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Capsid Proteins from Human Immunodeficiency Virus Type 1 and Simian Immunodeficiency Virus SIV_{mac} Can Coassemble into Mature Cores of Infectious Viruses[▽]

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We have recently shown that the Gag polyproteins from human immunodeficiency virus type 1 (HIV-1) and HIV-2 can coassemble and functionally complement each other. During virion maturation, the Gag polyproteins undergo proteolytic cleavage to release mature proteins including capsid (CA), which refolds and forms the outer shell of a cone-shaped mature core. Less than one-half of the CA proteins present within the HIV-1 virion are required to form the mature core. Therefore, it is unclear whether the mature core in virions containing both HIV-1 and HIV-2 Gag consists of CA proteins from a single virus or from both viruses. To determine whether CA proteins from two different viruses can coassemble into mature cores of infectious viruses, we exploited the specificity of the tripartite motif 5α protein from the rhesus monkey (rhTRIM5α) for cores containing HIV-1 CA (hCA) but not the simian immunodeficiency virus SIV_{mac} CA protein (sCA). If hCA and sCA cannot coassemble into the same core when equal amounts of sCA and hCA are coexpressed, the infectivities of such virus preparations in cells should be inhibited less than twofold by rhTRIM5α. However, if hCA and sCA can coassemble into the same core structure to form a mixed core, rhTRIM5α would be able to recognize such cores and significantly restrict virus infectivity. We examined the restriction phenotypes of viruses containing both hCA and sCA. Our results indicate that hCA and sCA can coassemble into the same mature core to produce infectious virus. To our knowledge, this is the first demonstration of functional coassembly of heterologous CA protein into the retroviral core.

Coinfection of two distinct retroviruses in the same host occurs in nature. For example, a significant human population is currently coinfected with human immunodeficiency virus type 1 (HIV-1) and HIV-2, two distinct lentiviruses (2, 19, 25). Phylogenetic analyses of the primate lentiviruses indicate that some of the viruses are recombinants of two distinct parental viruses (3, 37), indicating that the two parental viruses must coinfect the same host and infect the same target cells. Coinfection allows interactions between two viruses by several mechanisms including recombination and pseudotyping. Recombination generates a progeny different from the two parents; the best known example is that simian immunodeficiency virus SIV_{cpz}, the zoonotic precursor of HIV-1, is thought to be a recombinant of SIV_{rcm} and SIV_{gsn} (3). Pseudotyping refers to virions containing components from two different viruses. A commonly observed example of pseudotyping is the use of Env proteins from different viruses, although pseudotyping can occur with many other virion components. Pseudotyped viruses often have properties different from those of virions containing components from one virus; for example, Env-pseudotyped viruses can have a different host range (27, 40).

In all retroviruses, the major structural proteins are encoded by the *gag* gene, which is translated into the Gag polyprotein. During virus assembly, Gag coordinates the incorporation of other viral proteins and viral RNA and interacts with the host cell machinery to facilitate the release of viral particles (15, 20, 43). In orthoretroviruses, most of the newly released particles are "immature"; virus-encoded protease cleaves the Gag polyprotein during or soon after assembly to allow the transformation from immature to mature particles (1). This process, termed maturation, is required for the production of infectious virions. Although Gag polyproteins from different retroviruses might have only limited sequence homology, they share three similarly organized, conserved domains: matrix (MA), capsid (CA), and nucleocapsid (NC). Additionally, Gag from different retroviruses can have other domains. For example, the proteolytic cleavage of HIV-1 Gag also yields spacer peptide 1 (SP1 or p2), SP2 (p1), and p6 proteins. In the immature particles, Gag forms an approximately spherical shell underlying the membrane (51). After cleavage from the polyprotein, the HIV-1 CA (hCA) protein undergoes structural refolding and reassembles into a cone-shaped core that encloses the genomic RNA, NC, reverse transcriptase, integrase, and other viral and host components (1, 6, 8). It has been estimated that, although each immature HIV-1 particle contains around 5,000 Gag proteins, the mature shell of HIV-1 contains 1,000 to 1,500 CA proteins assembled into a mostly hexameric lattice (7). Therefore, only a fraction of CA proteins generated from Gag cleavage are used to form one mature virion core.

We previously demonstrated that Gag proteins from HIV-1 and HIV-2 can coassemble and complement each other's functions (5). Because only a fraction of the mature CA proteins are used to generate a core, it is possible that only the CA proteins from one of the viruses are used to form a core.

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Coassembly of heterologous CA proteins into a core has not previously been demonstrated; therefore, it is unclear whether such a core can be formed and whether it is capable of conducting all steps leading to a successful infection. The mature CA proteins in the virion core play important roles in the viral replication cycle (14, 44, 45, 50, 52). After entry into the cells, virus uncoating occurs; CA proteins play an important role in regulating the uncoating process. Mutations in CA can lead to acceleration or delay of the uncoating events (14); both alterations can abolish the infectivity of HIV-1. Additionally, CA can influence the ability of HIV-1 to infect nondividing cells, possibly by affecting nuclear import (12, 53). Taken together, the identities of the CA proteins can affect the biological properties of the virus, and it is possible that a coassembled core can have properties different from those of the two parental viruses.

We sought to determine whether CA proteins from two different primate lentiviruses can coassemble into a mature core to carry out all the steps necessary for infection. We envisioned three possibilities for virions with heterologous Gag proteins: first, mature heterologous CA proteins do not coassemble and the cores consist of CA proteins from one virus (pure CA cores); second, heterologous CA proteins coassemble into a core (mixed CA core) but viruses containing mixed cores are not infectious; and third, heterologous CA proteins coassemble into a mixed core and viruses containing mixed cores are infectious. To answer our experimental question, we exploited the inhibitory specificity of the tripartite motif 5α protein from the rhesus monkey (rhTRIM5α) for cores containing hCA but not SIV_{mac} CA (sCA).

TRIM5α is a member of the tripartite motif-containing family of proteins (35). The tripartite motif comprises a RING domain that includes two zinc finger motifs, one or two B-box domains, and a coiled-coil domain that mediates protein-protein interactions between TRIM family members (32, 41, 47). The rhTRIM5α protein restricts the replication of HIV-1 and HIV-2, but not SIV_{mac}, a virus closely related to HIV-2 (22, 41, 54, 55). Although the mechanism by which rhTRIM5α restricts HIV-1 replication is not fully elucidated, recent studies suggest that rhTRIM5α targets the incoming mature HIV-1 core to promote premature uncoating and possibly degradation of CA proteins (9, 42). However, the inhibition of HIV-1 infection imposed by TRIM5α can be saturated by overwhelming the system with restriction-sensitive mature viruses or virus-like particles (21, 39, 41, 54). Interestingly, TRIM5α restriction cannot be saturated by adding immature virions or particles with cores consisting of restriction-insensitive CA proteins (10, 13). Furthermore, saturation of TRIM5α restriction depends on the stability of the incoming HIV-1 capsid (39). These results strongly support the idea that TRIM5α recognizes CA that has undergone conformational changes or the tertiary structure of CA in the mature core.

N- and B-tropic murine leukemia viruses, two highly homologous virus strains that differ in their sensitivities to Fv-1 and TRIM5α, were used to demonstrate that coassembled viruses were sensitive to host Fv-1 restriction (26, 34, 46). The restriction imposed by rhTRIM5α is specific to hCA and is relieved in HIV-1 particles that have cores containing sCA proteins (33). We hypothesize that, if hCA and sCA can coassemble into the same core, TRIM5α can recognize hCA present in the mixed CA core and restrict the infection of such viruses; in contrast, a population of pure CA core viruses, some with sCA cores and some with hCA cores, would generate a different restriction pattern. To determine whether hCA and sCA can coassemble to form a mixed core, we examined the TRIM5α restriction phenotypes of viruses containing two different types of Gag proteins, one with hCA and the other with sCA. To ensure that most of the infection events observed were from coassembled viruses, we used two modified viruses that each harbored a debilitating *gag* mutation so that viruses derived from neither mutant could replicate efficiently. However, Gag proteins from two mutants could coassemble to allow functional complementation, thereby rescuing efficient virus replication. Our results indicate that hCA and sCA can coassemble into a mixed CA core to produce infectious virus.

MATERIALS AND METHODS

Plasmid construction. Plasmids were constructed with standard molecular cloning procedures (38). The general structures of all constructs were verified by restriction mapping, and PCR-amplified regions were further characterized by DNA sequencing to avoid inadvertent mutations. For clarity, pHDV-eGFP (48) and its derivative pHIV-Thy1 (36) are referred to as hCA-gfp and hCA-thy, respectively. For clarity, HIV-1-based vectors 1-NC-thy and 1-PTAP-gfp (5), containing mutations in the NC and PTAP regions of HIV-1 Gag, respectively, are referred to as hCA-NC*-thy and hCA-PTAP*-gfp, respectively. By overlapping PCR and cloning, most of the CA region (amino acids [aa] 1 to 204) in hCA-NC*-thy and hCA-PTAP*-gfp was replaced with sCA (aa 1 to 202) to generate sCA-NC*-thy and sCA-PTAP*-gfp, respectively. Vectors sCA-thy and sCA-gfp were constructed by replacing the AtaII-SbfI fragments in hCA-thy and hCA-gfp, respectively, with the corresponding fragment in NL43-sCA; NL43-sCA contains sCA (aa 1 to 202) in place of hCA (aa 1 to 204).

Cells, transfections, and infections. Cell line 293T and 293T cells expressing TRIM5α or empty plasmid have been previously described (41). These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, penicillin (50 U/ml), and streptomycin (50 U/ml). All cultured cells were maintained at 37°C with 5% CO_2.

To generate vector-derived viruses, 293T cells were transiently transfected with an HIV-1 vector and pHCMV-G, which expresses vesicular stomatitis virus G protein, using the MBS mammalian transfection kit (Stratagene). Viral supernatants were harvested 24 h later, clarified through a 0.45-μm-pore-size filter to remove cellular debris, and used immediately or stored at −80°C prior to infection.

For infection of 293T cells expressing TRIM5α or empty plasmid, 50,000 cells were plated in each well of a 24-well plate and infected 24 h later. Serial dilutions were generated from each viral stock and used to infect target cells in the presence of Polybrene at a final concentration of 10 μg/ml. Viruses were removed 2 h later, and fresh medium was added to the cells; target cells were processed 48 h postinfection, followed by flow cytometry analyses.

Antibody staining and flow cytometry analyses. Cells were stained with allophycocyanin-conjugated anti-Thy-1 antibody (eBioscience) and fixed with 2% paraformaldehyde prior to flow cytometry analyses. These assays were performed on a FACSCalibur apparatus (BD Biosciences), and data obtained were analyzed with Flowjo software (Tree Star). Infected cells were scored by expression of Thy or a green fluorescent protein (GFP) marker. Multiplicity of infection (MOI) was calculated from the number of infected cells obtained from flow cytometry as previously described (11).

RESULTS

Strategy to examine whether CA proteins from distinct primate lentiviruses can coassemble to form infectious cores. To determine whether CA proteins from two different viruses can coassemble into mature cores to form infectious particles, we exploited the specificity of rhTRIM5α to restrict the replication of viruses containing the hCA core but not the sCA core. We previously observed that HIV-1 and HIV-2 Gag proteins can coassemble. Therefore, we expected that HIV-1 Gag mol-

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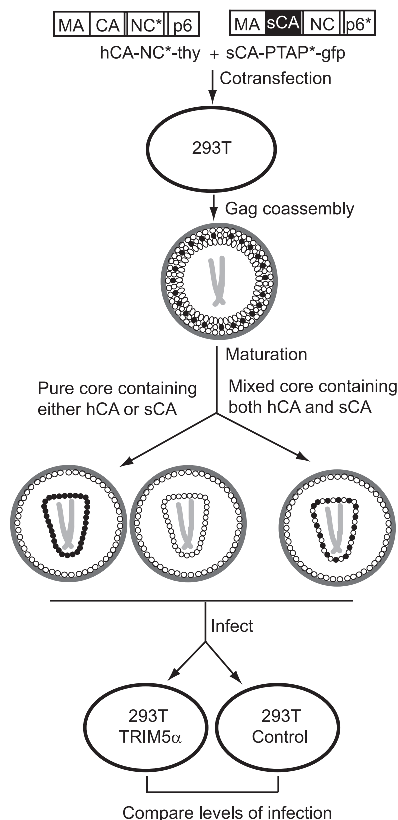

FIG. 1. Strategy to examine whether hCA and sCA can coassemble into infectious mature cores. HIV-1 vectors encoding hCA or sCA and containing either a debilitating NC or PTAP mutation were cotransfected into 293T cells. Coassembly and functional complementation of the two mutant Gag proteins result in the production of infectious virus particles. After proteolytic cleavage during virus maturation, the coassembled viruses contain both mature hCA and sCA. It is possible that hCA and sCA cannot coassemble into the same core to generate infectious viruses, in which case the infectious virus population is a mixture of particles with pure cores consisting of either hCA or sCA. It is also possible that hCA and sCA can coassemble into the same core and that viruses containing these cores are infectious. To distinguish between these two possibilities, the virus preparation was used to infect 293T cells expressing rhTRIM5α or control 293T cells expressing the empty plasmid and the levels of infection were compared to determine the effect of rhTRIM5α restriction on virus infectivities.

ecules containing hCA can coassemble with those containing sCA (Fig. 1) because of the high similarity between SIV_{mac} and HIV-2 (83% amino acid sequence identity in Gag). Coassembled viruses that contain both types of Gag proteins would have two different mature CA proteins, hCA and sCA, after proteolytic cleavage. If hCA and sCA cannot coassemble into the same core, the virus preparation would be a mixture of particles with pure CA cores. Viruses with hCA cores would be restricted by rhTRIM5α, whereas those with sCA would not. Therefore, the titer of such a virus preparation would be slightly decreased by rhTRIM5α. In contrast, if hCA and sCA can coassemble to form a mixed CA core, rhTRIM5α would recognize and significantly restrict the infection of particles containing these cores. By determining the phenotypes of the coassembled viruses, we could determine whether hCA and sCA are coassembled in the cores of infectious particles.

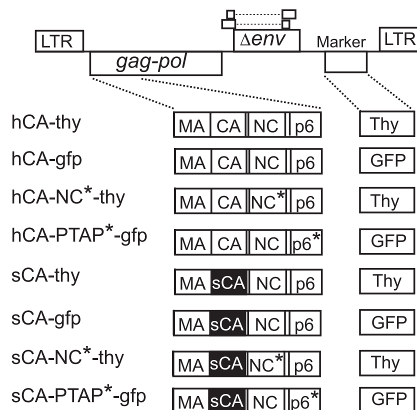

FIG. 2. General structure of HIV-1-based vectors. These vectors express *gag-pol*, *tat*, *rev*, and a marker gene, either *gfp* or *thy*. The Gag domains MA, CA, NC, and p6 are indicated. The asterisks indicate the locations of the mutations. LTR, long terminal repeat.

Characterization and complementation of the mutant constructs. To ensure that most of the infectious viruses contained both Gag proteins, we introduced mutations in either the NC domains or the PTAP motifs of our vectors. The NC mutation replaces the conserved CCHC/CCHC with CCHH/CCCC and drastically decreases the viral RNA encapsidation and virus titer (18). The PTAP mutation changes PTAP to LIRL and significantly reduces the virus production and virus titer (23). When coexpressed in the HIV-1 context, these two mutants can complement each other to generate infectious viruses with mostly restored infectivity (5).

To implement this strategy, we used several HIV-1-based vectors that contain *cis*-acting elements essential for viral replication and express *gag-pol*, *tat*, *rev*, and a marker gene, either *thy* or *gfp* (Fig. 2). The vector name indicates the identity of the CA, nature of the mutation, and encoded marker. For example, hCA-thy expresses wild-type HIV-1 *gag-pol*, sCA-thy expresses a functional chimeric *gag-pol* with most of the CA-encoding domain derived from SIV_{mac}, and hCA-NC*-thy expresses HIV-1 *gag-pol* encoding a mutation in NC; additionally, these three vectors express the *thy* marker.

To measure the viral titer generated by each vector, we transfected the vector plasmid into 293T cells along with pHCMV-G, which expresses vesicular stomatitis virus G protein. Viruses were harvested from transfected cells and used to infect fresh 293T cells; infected cells were later analyzed by flow cytometry. Representative flow cytometry analyses are shown in the top and middle panels of Fig. 3. As expected, the NC mutants and PTAP mutants generated drastically decreased titers compared with titers generated by hCA vectors and sCA vectors that contain functional *gag-pol*.

To ensure that Gag-containing hCA and sCA can coassemble and complement each other's function, we cotransfected two constructs, one with an NC mutation and one with a PTAP mutation, and examined the viral titers generated from these transfections. As shown in the bottom panels of Fig. 3, two hCA-containing or two sCA-containing Gag proteins can complement each other's function. Furthermore, hCA-containing and sCA-containing Gag proteins can also complement each other's function to generate robust viral titers. The

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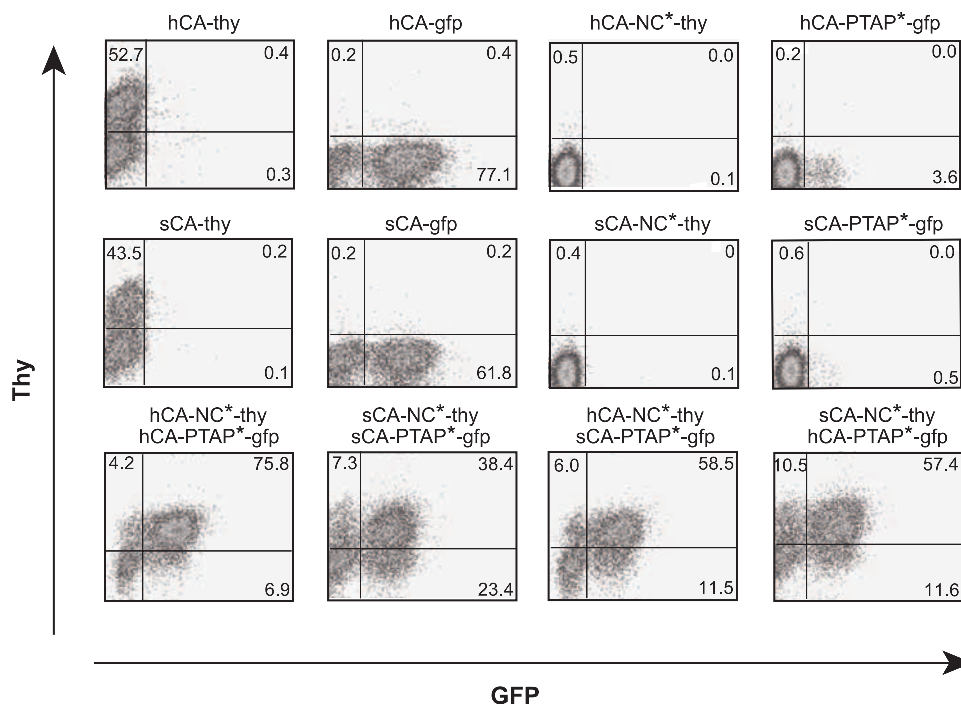

FIG. 3. Representative flow cytometry analyses of infection by various viruses. The axes indicate Thy expression and GFP expression. (Top) Infection by viruses generated by wild-type or mutant vectors containing hCA. (Middle) Infection by viruses generated by wild-type or mutant vectors containing sCA. (Bottom) Infection by viruses generated from cotransfection of vectors containing an NC or PTAP mutation.

successful complementation between vectors with heterologous CA domains provided the opportunity to study whether heterologous CA proteins coassemble into infectious viral cores.

TRIM5α restriction in viruses with pure CA cores consisting of either hCA or sCA. Several reports have demonstrated that TRIM5α restriction can be saturated and that the level of restriction depends on the amount of virus used for infection (4, 10, 13, 21, 39, 41, 54). To examine TRIM5α restriction of virus infection, we serially diluted each viral preparation and measured viral titers generated from infecting 293T cells expressing rhTRIM5α and control 293T cells expressing an empty vector; for simplicity, these cells will be referred to as TRIM5α cells and control cells, respectively. We then converted the percentage of infection to MOI and calculated the level of restriction by dividing the MOI in control cells by the MOI in TRIM5α cells at a given dilution of a viral stock. Three independent experiments were performed and generated similar results; representative results of restriction in viruses derived from hCA-gfp or sCA-thy are shown in Fig. 4A and Table 1. Viruses generated from hCA-gfp alone (Fig. 4A) were greatly restricted in TRIM5α cells compared with those in control cells; consistent with the previous observations, the levels of restriction were affected by the amount of the input virus. Using serially diluted hCA-gfp viruses, we determined that the percentages of infection for TRIM5α cells compared with those for control cells were 24% versus 95.3%, 4% versus 73.2%, and 1.3% versus 46.3%, which resulted in an 11- to 48-fold restriction. In contrast, sCA-thy-derived viruses (Fig. 4A) generated similar percentages of infection in TRIM5α and control cells: 72.2% versus 73.8%, 52.5% versus 56.50%, 40.50% versus 40.90%, 24.8% versus 28.6%, and 11.4% versus 14% (Fig. 4A and Table 1).

We then tested the effect of infecting cells with a mixture of viruses, some containing hCA cores and the others sCA cores. Virus stocks were generated separately, derived either from hCA-gfp or from sCA-thy; these two virus stocks were then added together at various ratios and used to infect TRIM5α and control cells; the infected cells in both cell lines were measured, and levels of TRIM5α restriction were determined. When viral stocks of hCA-gfp and sCA-thy were mixed at equal amounts, a 2- to 2.5-fold restriction was observed, whereas when viral stocks of hCA-gfp and sCA-thy were mixed at 1:2 and 1:4 ratios, very little restriction was observed in both mixtures. We reasoned that the virus population with sCA would infect both TRIM5α cells and control cells equally well, whereas the virus population with hCA cores would infect TRIM5α cells poorly compared with control cells. When the effects of the two populations were added together, we should observe a very mild restriction. A theoretical simulation of this scenario is shown in Table 2. For example, if we have a mixture of viruses and half of the viruses contain hCA cores and half contain sCA cores, each infecting 5% of control cells, then infection of 10% of the control cells should be detected. If hCA virus is restricted by 50-fold in the TRIM5α cells, it would yield 0.2% infection. Because sCA virus is not restricted by rhTRIM5α, it would yield 5% infection; together, these infection levels imply that a total of 5.2% of the TRIM5α cells should be infected. The level of restriction is calculated by comparing the control cell infection (10%) with the TRIM5α cell infection (5.2%), which yields approximately twofold restriction. The level of restriction would decrease when the

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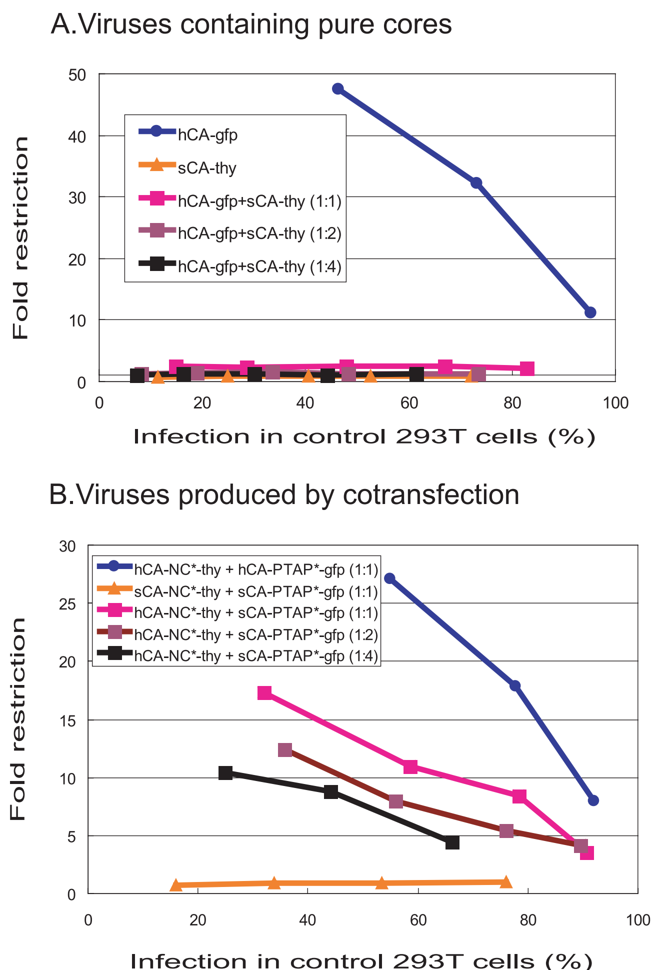

FIG. 4. Restriction patterns of viruses. (A) Representative results of TRIM5α restriction for viruses containing pure cores. Separately produced viruses were mixed at various ratios, and the restriction levels of such virus mixtures were determined. Three sets of independent experiments were performed and yielded similar results; in all experiments, the mixed viruses never achieved more than threefold restriction. (B) Representative results of TRIM5α restriction in viruses produced by cotransfection of NC or PTAP mutants with homologous or heterologous CA proteins. All three independent experiments generated similar results. In each experiment, viruses containing only hCA had the highest restriction, whereas viruses containing only sCA did not have significant restriction. Among the mixed viruses, restriction decreased with smaller amounts of hCA. In the three sets of experiments, when the control cells were infected at 50% and hCA- and sCA-encoding plasmids were transfected at 1:1, 1:2, and 1:4 ratios, we estimated that TRIM5α restricted the virus titers 11- to 13-fold, 7- to 9-fold, and 5- to 8-fold, respectively. Restriction values were calculated by dividing the MOI in control cells by the MOI in rhTRIM5α cells.

relative amounts of sCA virus increase (Table 2). These predictions are consistent with our results for virus populations containing pure CA cores.

TRIM5α restriction phenotypes of viruses containing both hCA and sCA proteins. To determine whether heterologous CA proteins can coassemble into cores of infectious viruses, we examined the restriction phenotypes of viruses generated by cotransfected vectors hCA-NC*-thy and sCA-PTAP*-gfp. A total of three independent experiments were performed, which yielded similar results; data from one set of representative experiments are shown in Table 3 and Fig. 4B. For controls, we also examined the restriction phenotypes of viruses generated from cotransfecting at a 1:1 ratio either two hCA-containing vectors (hCA-NC*-thy and sCA-PTAP*-gfp) or two sCA-containing vectors (sCA-NC*-thy and sCA-PTAP*-gfp). As expected, we observed strong rhTRIM5α restriction when both vectors used in the complementation system contained hCA and little, if any, restriction when both vectors contained sCA (Fig. 4B).

The phenotypes of viruses containing both hCA and sCA proteins are also shown in Fig. 4B. We observed significant restriction in viruses generated by cotransfecting hCA-NC*-thy and sCA-PTAP*-gfp. However, the levels of restriction are lower than those in viruses containing only hCA proteins (Fig. 4B). When hCA- and sCA-expressing vectors were mixed at equal amounts, the resulting viruses were restricted 3.6- to 17.3-fold by rhTRIM5α cells, depending on the titer of infection (Fig. 4B). Even when hCA-expressing vectors were present at 20% of the mixture, the resulting viruses were restricted 4.4- to 10.5-fold by rhTRIM5α cells depending on the titer of infection (Fig. 4B). These results are in sharp contrast to those shown in Fig. 4A, when pure CA core viruses from separate transfection were mixed prior to infection. First, these coassembled Gag viruses had far greater levels of restriction than the pure CA core viruses in Fig. 4A, which had a maximum of only 2- to 2.5-fold restriction in the 1:1 (hCA-sCA) mixture. Additionally, the levels of restriction of coassembled Gag viruses are heavily influenced by the amounts of input viruses, similar to the restriction kinetics of HIV-1, and are very different from those of pure CA core viruses (Fig. 4A). These results support the conclusion that hCA and sCA can coassemble into the same mature cores and that such cores are susceptible to rhTRIM5α restriction. Furthermore, cores containing coassembled hCA and sCA are capable of completing steps of HIV-1 replication to lead to successful infection.

DISCUSSION

In this study, we exploited the specificity of rhTRIM5α for hCA in the mature viral core to demonstrate that CA proteins from HIV-1 and SIV_{mac} can coassemble into the same core to generate infectious viruses. To our knowledge, this is the first demonstration that CA proteins from distinct retroviruses can coassemble into an infectious mature core.

Primate lentiviruses consist of HIV-1, HIV-2, and various simian immunodeficiency virus strains isolated from more than 30 nonhuman primate species; most of these viruses can be assigned to one of the six approximately equidistant phylogenetic lineages (37). Generally, the Gag proteins from viruses of different phylogenetic lineages are around 50% identical in amino acid sequences, whereas the Gag proteins from viruses of the same phylogenetic lineage are usually more than 80% identical in amino acid sequences (HIV databases, http://www.hiv.lanl.gov). HIV-2 and SIV_{mac} belong to the same phylogenetic lineage, whereas HIV-1 belongs to a different lineage. In terms of amino acid sequence, the Gag proteins of HIV-2 and SIV_{mac} share 83% identity, whereas these two proteins share 54% and 53% identity with HIV-1 Gag, respectively. Among the Gag domains of primate lentiviruses, CA domains usually have the highest similarity. The CA proteins of HIV-2 and

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TABLE 1. TRIM5α restriction of a mixture of separately produced viruses

Virus stock	Dilution	TRIM5α cells		Control cells		Fold restriction
		% Infection[a]	MOI	% Infection[a]	MOI	
hCA-gfp virus	1	24.0	0.27	95.3	3.05	11.1
	2	4.0	0.04	73.2	1.32	32.3
	4	1.3	0.01	46.3	0.62	47.5
sCA-Thy virus	1	73.8	1.34	72.2	1.28	1.0
	2	56.5	0.83	52.5	0.74	0.9
	4	40.9	0.53	40.5	0.52	1.0
	8	28.6	0.34	24.8	0.28	0.9
	16	14.0	0.15	11.4	0.12	0.8
hCA-gfp virus + sCA-thy virus at 1:1 ratio	1	56.2	0.83	82.8	1.76	2.1
	2	36.1	0.45	66.9	1.11	2.5
	4	23.0	0.26	47.9	0.65	2.5
	8	13.3	0.14	28.6	0.34	2.4
	16	6.3	0.07	14.7	0.16	2.4
hCA-gfp virus + sCA-thy virus at 1:2 ratio	1	66.6	1.10	73.3	1.32	1.2
	2	42.0	0.54	48.3	0.66	1.2
	4	23.3	0.27	33.5	0.41	1.5
	8	13.4	0.14	18.9	0.21	1.5
	16	6.2	0.06	8.2	0.09	1.3
hCA-gfp virus + sCA-thy virus at 1:4 ratio	1	53.7	0.77	61.3	0.95	1.2
	2	41.7	0.54	44.2	0.58	1.1
	4	25.9	0.30	30.1	0.36	1.2
	8	13.9	0.15	16.3	0.18	1.2
	16	6.3	0.07	7.3	0.08	1.2

[a] Infected cells were scored by the expression of either Thy or GFP markers.

SIV_{mac} share 90% amino acid identity, whereas these two proteins share 68% and 66% identity with HIV-1 CA protein. The overall higher conservation of the CA domain relative to other domains is probably due to the evolutionary constraints imposed by the multiple roles that CA plays during retrovirus replication, both as a Gag domain and as a mature protein.

Our current understanding of virus maturation is that core formation is not simply condensation of the CA domain of the Gag lattice after proteolytic cleavage. Rather, a second assembly step, including the refolding of the mature CA protein and formation of a hexamer CA lattice, is involved in the formation of the cone-shaped core (49). The structure and protein-protein interactions of mature retrovirus cores are just beginning to be elucidated. The core of HIV-1 is proposed to be a fullerene structure composed of 200 to 300 CA hexamers and 12 CA pentamers (17, 30). Recent structural analyses revealed that there are three important protein-protein interaction interfaces in the mature core (16, 28, 29). Helices 1 to 3 in the N-terminal domain of CA mediate the N-terminal–N-terminal hexamerization interaction interface. Helix 9 in the C-terminal domain primarily mediates the C-terminal–C-terminal dimerization interaction. Helix 4 in the N-terminal domain and helices 8 to 11 in the C-terminal domain are involved in the N-terminal-C-terminal interaction. Therefore, extensive interactions between neighboring CA molecules in the hexameric lattice are required in forming a mature core. Although our current knowledge does not extend to the detailed CA-CA side chain interactions in immature or mature particles, mutagenesis analyses have identified a number of amino acids potentially involved in these interactions. Mutations R18A/N21A, A22D, E28A/E29A, M39D, A42D, D51A, T54A/N57A, and K70A have been shown to reduce or alter mature core formation, whereas mutations E75A/E76A, R100A/S102A, T107A/T108A, T110A/Q112A, K158ADQ, W184A, M18A, and D197AEN di-

TABLE 2. Simulation of restriction in mixed viral populations containing pure cores

hCA virus/ sCA virus ratio	% Infection in:						Fold restriction[b] (control cells/ TRIM5α cells)
	Control cells			TRIM5α-expressing cells[a]			
	From hCA[c]	From sCA	Total	From hCA	From sCA	Total	
1:1	5	5	10	0.125	5	5.125	~2
1:2	5	10	15	0.125	10	10.125	~1.5
1:4	5	20	25	0.125	20	20.125	~1.125

[a] An assumption of 50-fold restriction is made.
[b] For simplicity, percentages of infected cells instead of MOIs are used to calculate restriction in these examples.
[c] From a virus containing hCA cores.

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TABLE 3. TRIM5α restriction of viruses generated from cotransfection of two vectors

Cotransfected viral vectors (ratio)	Dilution	TRIM5α cells		Control cells		Fold restriction
		% Infection[a]	MOI	% Infection[a]	MOI	
hCA-NC*-thy + hCA-PTAP*-gfp (1:1)	1	27.2	0.32	92.1	2.54	8.0
	2	8.1	0.08	77.8	1.50	17.8
	4	2.9	0.03	54.9	0.80	27.1
sCA-NC*-thy + sCA-PTAP*-gfp (1:1)	1	77.6	1.50	76.1	1.43	1.0
	2	56.3	0.83	53.4	0.76	0.9
	4	36.1	0.45	33.8	0.41	0.9
	8	21.8	0.25	16.0	0.17	0.7
hCA-NC*-thy + sCA-PTAP*-gfp (1:1)	1	48.8	0.67	90.7	2.38	3.6
	2	16.6	0.18	78.4	1.53	8.4
	4	7.7	0.08	58.5	0.88	11.0
	8	2.2	0.02	31.9	0.38	17.3
hCA-NC*-thy + sCA-PTAP*-gfp (1:2)	1	42.1	0.55	89.5	2.25	4.1
	2	23.3	0.27	76.1	1.43	5.4
	4	9.7	0.10	55.8	0.82	8.0
	8	3.5	0.04	35.8	0.44	12.4
hCA-NC*-thy + sCA-PTAP*-gfp (1:4)	1	21.7	0.24	66.2	1.08	4.4
	2	6.4	0.07	44.2	0.58	8.8
	4	2.7	0.03	24.9	0.29	10.5

[a] Infected cells were scored by the expression of either Thy or GFP markers.

minish immature particle production (50). Most of these amino acids are conserved between hCA and sCA. However, M39, T54, K70, R100, T110, and Q112 in hCA are changed to G, Q, R, S, S, and D in sCA, respectively. These differences appear to be tolerated and do not prevent the coassembly of Gag proteins containing CA domains from HIV-1 and SIV_{mac} or the formation of mature cores from these heterologous CA proteins. What remains unknown is the arrangement of these heterologous CA proteins in the mature core. For example, it is not clear whether these two different CA proteins are evenly distributed in the core and whether these two different CA proteins form mixed hexamers.

We demonstrated that viruses containing hCA and sCA were restricted by rhTRIM5α in a virus titer-dependent manner. However, the level of rhTRIM5α restriction in these viruses was less than that in viruses containing pure hCA cores at a comparable infection rate (Fig. 4B). Furthermore, the levels of restriction are directly correlated with the amount of hCA-containing vector used in transfection. For example, when control cells were infected at about the 55% level, the restriction in hCA cores is about 27-fold (Fig. 4B and Table 3); when the hCA and sCA ratios are 1:1, and 1:2, the restriction levels are about 11-fold and 8-fold, respectively; and when the hCA and sCA ratio is 1:4, the restriction is estimated to be 6-fold. Therefore, these mixed-core viruses have an intermediate phenotype and the restriction levels are in proportion to the amounts of hCA in the mixture. These results reveal that the number of TRIM5α binding sites in the cores is an important limiting factor of restriction. It is possible that multiple TRIM5α binding is required to achieve restriction or that TRIM5α finding the target hCA is a rate-limiting step. Therefore, decreasing the number of target hCA proteins causes the reduction of TRIM5α restriction efficiency. Experimental evidence indicates that TRIM5α is a trimer, and it has been proposed that the three molecules of the trimer can fit into three binding pockets in a CA hexamer or between hexamers (24, 31). If each of the molecules in the trimer needs to bind a pocket to be functional and hCA and sCA assemble randomly, then the probability of a TRIM5α trimer finding all three binding pockets to be suitable should be reduced nonlinearly with the decrease in the amounts of hCA. For example, when hCA and sCA are mixed in 1:1, 1:2, and 1:4 ratios, the probability of a trimer finding three suitable binding sites should be 1 in 8 (2^3), 1 in 27 (3^3), and 1 in 125 (5^3), respectively. These probabilities are reduced even more drastically if more than one hCA protein is required to form each binding pocket. In either scenario, the numbers of sites for binding to TRIM5α trimers in the core are not linear to the amounts of hCA proteins in the core. Our data indicate a nearly linear relationship between the proportion of hCA in the virus and the restriction level, thereby suggesting either that all three TRIM5α molecules in the trimer are not required to bind to the core to be functional or that hCA and sCA are not distributed randomly in the core because homologous CA proteins have stronger affinity to each other than to heterologous CA proteins. To distinguish between these two possibilities requires further advances in our understanding of TRIM5α-CA binding and the assembly of heterologous CA proteins in virus cores.

In this report, we illustrate that sufficient features are conserved between CA proteins from HIV-1 and SIV_{mac} to allow coassembly of infectious virus cores. The mixed cores have an intermediate phenotype in terms of restriction by TRIM5α. One possible implication is that virus could use this mechanism to lower the efficiency of the host innate defense system to initiate infection. These experiments are the first to demonstrate that heterologous CA proteins can coassemble into mature cores of infectious viruses and have implications for virus

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evolution. These studies also provide insights into the mechanisms of TRIM5α restriction and virus assembly.

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