also blocked CA assembly in vitro and virion core condensation in vivo. However, these defects were restored by the introduction of upstream I26V and/or downstream I71V substitutions in CA. These findings demonstrate how flanking compensatory amino acid substitutions can facilitate viral escape from a dominant epitope-specific CTL response through the effects of these associated mutations on the structural integrity of SIV/SHIV.

Viral sequence evolution plays a central role in AIDS immunopathogenesis. Mutations within cytotoxic T-lymphocyte (CTL) epitopes of human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) lead to uncontrolled viral replication and disease progression (2, 3, 16). Interestingly, the rate at which particular immunodominant CTL responses select for HIV/SIV escape mutations can vary dramatically. For example, viral escape from CTL at certain epitopes, such as the HLA-B44-restricted Env and the HLA-B8-restricted Nef epitopes in HIV-1, as well as the Mamu-A*01-restricted Tat SL8 epitope in SIV, occurs early after infection (1, 5, 10, 14, 21, 27, 30). In contrast, viral escape from CTL at other epitopes, such as the HLA-B26-restricted HIV-1 Gag and Mamu-A*01-restricted SIV/simian-human immunodeficiency virus (SHIV) Gag p11C, C-M epitopes, occurs infrequently and only after years of persistent viral replication (3, 11, 28). Clarification of the precise mechanisms that facilitate HIV/SIV escape from CTL is needed for an understanding of this central event in AIDS pathogenesis.

The Gag p11C, C-M epitope (CTPYDINQM) is an immunodominant SIV/SHIV Gag CTL epitope in Mamu-A*01$^+$ rhesus monkeys that rarely develops escape mutations. This epitope is embedded in a highly conserved region of the SIV capsid protein (CA) that is required for virion maturation (15). High-resolution three-dimensional structural studies have revealed that the HIV-1 CA NH_2-terminal domain is composed of seven α-helices, two β-hairpins, and an exposed proline-rich loop (4, 12, 15, 24). Upon proteolytic processing of the matrix-capsid junction during HIV-1 maturation, the conserved amino-terminal proline residue (Pro^1) forms a salt bridge with a buried aspartate residue (Asp^{51}) in helix 3 of the NH_2-terminal domain to form the terminal β-hairpin (36). This hairpin is important for triggering condensation of the core particle and dimerization of the CA monomers. The salt bridge needed for the formation of this hairpin requires stabilization by a hydrogen bond with the neighboring threonine in position 48 (Thr^{48}) (28, 32, 33). Importantly, the HIV-1 Thr^{48} and Asp^{51} residues are equivalent to Thr^{47} and Asp^{50} in the SIV CA, and these threonine and aspartate residues are present in positions 2 and 5 of the Mamu-A*01-restricted Gag p11C, C-M epitope. Since this region of the CA is critical for the formation of the β-hairpin, it is likely that mutations within this epitope which disrupt either the salt bridge or the neighboring hydrogen bonds will interfere with maturation of viral particles.

We and others have previously reported the rare emergence of a position 2 p11C, C-M epitope substitution in SIV- and SHIV-89.6P-infected Mamu-A*01$^+$ monkeys, a threonine-to-isoleucine mutation in position 47 of CA (T47I) (3, 11, 28). This epitope mutation allowed the resulting variant viruses to escape from recognition by p11C, C-M-specific CTL, leading to clinical disease progression in a monkey that was immunized with interleukin-2 (IL-2)-augmented DNA vaccines and then challenged with SHIV-89.6P (monkey 798) (3). These variant viruses were observed infrequently and were slow to emerge because a compensatory mutation, an isoleucine-to-valine substitution in position 71 (I71V), was required to facilitate the residue 2 epitope escape mutation. We showed that the T47I mutation can significantly decrease viral protein expression, infectivity, and replication. Furthermore, we demonstrated

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that when mutations in this epitope occur, they are only tolerated by the virus if additional downstream compensatory flanking mutations arise (28). In the present study, we further evaluated the effect of an upstream mutation on Gag expression and viral replication, as well as the morphological ramifications of the position 2 amino acid mutation in the p11C, C-M epitope, and sought to determine how flanking mutations are able to rescue the defect caused by this CTL escape mutation.

MATERIALS AND METHODS

Gag point mutations and cloning. The various Gag mutations were introduced into the plasmid p239SpSp5′ obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), from Ronald Desrosiers. *gag* mutants I26V, T47I, I71V, I26V/T47I, T47I/I71V, I26V/T47I/I71V, T47A, I26V/T47A, T47A/I71V, and I26V/T47A/I71V were created by PCR mutagenesis with a QuikChange kit (Stratagene, La Jolla, Calif.). Oligonucleotide primers I26VF (5′-CTTCTGCTCCAAATTTCTTTTCCTCTACCA ATTTTACCCAGGCATTTAATGTTC-3′) and I26VR (5′-GAACATTAAAT GCCTGGGTAAAATTGGTAGAGGAAAAGAAATTTGGAGCAGAAG-3′) were used to change Ile (ATA) to Val (GTA). Primers T47IF (5′-CAGGCAC TGTCAGAAGGTTGCATCCCCTATGACATTAATCAGATGTTAAATTG-3′) and T47IR (5′-CAATTTAACATCTGATTAATGTCATAGGGGATGCAACCT TCTGACAGTGCCTG-3′) were used to change Thr (ACC) to Ile (ATC). Primers I71VF (5′-GCGGCTATGCAGATTATCAGAGATGTTATAAACGAGGAGGC TGCAGATTG-3′) and I71VR (5′-CAATCTGCAGCCTCCTCGTTTATAACAT CTCTGATAATCTGCATAGCCGC-3′) were used to change Ile (ATT) to Val (GTT). Primers T47AF (5′-CAGGCACTGTCAGAAGGTTGCGCCCCCTATG ACATTAATCAGATGTTAAATTG-3′) and T47AR (5′-CAATTTAACATCTG ATTAATGTCATAGGGGGCGCAACCTTCTGACAGTGCCTG-3′) were used to change Thr (ACC) to Ala (GCC).

Plasmids. *gag* expression plasmids were constructed by PCR amplification of the entire *gag* gene with primers *gag*F (5′-AAGAGATCTGCCACCATGGGC GTGAGAAACTCCGTCTTGTCAGG-3′) and *gag*R (5′-GAGAGATCTCTAC TGGTCTCCTCCAAAGAGAGAATTGAGG-3′) from the wild-type (WT) and mutant p239SpSp5′ plasmids. To obtain high-level protein expression, a Kozak sequence (GCCACC) was engineered into primer *gag*F. The amplified *gag* gene was cloned into expression vector pVRC, provided by G. Nabel (Vaccine Research Center, NIAID, NIH), by using BglII restriction sites that were incorporated into both primers *gag*F and *gag*R.

Protein quantification. 293T cells were transfected with 1 μg of purified *gag* expression plasmid by the calcium phosphate method (Invitrogen, Carlsbad, Calif.). Forty-eight hours later, cell supernatants were collected and the cells were lysed for 20 min with 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–1% Nonidet P40–0.5% sodium deoxycholate–protease inhibitors (Roche, Mannheim, Germany). Cell lysates were centrifuged for 20 min at 16,000 × *g*, and the soluble fraction was separated from the insoluble pellet. To normalize samples, total protein content was quantified by using a Bio-Rad protein assay (Hercules, Calif.). Concentrations of SIV p27 in the supernatant were quantified using the SIV core antigen enzyme-linked immunosorbent assay (ELISA) (Coulter, Miami, Fla.). The soluble cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes for Western blotting.

Western blots. An anti-SIV of macaques (SIVmac) p27 monoclonal antibody (55-2F12) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Niels Pederson. The primary detection antibody 2F12 was used at a 1:2,000 dilution and detected by using a SuperSignal West Pico mouse immunoglobulin G detection kit (Pierce, Rockford, Ill.).

Viruses and cells. Mutant SIVmac239 proviral plasmids were produced by shuttling modified *gag* genes from the p239SpSp5′ plasmid into full-length WT SIVmac239 proviral constructs. A full-length SIVmac239 proviral plasmid was obtained as a gift from Heinrich Gottlinger (University of Massachusetts, Worcester, Mass.). To produce recombinant viruses for viral replication studies, proviral plasmids were transfected into CEMx174 cells (American Type Culture Collection) by the DEAE-dextran method. Cultures were monitored for p27 expression using a SIV core antigen assay and for reverse transcriptase (RT) activity by a colorimetric ELISA (Roche). Following transfection, viruses were harvested at day 7 for use in subsequent infection studies with rhesus monkey peripheral blood mononuclear cells (PBMCs).

Rhesus monkey PBMCs were purified by Ficoll-Paque density gradient centrifugation. Contaminating erythrocytes were lysed with a solution containing 150 mM NH_4Cl and 7 mM $NaHCO_3$ (pH 7.2). Cells were washed three times with phosphate-buffered saline and resuspended in RPMI 1640 complete medium with 10% fetal calf serum, penicillin, and streptomycin. Cells were then stimulated for 2 days with 6.25 μg of concanavalin A per ml and 20 U of IL-2 per ml 24 h prior to infection. Equivalent amounts of WT and mutant SIV viruses, as measured in p27 units, were used to infect 2×10^6 rhesus monkey PBMCs and macrophages overnight. Supernatants were monitored for p27 activity from day 1 through day 13.

Virus sequencing. Viruses were isolated from cell-free supernatants by using a viral RNA isolation kit (QIAGEN, Valencia, Calif.). Virus sequence analyses were performed on a 749-bp region of *gag* by amplification with an OneStep RT-PCR kit (QIAGEN) and the primers *gag*-fwd (5′-CTTTCGGTCTTAGCT CCATTAGTGCC-3′) and *gag*-rev (5′TGTCTGTTCTGCTCTTAAGCTTTTG TAG-3′). RT-PCR products were purified in agarose gels, the PCR fragment was cloned into pGEM-T Easy vector (Promega, Madison, Wis.), and individual transformed colonies were subjected to T7/SP6 dideoxy sequencing.

SIV recombinant capsid protein expression and purification. Procedures used to express and purify the WT and mutant CA proteins were similar and are therefore described in detail only for the WT protein (37). The SIVmac239 capsid gene was amplified from p239SpSp5′ DNA by PCR. Primers were designed to introduce NdeI and HindIII cloning sites, a stop codon, and optimized *Escherichia coli* codons for the first three amino acid residues. The amplified gene was subcloned into the phage T7 RNA polymerase-based expression vector, pET23*a* (Novagen, San Diego, Calif.), and the resulting plasmid was confirmed by DNA sequencing.

E. coli BL21(DE3)-RIL cells (Stratagene) containing various expression plasmids were grown in LB medium. Protein expression was induced late in log-phase growth (A_{600} of ~0.6) by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Soluble CA accumulated to very high levels during a 4-h incubation period. All steps in the protein purification were performed at 4°C. The cells were harvested and resuspended in 10 ml of buffer A (25 mM Tris-HCl [pH 8.00], 5 mM β-mercaptoethanol [β-ME], 1 tablet of protease inhibitor cocktail [Boehringer Mannheim]), and 50 mM NaCl. Cells were then lysed by lysozyme and sonicated to reduce viscosity. Insoluble material was removed by centrifugation at 40,000 × *g* for 1 h. The CA protein was precipitated by the addition of 20% (vol/vol) saturated ammonium sulfate, stirred on ice for 45 min, and collected by centrifugation for 10 min at 3,000 × *g*. The CA pellet was redissolved in 7 ml of buffer A and dialyzed against 1 liter of buffer A to desalt. The CA was then chromatographed on Q Sepharose (Amersham, Piscataway, N.J.) using a linear gradient from 0 to 1 M NaCl in buffer A. The protein was eluted with ~300 mM NaCl and dialyzed overnight against buffer A.

Following purification, all CA proteins were concentrated by centrifugal filtration through a Centricon filter, flash frozen in liquid N_2, and stored at −70°C prior to analysis. Protein concentrations were quantified by using a Bio-Rad protein assay. The identities of all mutants were confirmed by N-terminal sequencing and mass spectrometry. Recombinant CA proteins lost their amino-terminal methionines during expression and thus corresponded exactly to the authentic CA sequences.

To ascertain their purities, the recombinant proteins were then subjected to SDS-PAGE for Coomassie staining and Western blotting.

Assembly and analyses of recombinant SIV CA proteins. For all WT and mutant recombinant SIV CA proteins, cylinders were assembled in vitro under the following conditions: 20 μl of protein solution (5, 10, and 15 mg/ml) was incubated for 16 h at 4°C in 50 mM Tris-HCl (pH 7.4), 1 M NaCl, and 5 mM β-ME. For negatively stained transmission electron microscopy images, the assembled particles were adsorbed onto glow-discharged Formvar carbon-coated copper grids by floating the grids on 10 μl of each sample for 60 s. Whatman filter paper was then touched with the grids and stained with 2% uranyl acetate and dried on filter paper. Transmission electron micrographs were taken at optical magnifications of ×20,000, ×40,000, and ×100,000. Assembly reactions were analyzed at three different protein concentrations (5, 10, and 15 mg/ml) for each CA mutant. Multiple negatively stained samples were examined for each reaction, and assembly reactions for each mutant were repeated at least three times with proteins from three separate purifications.

Production and visualization of recombinant SIVmac239 viral particles. For the production of recombinant viruses for electron microscopy, 10-μg samples of WT and mutant SIVmac239 proviral plasmid DNA were transfected into 293T cells by the calcium phosphate method. Cell culture supernatants were harvested 48 h later, and cell-free virions were purified and concentrated through a 0.45-μm-pore-size filter, followed by ultracentrifugation (100,000 × *g* for 4 h at 4°C)

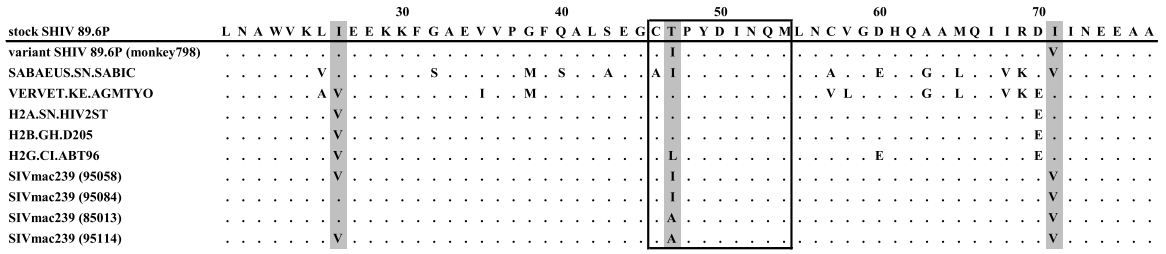

FIG. 1. Amino acid sequence alignment of representative SIV and HIV-2 CA proteins. Sequences were obtained from the Los Alamos HIV Sequence Database (http://www.hiv.lanl.gov) and previously published data. Threonine in position 47 corresponds to position 2 of the immunodominant Mamu-A*01-restricted Gag p11C, C-M CTL epitope. The SIV p11C, C-M epitope is outlined in a box. Positions 26 and 71, highlighted in gray, are extraepitopic amino acid positions with substitutions that are associated with epitope mutations in position 47. The following representative viral sequences are shown: SHIV-89.6P (accession no. U89134), SAB1C (accession no. U04005), AGMTYO (accession no. AF395567), HIV2ST (accession no. U81836), D205 (accession no. X16109), and ABT96 (accession no. AF208027). The SIVmac239 strains represent sequences that were derived from Mamu-A*01$^+$ chronically infected rhesus monkeys, as published by Friedrich et al. (11).

through a cushion of 30% sucrose (wt/vol) in STE buffer (10 mM Tris-HCl [pH 7.3], 100 mM NaCl, 1 mM EDTA). Viral pellets were fixed in 2% glutaraldehyde–2% paraformaldehyde overnight at 4°C. The pellets were washed thoroughly with buffer, postfixed with osmium, rinsed with buffer and water, stained en bloc with a saturated aqueous solution of uranyl acetate, dehydrated in a graded acetone series, and embedded in epoxy resin. Thin sections were picked up on copper grids and stained with saturated uranyl acetate. Electron micrographs were taken on a Hitachi transmission electron microscope at an accelerating voltage of 80 kV and optical magnifications of ×20,000 to ×50,000.

Comparison of virion morphology. The proportion of mature WT and mutant SIVmac239 virions with conical and centric cores was determined by the following method. Virions in 50 photomicrographs of each virus sample were assessed for conical, centric, aberrantly condensed, and immature cores. To minimize bias in the study, three individuals who were blinded to the identity of each sample analyzed the photomicrographs separately. The proportion of mature conical and centric cores for each mutant genotype was compared with the proportion observed for the WT viruses by using a one-sided test for difference of proportions (31).

RESULTS

Sequence alignment of retroviral capsid regions flanking the p11C, C-M CTL epitope. Although the Gag p11C, C-M-specific CTL response is immunodominant in SIV- and SHIV-infected Mamu-A*01$^+$ rhesus monkeys, mutations in this epitope are rare and occur only in association with flanking amino acid substitutions (3, 11, 28). We previously showed that a residue 2 epitope mutation can occur in association with a single downstream amino acid substitution, I71V (28). To determine whether other amino acid substitutions are also associated with this residue 2 p11C, C-M epitope mutation, we evaluated reported sequences of the CA protein of SIVs and other retroviruses. We aligned full-length Gag protein sequences from consensus SIV and HIV-2 strains reported in the Los Alamos HIV Sequence Database and published studies. Of the 100 predicted amino acid sequences, we observed that two amino acid substitutions, isoleucine to valine in position 26 (I26V) and isoleucine to valine in position 71 (I71V), are consistently associated with position 2 p11C, C-M epitope mutations. We previously showed that there is a significant association between T47X and I71V (28). In our current analysis, 24 sequences contain the T47X mutation, only 10 of which do not include the I26V mutation. Statistical analysis (Fisher's exact test) revealed a significant association between T47X and I26V ($P < 0.0001$). Therefore, our viral sequence analysis demonstrated that mutations at position 2 of the p11C, C-M epitope (T47X) are highly associated with an upstream I26V mutation as well as a downstream I71V mutation. However, it is also important to note that the I26V mutation was found to occur in the absence of the T47X mutation in 16 sequences. Figure 1 highlights representative retroviral strains that contain the epitope and flanking amino acid substitutions.

While the I26V substitution was not observed in the mutant SHIV-89.6P that emerged in monkey 798, the present sequence analysis revealed a significant linkage in reported SIV sequences between any position 2 p11C, C-M epitope mutation and the I26V substitution. This I26V mutation occurred either alone or in association with T47I and/or I71V substitutions. Interestingly, the I71V substitution never occurred in the absence of the T47X mutation, while the I26V substitution was observed independent of the p11C, C-M mutation. The association between these flanking amino acid substitutions and the p11C, C-M epitope mutation in related retroviruses suggests that these extraepitopic substitutions were selected with the Gag p11C, C-M escape mutations. These findings suggest that the I26V and I71V mutations may facilitate SIV escape from p11C, C-M-specific CTL by compensating for deleterious effects of the epitope mutation. In addition, these associations indicate that there may be a further benefit for viral fitness in having both flanking mutations.

Contribution of the flanking mutations to viral protein expression. Because of the association between the T47I, I26V, and I71V mutations, we hypothesized that a selective advantage may exist for epitope-mutant viruses with these flanking substitutions. To explore the functional consequences of these mutations, we generated plasmid *gag* constructs expressing the p11C, C-M mutation alone (T47I), as well as this mutation in combination with the upstream mutation (I26V), the downstream mutation (I71V), or both mutations. We transfected 293T cells with these *gag* expression plasmids and 48 h later assessed Gag p27 expression in supernatants and soluble cell lysates. As was previously reported, cells transfected with the T47I plasmid had significantly lower p27 expression as assayed by ELISA and Western blot analysis than did cells transfected with the WT plasmid (28). However, protein expression was restored to WT levels when this mutation was combined with either the I26V or I71V mutation (Fig. 2A and B). Although having both flanking mutations appeared to restore in vitro

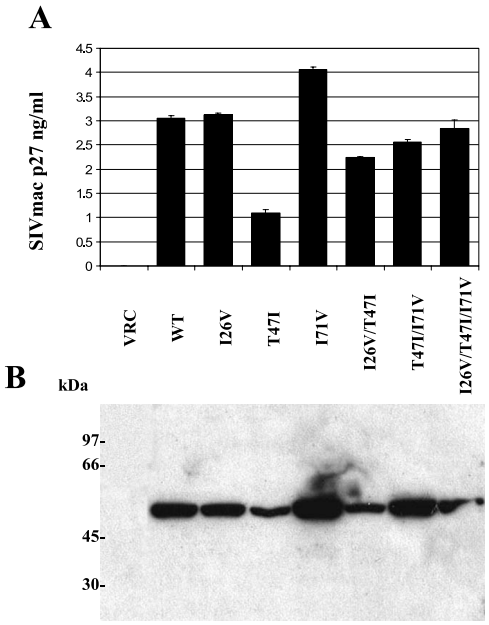

FIG. 2. Mutations I26V and I71V in *gag* restore the level of protein production to that of WT *gag*, 293T cells were transfected with each noted plasmid and supernatant, and cells were harvested 48 h later. SIV Gag p27 levels in supernatants (A) were determined by ELISA. Western blotting was performed to assess the amount of Gag in soluble cell lysates (B). Full-length unprocessed Gag is 55 kDa.

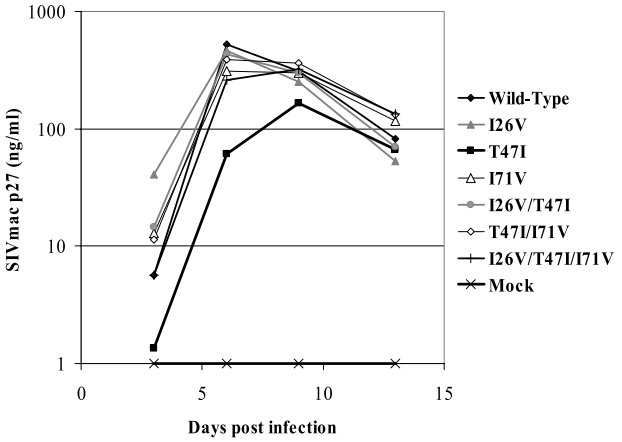

FIG. 3. Mutations I26V and I71V restore the replication rate to that of the WT SIV. Rhesus monkey PBMCs were infected with equal amounts of various SIVmac239 clones (WT, I26V, T47I, I71V, I26V/T47I, T47I/I71V, and I26V/T47I/I71V) and supernatants were monitored for SIV p27 antigen. Values illustrated are means of duplicates.

Gag protein production closer to WT levels, the levels of expression by plasmids containing one or two flanking mutations could not be differentiated.

Contribution of the flanking mutations to viral replication kinetics. Next, we analyzed the infectivity and replication kinetics of SIVmac239 clones incorporating the *gag* mutations. Rhesus monkey PBMCs were infected with SIVmac239 mutants, and supernatants were analyzed for Gag p27. Cells infected with WT SIVmac239 exhibited a peak in Gag p27 production on day 6 of culture, while cells infected with the T47I mutant virus exhibited a delay in the peak production of Gag p27. However, by day 13, the level of p27 production by the T47I mutant virus was indistinguishable from that of the WT (Fig. 3). In contrast, cells infected with the cloned virus incorporating either the I26V, I71V, or both substitutions in association with the T47I mutation demonstrated replication kinetics indistinguishable from those of cells infected with WT SIVmac239. Because viral mutations can arise quickly in vitro, we sequenced a 749-bp region of the *gag* gene in all of the viruses that were harvested at days 0 and 13 and found no additional accumulation of mutations or any reversion back to WT (data not shown). Thus, the single mutation T47I was associated with decreased viral fitness and delayed replication kinetics, while viruses that incorporate either or both flanking mutations exhibited replication kinetics comparable to those of the WT virus.

Mutations in the p11C, C-M CTL epitope inhibit SIV capsid assembly in vitro. The p11C, C-M epitope is situated in a domain of the SIV Gag protein that is homologous to a highly conserved region of HIV-1 Gag which is critical for maintaining the structure of the mature N-terminus CA protein (15, 36). Because of the importance of this region for CA structure, we hypothesized that a p11C, C-M epitope mutation diminishes SIV infectivity and replication kinetics at the level of virion assembly. To explore this hypothesis, we sought to determine whether CA morphology is affected by position 47 amino acid epitope substitutions and whether one or more extraepitopic mutations can compensate for the epitope substitution-associated morphological changes. Specifically, we examined both the ability of CA to assemble in vitro and the core morphology of viruses grown in tissue culture in order to assess the effects of these mutations on viral core formation.

The HIV-1 WT CA assemble in vitro into long hollow cylinders under conditions of high salt concentration (7, 9, 13, 17, 18). These cylinders are composed of helical arrays of CA hexamers. Since similar hexamers have also been observed in Fourier-filtered images of cylindrical cores isolated from HIV-1 virions, the cylinders formed in vitro are presumed to require many of the same CA-CA interactions that are essential for the formation of the viral core (6, 22). Therefore, we employed an in vitro CA assembly assay to evaluate the ramifications of the p11C, C-M epitope and flanking amino acid substitutions on virion structure.

We sought to assess the ability of SIV CA proteins that incorporate the I26V, T47I, I71V, I26V/T47I, T47I/I71V, and I26V/T47I/I71V mutations to assemble into complex structures in vitro. To this end, we generated a series of recombinant SIV CA proteins with amino acid substitutions at positions 26, 47, and 71. Expression levels and purified samples of the WT CA proteins are shown in Fig. 4A and B. All mutant CA showed similar patterns of expression and purification to those of the WT. The WT and mutant CA were soluble in *E. coli* and purified to high levels of homogeneity. The identity of the SIV CA was verified by Western blotting (Fig. 4C) as well as mass spectrometry and N-terminal amino acid sequencing. The purified WT protein preparation contained a mixture of full-length and degraded products, as shown by the smear of bands in the induced lane in Fig. 4C.

The ability of recombinant SIV CA to assemble into complex

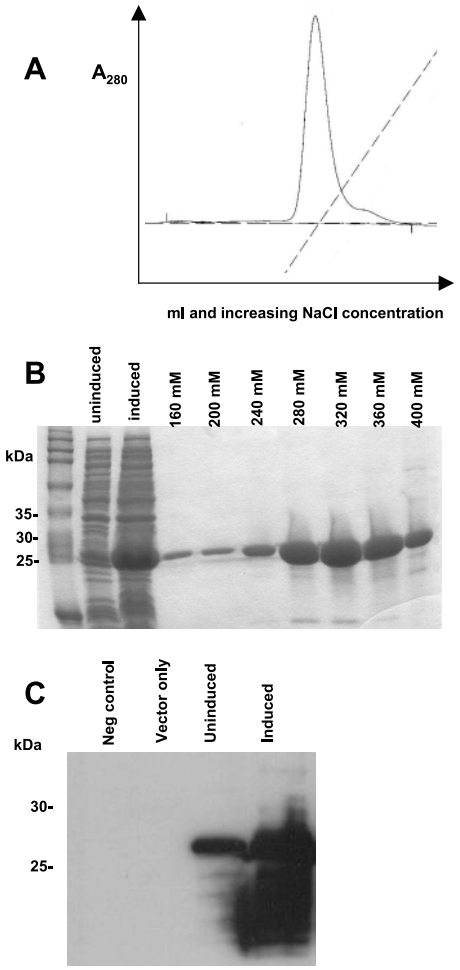

FIG. 4. Expression and purification of recombinant WT SIV capsid proteins. (A) Proteins were purified from *E. coli* by anion-exchange chromatography and eluted with a 0 to 1 M NaCl gradient as indicated by the dashed line. (B) Coomassie blue stain of the SDS-PAGE analysis of the following: lane 1, total cellular BL21(DE3)-RIL *E. coli* proteins prior to induced expression of the capsid protein; lane 2, total cellular bacterial proteins following induction; lanes 3 to 9, SIV CA fractions that were eluted, pooled, and concentrated. The 27-kDa SIV WT CA was optimally eluted at 280 to 320 mM NaCl. The protein preparations were >90% homogeneous. (C) The identity of the proteins was verified by Western blotting using a mouse monoclonal antibody, 55-2F12, directed against SIV CA. The SIV CA is 27 kDa. The positions of migration and molecular mass in kDa are indicated on the left.

structures was assessed by incubating various concentrations of purified proteins in 1 M NaCl solution and then visualizing cylinder formation by negative staining under transmission electron microscopy. The WT SIV CA formed long and irregularly shaped tubules with bends on the ends (Fig. 5A and B). In contrast, the T47I mutation alone completely abrogated the ability of CA to form large complexes, as seen in Fig. 5C. Given that the threonine in position 2 of the Gag p11C, C-M epitope is crucial for the proper folding of monomeric HIV CA, it is not surprising that the T47I substitution prevented mutant proteins from polymerizing into tubules. The recombinant CA containing the I71V substitution alone displayed a similar morphology.

Strikingly, while the addition of I71V to a CA protein containing the T47I mutation restored the ability of the mutant SIV CA to assemble, these proteins assembled into complexes with a novel morphology (Fig. 5E and F). While the WT SIV CA formed long and apparently flexible tubules, the CA with both amino acid substitutions (T47I/I71V) formed short and rigid rods. The morphological differences between the WT and mutant CA proteins were consistently observed in multiple protein preparations. Interestingly, CA containing only the I26V mutation formed long and rigid tubules, suggesting that CA with this mutation alone is capable of forming mature virions (Fig. 5D). This is not surprising since many naturally occurring, replication-competent retroviral strains have this upstream amino acid substitution alone in the absence of the p11C, C-M epitope mutation (Fig. 1). Finally, CA containing I26V in combination with either T47I or T47I/I71V also formed rod-like structures similar to those formed by I26V alone. Together, these micrographs demonstrate that the T47I and I71V substitutions by themselves disrupt SIV CA assembly and that the addition of either I26V or I71V facilitates the assembly of viral CA. The novel morphologies of the cylinders formed by proteins with the epitope and flanking substitutions suggest that the properties of the assembled complexes have changed, and the mechanism of compensation may not simply be a restoration of the structural change caused by T47I.

Capsid mutants with epitope substitutions do not form conical viral cores. To complement the in vitro studies, we also examined the effects of the SIV Gag p11C, C-M epitope and flanking mutations on viral core morphology in viruses grown in tissue culture. We transfected 293T cells with full-length proviral SIVmac239 plasmids containing the I26V, T47X, or I71V mutation alone, harvested cell-free virions 48 h later, and visualized these virions by transmission electron microscopy. Viruses prepared by this method should harbor no additional mutations, nor should they revert to WT, since 293T cells do not support subsequent rounds of viral replication. All viruses produced by this method contained SIV Gag p27 and intact reverse transcriptase as determined qualitatively by colorimetric ELISA (Table 1).

Viral core assembly is driven by polymerization of the unprocessed Gag polyproteins, which assemble beneath the plasma membrane and then bud from infected cells as immature virions. Concomitant with budding, the proteolytic processing of the Gag polyprotein liberates the CA, thereby triggering a structural rearrangement in which CA proteins condense from the spherical shell of the immature virus (Fig. 6, panel 4) into the conical core of the mature infectious virion (Fig. 6, panels 1 and 2) (36). In the electron micrograph of WT virions in Fig. 6A, we observed predominantly mature virus particles with condensed cores having two appearances: conical (cone-shaped cores when virions are sectioned longitudinally, as depicted in Fig. 6, panel 1) and centric (small round cores when virions are cut cross-sectionally, as magnified in Fig. 6, panel 2). Similarly, virions containing the I26V alone had a high proportion of mature, condensed cores. In contrast, viruses with the T47I or the downstream I71V mutation alone were morphologically abnormal (Fig. 6B and C, respectively). The cores of the majority of viruses containing either of these mutations were large and irregularly shaped (as magnified in Fig. 6, panel 3), suggesting that these mutations disrupt the

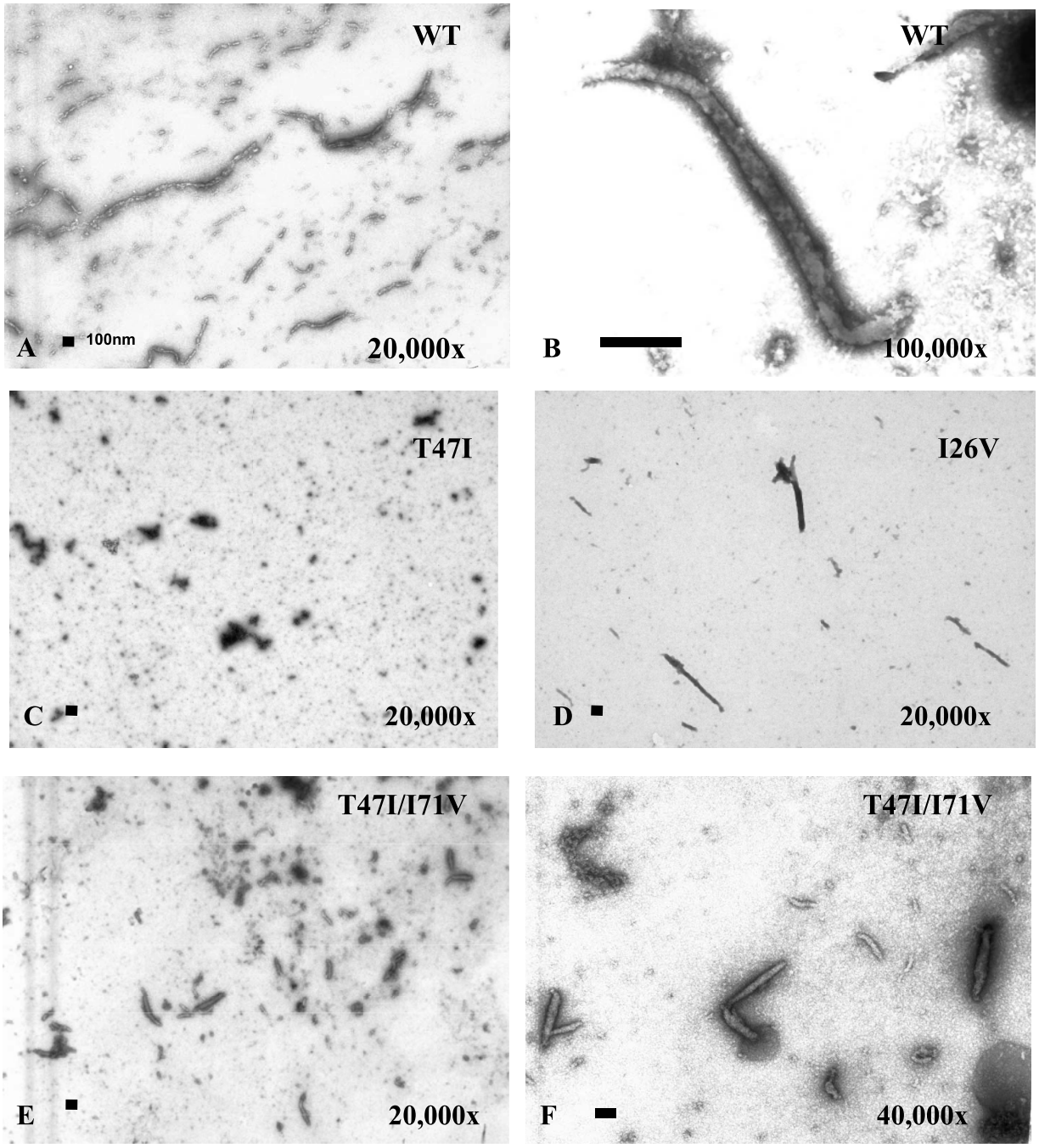

FIG. 5. In vitro assembly of mutant SIV capsid proteins. These panels show representative transmission electron micrographs of negatively stained structures formed by WT CA and CA containing p11C, C-M epitope and extraepitopic mutations. The WT CA polymerized into long and apparently flexible tubules that bend (A and B). Very little assembly was observed with the T47I (C) or I71V CA. The CA containing I26V alone consistently formed long, straight rods (D). I26V/T47I and I26V/T47I/I71V CA formed similar long rods. In contrast, SIV CA containing the T47I/I71V substitutions formed short, rigid rods (E and F). Photographs are shown at the indicated optical magnifications. Scale bars are 100 nm.

process of core condensation to cause aberrant core morphology. However, unlike the HIV-1 CA D51 mutation that abrogated virion core formation (36), the T47I mutation has a less profound effect on viral replication and virion assembly in that a few viruses harboring T47I have conical cores.

Having demonstrated that the T47I mutation disrupts core condensation, we explored the functional sequelae of other amino acid substitutions in position 2 of the Gag p11C, C-M epitope. To this end, we constructed full-length proviral SIVmac239 proviral constructs that contained an alanine residue in position 2 of the p11C, C-M epitope alone (T47A) and in combination with I26V and I71V substitutions. We chose this amino acid substitution because the alanine and flanking mutations have been shown to occur in in vitro cell culture sys-

TABLE 1. Summary of the phenotypes of SIV virions with various capsid mutations

WT or mutant capsid	Result by:					Core morphology[c]
	RT/p27[a]	Infectivity	Replication kinetics	In vitro cylinders	Particle production[b]	
WT	+/+	++	+++	++	+++	Cones and centric cores
I26V	+/+	++	+++	Altered/+	+++	Cones and centric cores
T47I	+/+	+	++	−	++	Fewer mature cores, many aberrant cores
I71V	+/+	++	+++	−	++	Aberrantly condensed cores
I26V, T47I	+/+	++	+++	Altered/+	+++	Cones and centric cores
T47I, I71V	+/+	++	+++	Altered/++	+++	Cones and centric cores
I26V, T47I, I71V	+/+	++	+++	Altered/+	+++	Cones and centric cores
T47A	+/+	ND	ND	ND[d]	+	Immature, uncondensed cores
I26V, T47A	+/+	ND	ND	ND	++	Mostly uncondensed cores, few mature cores
T47A, I71V	+/+	ND	ND	ND	++	Mostly uncondensed cores, few mature cores
I26V, T47A, I71V	+/+	ND	ND	ND	+++	Cones and centric cores

[a] Virus production from transfected 293T cell as assayed by reverse transcriptase activity and SIV p27 CA antigen levels in the culture supernatant.
[b] Virion particles were pelleted through 30% sucrose and quantified by both electron microscopy and p27 ELISA.
[c] Core morphologies were observed by transmission electron microscopy in thin sections of concentrated virions.
[d] ND, not done.

tems, as well as in Mamu-A*01$^+$ monkeys that are chronically infected with SIVmac239/251 (8, 11, 29). Interestingly, although the majority of viruses containing the T47A mutation alone were able to bud, almost all of these particles had immature, uncondensed cores (Fig. 6D). In addition, there appeared to be many extracellular immature virions that were tethered to larger membranes. We also observed an associated defect in the level of virus production in proviral plasmids that carried the T47A mutation as compared to WT viruses (Table 1). These observations are consistent with the previously reported phenotype associated with the CA T47A mutation in SIV (32).

Viruses containing both epitope and extraepitopic substitutions have phenotypes similar to wild-type virus. We then determined the contribution of extraepitopic amino acid substitutions on the morphology of virions harboring p11C, C-M mutations. Preparations of SIVmac239 viruses with substitutions including T47I/I71V, I26V/T47I, I26V/T47I/I71V, and I26V/T47A/I71V demonstrated numerous mature virions with conical and centric cores. Figure 6E shows an electron micrograph of recombinant viruses containing I26V/T47I/I71V substitutions. The percentage of mature virions with conical and centric cores produced by these mutant viruses was similar to that seen for WT SIVmac239. These findings suggest that the extraepitopic substitutions have the ability to restore the condensation defect associated with the T47I or T47A mutations.

To assess quantitatively whether these particular mutant viruses produced more virions with irregular cores than did the WT virus, we determined the percentage of mature cores for each mutant virus and compared this percentage to that determined for WT proviral samples by statistical analysis. As summarized in Table 2, viruses with single-amino-acid substitution T47I, T47A, or I71V produced fewer mature, condensed cores than did WT viruses. However, this morphological abnormality was rescued by the addition of either upstream or downstream mutations, as reflected by the proportions of mature cores in I26V/T47I, T47I/I71V, and I26V/T47I/I71V viruses when compared to WT viruses. Furthermore, the proportions of mature cores were not appreciably different between viruses that contained the T47I and one flanking mutation and viruses that harbored the epitope and two flanking mutations. This suggests that there may not be a structural advantage for viruses to accrue multiple compensatory mutations. However, it is possible that the assays we have employed are not sensitive enough to detect what may be a subtle increase in the viability of T47I mutant cores associated with the addition of a second flanking mutation.

Interestingly, in contrast to what was observed for the T47I viruses, the number of mature cores in T47A viruses was not restored by only one flanking mutation at either the downstream or upstream position. The T47A substitution at position 2 of the epitope required two flanking substitutions in order to rescue the defect caused by this particular amino acid substitution in the p11C, C-M epitope (Table 2). This difference between the T47A and I47I viruses could reflect the fact that the T47A mutation is more disruptive for the formation of the viral core and thus required multiple compensatory substitutions in order to yield replication-competent viruses.

DISCUSSION

Accumulating data indicate that the ability of HIV-1 or SIV to escape from CTL recognition can sometimes be constrained by the limited plasticity of particular regions of viral proteins. The SIV Gag p11C, C-M epitope is located in a region of the capsid that is required for capsid self-association and conformational assembly. Therefore, escape from CTL recognition at the immunodominant SIV/SHIV-89.6P Gag p11C, C-M epitope offers an important window into the interactions between immune cells and evolving domains of retroviruses that are structurally constrained.

In the present structure-function analysis of the SIV CA, we have demonstrated that mutations in the Mamu-A*01-restricted immunodominant Gag p11C, C-M epitope disrupt CA protein expression, viral replication kinetics, and virion core assembly. In addition, we defined the morphological ramifications of associated mutations that facilitate the generation of viable viruses. We showed that viruses with amino acid substitutions in this epitope can only be formed if flanking mutations are also present to compensate for epitope mutation-associ-

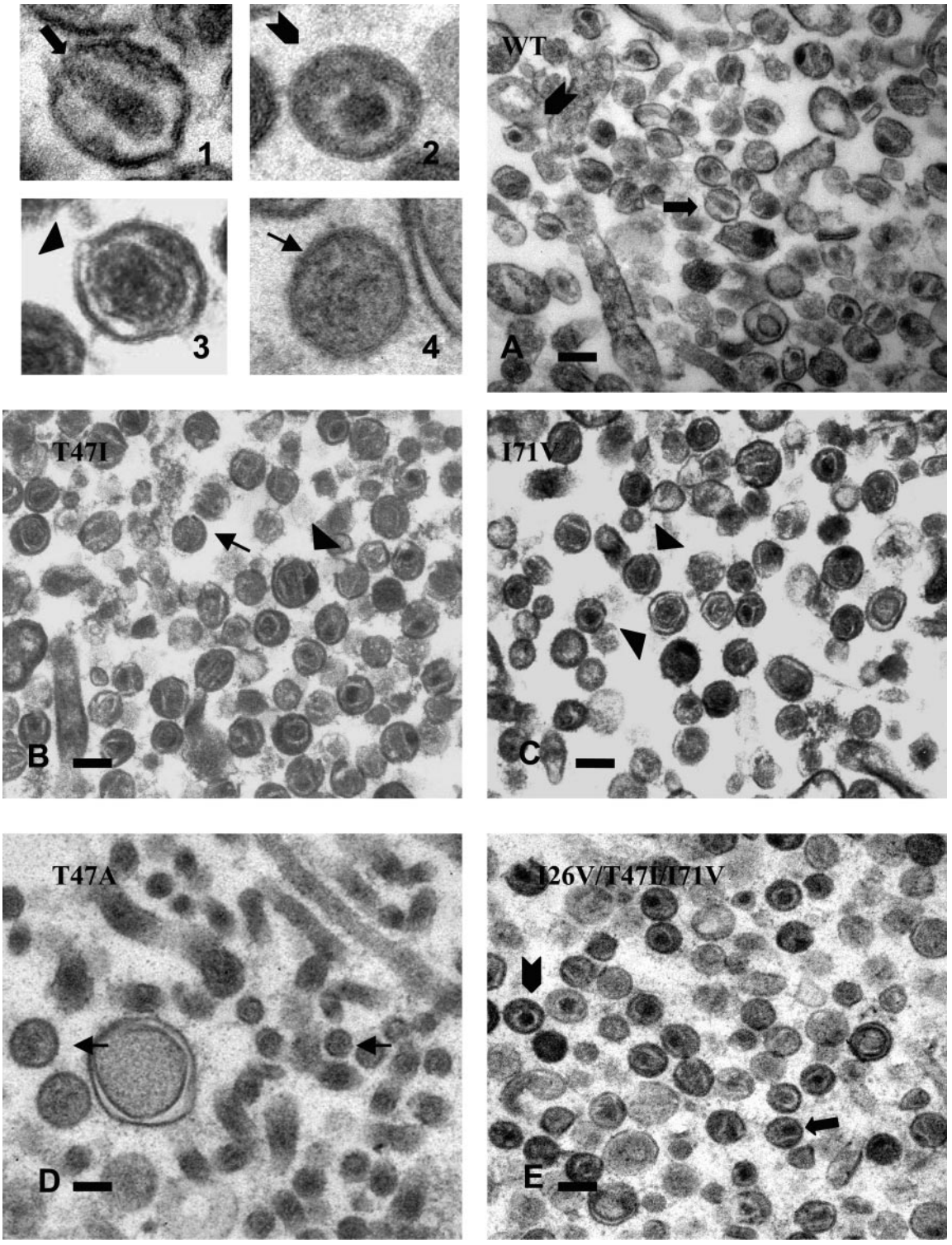

FIG. 6. Transmission electron micrographs of WT and mutant SIVmac239 virions with Gag p11C, C-M and compensatory substitutions. Panels 1 to 4 show detailed magnified views of mature conical cores (panel 1), mature centric cores (panel 2), aberrantly condensed cores (panel 3), and immature/uncondensed cores (panel 4). Panel A shows an overview of a field containing WT viruses. Panel B shows viruses that contain the T47I mutation alone. Note that the majority of viruses contain aberrantly condensed cores, and there are fewer mature conical and centric cores compared to WT viruses. Panel C depicts viruses containing the I71V mutation alone, with the majority of virions containing large cores. Panel D illustrates immature, uncondensed viruses containing the T47A mutation alone, which have a tendency to tether to membranes. Panel E depicts viruses containing the I26V/T47I/I71V mutations. Viruses with I26V/T47I, T47I/I71V, and I26V/T47A/I71V mutations show similar core morphology to what is shown in panel E. Note that the proportion of virions that are immature and contain abnormally large cores is higher in panels B and C than in panels A and E. Thick solid arrows show representative conical cores, bullet arrows indicate representative centric cores, arrowheads point to aberrantly condensed cores, and arrows with thin shafts highlight immature, uncondensed cores. Scale bars are 100 nm.

TABLE 2. Proportion of mature cores observed for wild-type and capsid mutant SIVmac239 strains

WT or mutant	% of mature cores (conical or centric)	*P* value[a]
WT	67	NA[b]
I26V	79	0.009
T47I	46	<0.001
I71V	14	<0.001
I26V/T47I	67	0.596
T47I/I71V	70	0.461
I26V/T47I/I71V	60	0.078
T47A	45	<0.001
I26V/T47A	44	<0.001
T47A/I71V	47	<0.001
I26V/T47A/I71V	71	0.478

[a] *P* values were obtained by using a one-sided test for differences in the proportion of mature cores in viruses with the designated mutant CA type relative to viruses with WT CA.
[b] NA, not applicable.

ated perturbations in the viral core structure. Specifically, we demonstrated that the T47I in position 2 of the p11C, C-M epitope interferes with CA protein production, replication fitness, and viral core formation and that these defects are reversed with the introduction of amino acid substitutions in specific upstream and/or downstream positions. These compensatory substitutions were able to restore the ability of viral CA containing a p11C, C-M mutation to generate cylinders in vitro and mature viral cores in cell culture. These results indicate that either a downstream or upstream substitution is sufficient to restore the ability of viruses containing the T47I mutation to condense its core. Although we did not observe an obvious structural advantage for viral CA to have multiple flanking mutations in association with T47I, the consistent emergence of both substitutions associated with the Gag p11C, C-M epitope in our sequence analysis suggests that there may be a selective advantage for accruing more than one flanking amino acid substitution in viruses with certain epitope escape mutations. This was demonstrated experimentally with the T47A escape mutation, where two compensatory mutations are needed to restore proper viral assembly.

Consistent with proposed models of HIV-1 CA assembly, our studies suggest that interactions between the β-hairpins of CA monomers facilitate capsid multimerization and viral core assembly. Using the coordinates of the HIV-1 CA N-terminal domain published by Gitti and colleagues (15), we constructed a model of the SIV CA that predicts the elimination of the hydrogen bond involving Asp^{50} when Thr^{47} is mutated to Ile^{47} (28). Our results suggest that the molecular interactions between Thr^{48} and Asp^{51} in HIV-1 are conserved in SIV and that the T47I mutation interferes with stabilization of the essential β-hairpin. It is interesting to note that the T47I substitution only appears to cause a delay in virus replication and a decrease in the amount of mature virions, in contrast to the complete abrogation of viral replication and core formation caused by the D51 mutation in HIV-1 CA (36). Our results suggest that the hydrogen bond between Thr^{47} and Asp^{50} may position Asp^{50} so that it can optimally participate in another requisite interaction. Thus, mutations involving Thr^{47} will adversely influence the ability of viruses to form properly condensed capsid cores but will not completely abolish virion formation. This model is consistent with the findings of other studies showing that viruses containing mutations in HIV-1 Asp^{51} and SIV Asp^{50} have maturation defects (32, 36).

Our present analysis was restricted to isoleucine and alanine substitutions in position 2 of the Gag p11C, C-M epitope. We chose to limit our studies to these epitope substitutions for a number of reasons. A position 2 mutation is the only epitope mutation that is both tolerated by the virus and not recognized by p11C, C-M-specific CTL (29). In addition, the threonine-to-isoleucine and threonine-to-alanine substitutions appeared consistently in sequence databases as well as in numerous SIVmac239/251, SIVsmE660, and SHIV-89.6P isolates (2, 8, 11). Finally, the use of alanine substitution is a well-defined approach for studying the importance of individual amino acids in protein structure and function. However, it is important to note that the T47A mutation does not appear in naturally occurring retroviral strains in the Los Alamos HIV Sequence Database. Our data provide a potential explanation for this observation. In our studies, the alanine substitution had a profound effect on viral core formation. The defect caused by alanine was not rescued by a single compensatory mutation, as was the case for the T47I substitution. Rather, additional substitutions were needed to restore the viral phenotype to WT. Thus, viruses that carry the T47A mutation may not replicate to a level high enough in vivo to allow the accumulation of multiple compensatory mutations.

CTL that recognize structurally constrained epitopes can be associated with long-term survival in HIV-1- and SIV-infected individuals (20, 23, 25, 27), while CTL specific for less-conserved regions of the viruses can be associated with rapid progression (19, 26, 34, 35). A major contributing factor to the delayed disease progression in rhesus monkeys that mount a robust CTL response against the immunodominant Gag p11C, C-M epitope may be the inability of the virus to accumulate escape mutations in the region of the capsid containing this epitope. The present work elucidates the functional and structural constraints on the ability of HIV/SIV to escape from CTL pressure. Ultimately, an understanding of why certain epitopes are highly conserved and delineating strategies that retroviruses employ to evade immune surveillance will facilitate the design of novel immunization strategies that can prevent the evolution of viral escape mutations.

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