Biological, Molecular, and Structural Analysis of a Cytopathic Variant from a Molecularly Cloned Simian Immunodeficiency Virus

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Some isolates of simian immunodeficiency virus (SIV) have been shown to infect Sup-T1 cells with slow kinetics and in the absence of cytopathic effects, including cell fusion or CD4 down-modulation (J. A. Hoxie, B. S. Haggarty, S. Bonser, J. Rackowski, H. Shan, and P. Kanki, *J. Virol.* 62:2557–2568, 1988). In the present study, we describe the isolation and characterization of a SIVmac variant, derived from the BK28 infectious molecular clone, that became highly cytopathic for Sup-T1 cells. This variant, termed CP-MAC, exhibited a number of differences from BK28, including (i) an altered tropism which largely restricted its host range to Sup-T1 cells, (ii) the ability to induce cell fusion and CD4 down-modulation, and (iii) a highly stable interaction of its external (SU) and transmembrane (TM) envelope glycoproteins. In addition, a marked increase in the level of surface envelope glycoproteins was observed both on CP-MAC-infected cells and on virions. The CP-MAC *env* gene was PCR amplified from infected cells, and sequence analysis identified five amino acid changes in SU and six in TM compared with BK28. The introduction of these changes into BK28 was shown to fully reconstitute the biological and morphological properties of CP-MAC. The limited number of mutations in CP-MAC should enable the molecular determinants to be more precisely defined and help to identify the underlying mechanisms responsible for the striking biological and structural alterations exhibited by this virus.

Human and simian immunodeficiency viruses (HIV and SIV, respectively) exhibit a remarkable degree of biological diversity in vitro in their host range (9, 42, 56), infection kinetics (10, 22), and the extent to which they induce cytopathic effects during infection (10, 20, 22, 33, 43). For HIV type 1 (HIV-1), many of these biological differences have been shown to correlate with the clinical stage of infection (10, 22, 70) and with the tissue from which a virus is isolated (12, 42, 46). In addition, for SIV, in which an animal model enables correlations to be made between biological properties of a virus in vitro and pathogenicity in vivo, the biological phenotype can be associated with distinct clinical syndromes in infected animals (16, 29, 65). Although findings with the human-PBL-SCID mouse model have suggested that caution be exercised in interpreting the significance of in vitro parameters of cytopathicity (55), it is likely that an understanding of the molecular and structural bases for HIV and SIV biological diversity will be highly relevant to the complex natural history of disease in infected individuals.

Although a number of viral gene products can contribute to this biological diversity, it is clear that the viral envelope glycoproteins contain major determinants for cell tropism (4, 6, 29, 34, 35, 53), infection kinetics (11, 15, 43), and cytopathic effects (11, 15, 41). The mechanisms responsible for these differences likely involve a complex interplay of viral and cellular factors (50). Factors at the level of cell entry include differences in the affinity of the viral envelope for CD4 (32, 51,

71), the extent to which conformational changes in envelope occur following CD4 binding (2, 62, 63, 76), and the utilization of CD4-associated molecules (33, 68) or alternate receptors (26). Alternatively, poorly understood events following viral entry could also be involved (52). Nonetheless, even when determinants for different biological phenotypes can be mapped to specific regions or single amino acid changes, little is known about the mechanisms responsible for these effects (4, 11, 13, 29, 35, 53, 66, 69). Model systems are clearly needed in which the molecular determinants that produce differences in tropism, infection kinetics, and cytopathic effects are associated with defined alterations in envelope structure and function.

Our laboratory has reported the biological characterization of an uncloned SIVmac that was remarkable for its restricted host range among CD4-positive T-cell lines (33). Although this virus was able to infect HUT-78 cells with rapid kinetics, syncytium formation, cell killing, and CD4 down-modulation, it infected Sup-T1 cells with markedly retarded kinetics and without causing cell killing or fusion. In chronically infected Sup-T1 cells, this SIV also failed to down-modulate CD4 or block epitopes on CD4 normally involved in envelope binding. Our laboratory has subsequently repeated these experiments using virus derived from the BK28 molecular clone of SIVmac and has found the biological phenotype to be identical to that described for the original uncloned SIV. We have termed the virus derived from BK28-infected Sup-T1 cells NC-MAC for noncytopathic SIVmac.

In the present study, we describe the derivation and characterization of a highly cytopathic variant of NC-MAC, termed CP-MAC, that evolved during long-term propagation in Sup-T1 cells. In contrast to NC-MAC, CP-MAC infected Sup-T1 cells with rapid kinetics, produced cell fusion, and

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completely down-modulated CD4. CP-MAC also exhibited a remarkable alteration in its host range in that, although it was highly infectious for Sup-T1 cells, it was unable to infect other cell lines that were susceptible to both BK28 and NC-MAC. Biochemical and morphological analyses showed a number of other differences for CP-MAC, including (i) a highly stable association of its transmembrane (TM) and external (SU) envelope glycoproteins, (ii) an increased level of envelope molecules on the surface of infected cells, and (iii) increased numbers of envelope spikes on virions. These properties could be attributed to 11 amino acid changes in the CP-MAC *env* gene (5 in SU and 6 in TM). The remarkable differences conferred by these mutations on the viral host range, on cytopathicity, and on envelope structure should make this an important model for understanding the underlying mechanisms that mediate these characteristics.

MATERIALS AND METHODS

Viruses and cell lines. The proviral clone of SIVmac, pBK28, was obtained from the AIDS Reference and Reagent Repository (courtesy of J. Mullins). HUT-78 cells chronically infected by BK28 were provided by J. Mullins. Immortalized T-cell lines used for host range studies were CEM and CEMx174 (provided by P. Cresswell), Molt4-clone 8 (provided by B. Hahn), HUT-78 (provided by J. Levy), and Sup-T1 (provided by K. Huebner). The RD rhabdomyosarcoma cell line used for transfection protocols was provided by A. Srinivasan.

MAbs and antisera. Murine monoclonal antibodies (MAbs) used for detection of SIV proteins included 3A8, reactive with SIVmac p27^{gag} (provided by J. McClure); 1H8 (59) and 43.1, reactive with SIVmac TM (provided by J. McClure and C. Thiriart, respectively); and 101.1, reactive with SIVmac SU (provided by C. Thiriart). Anti-CD4 MAbs OKT4 and OKT4A and the anti-CD8 MAb OKT8 were obtained from Ortho Diagnostics (Raritan, N.J.). The murine immunoglobulin G2b MAb, P3X63, was used as a negative control. Antisera from SIVmac-infected rhesus macaques were provided by J. Allan and P. Fultz. Rabbit serum reactive with the SIVmac SU glycoprotein was provided by R. Sweet.

PCR and sequencing. Envelope coding regions of NC-MAC and CP-MAC viruses were amplified from genomic DNAs by PCR in two fragments. SU was amplified using the forward primer 5'-GAGCAGTCACGAAAG (nucleotides [nt] 6506 to 6520) and the reverse primer 5'-ACCTGCCGTTGCGA (nt 8205 to 8192). For cloning purposes, a *Bam*HI site was added to the 5' end of the sense primer (5'-GCTGGATCC), and a *Sac*I site was added to the 5' end of the antisense primer (5'-CTGGAGCTC). TM was amplified using the forward primer 5'-GTGGCAGAACTGTATCGATTGGAGT (nt 8041 to 8073) and the reverse primer 5'-GCCTCTGCAGTATTG GTTGA (nt 8998 to 8978). Cloning of the TM fragment was achieved using a *Cla*I site inherent in the forward primer and an *Xho*I site introduced into the reverse primer by changing a G to a C (underlined). PCR-amplified envelope fragments were sequenced either uncloned from bulk PCR DNA or after cloning into pBluescript (Stratagene) by the dideoxy chain termination method (Sequenase; U.S. Biochemicals).

Mutant envelope construction and virus production. Molecularly derived viruses were produced by cotransfecting an internal 3.5-kb *Sac*I fragment containing the *env* gene and the pBK28 plasmid from which this corresponding *Sac*I fragment had been deleted. A *Sac*I fragment containing the *env* mutations of CP-MAC was constructed using the 3.5-kb *Sac*I BK28 fragment subcloned into pBluescript SK⁺ (Stratagene). Mul-

tip cloning sites in the polylinker of the plasmid were removed by digesting with *Xho*I and *Not*I and creating blunt ends with mung bean nuclease for religation. This plasmid containing the BK28 *Sac*I fragment was digested with *Hind*III and *Pst*I and ligated to the 1.2-kb *Hind*III-to-*Cla*I fragment from CP-MAC.2 SU PCR clone 3 (see Fig. 2) and the 600-bp *Cla*I-to-*Pst*I fragment from CP-MAC.1 TM PCR clone 10 (Fig. 2). The resulting plasmid was then digested with *Hind*III and ligated with the 500-bp *Hind*III fragment of BK28 (nt 6215 to 6798) that had been removed by the initial *Hind*III digestion (see Fig. 3). Correct orientation of this *Hind*III fragment was confirmed by sequencing. Recombinant viruses were generated by coprecipitating 5 µg of the gel-purified *Sac*I fragment and 5 µg of the *Sac*-deleted pBK28 construct, resuspending in 100 µl of Tris-EDTA (TE) buffer, and transfecting into RD cells by the calcium phosphate method. After a glycerol shock, RD cells were cocultured with Sup-T1 cells, and the nonadherent lymphoid cells were passaged and monitored for viral replication, as described below.

Infection assays. Supernatants from Sup-T1 cells chronically infected with NC-MAC or CP-MAC were clarified by centrifugation (2,000 × *g* for 10 min), filtered (0.2-µm-pore-size filter), and normalized for reverse transcriptase (RT) activity prior to inoculation onto uninfected cells. RT activity was measured on pelleted virus (100,000 × *g* for 30 min), as described previously (33). For infection protocols, 1.8 ml of viral supernatant was cultured for 18 h with 200 µl of a cell suspension containing 2 × 10⁶ cells. The cells were then washed twice to remove input virus and were maintained in RPMI-10% fetal calf serum. Infection was monitored by serial RT determinations of culture supernatant, and the percentages of cells producing viral p27^{gag} were monitored by immunofluorescence microscopy (IFA) on methanol-acetone-fixed cells using the 3A8 MAb.

FACS analysis of cell surface antigens. Cell surface expression of viral and cellular antigens was detected with a fluorescence-activated cell sorter (FACS) using a FACScan flow cytometer (Becton Dickinson), as described previously (31, 33). Briefly, cells were centrifuged (2,000 × *g* for 5 min) and resuspended in ice-cold staining buffer (0.1% bovine serum albumin-0.02% sodium azide-phosphate-buffered saline [PBS]), and the suspensions were incubated with saturating concentrations of either MAbs or serum (30 min for 4°C) followed by a fluorescein isothiocyanate-labeled secondary antibody (30 min for 4°C). Cells were washed and fixed in 4% paraformaldehyde-PBS prior to analysis. Viral SU and TM were detected by MAbs 101.1 and 43.1, respectively, or by antiserum from a SIVmac-infected rhesus macaque. Negative-control antibodies were either P3X63 or normal human serum. Secondary antibodies were fluorescein isothiocyanate-conjugated F(ab')₂ goat anti-mouse or goat anti-human immunoglobulin G (Tago Laboratories, Burlingame, Calif.).

Western immunoblot analysis. Virus was pelleted (100,000 × *g* for 30 min) and lysed for 30 min on ice in lysis buffer (0.02 M Tris [pH 8.0], 0.12 M NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid [EGTA], 0.2 mM NaF, 5 µg of aprotinin per ml, 0.2% sodium deoxycholate, 0.5% Nonidet P-40). Viral proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to nitrocellulose, and detected with optimal concentrations of either anti-SIV serum or MAb 1H8 or 101.1. Secondary antibodies were peroxidase-conjugated anti-mouse or anti-human immunoglobulin G (Fisher-Biotech), and diaminobenzidine was used as the colorimetric substrate (Sigma).

Radioimmunoprecipitation and pulse-chase protocols. Im-

TABLE 1. CD4 expression on SIV-infected Sup-T1 cells

Cell line	Mean channel fluorescence value ^a			
	P3X63	OKT4	OKT4A	OKT8
NC-MAC.3 ^b				
Day 0	6	120	153	98
Day 15	11	96	148	81
Day 35	6	78	68	89
Day 48	6	47	38	50
Day 74	7	12	10	63
Day 110	6	6	7	97
Sup-T1 (uninfected) ^c	5	177	168	ND ^d
NC-MAC.1 ^c	5	149	88	ND
CP-MAC ^c	5	21	6	ND
Sup-T1 ^c	9	147	189	89
NC-MAC.1 ^c	6	120	172	89
CP-MAC.1 ^c	3	22	4	89
BK/CP-env ^c	5	21	3	84

^a Sup-T1 cells, uninfected or infected with the viruses indicated, were stained for FACS analysis with MAbs to CD4 (OKT4 and OKT4A), CD8 (OKT8), or a negative-control MAb (P3X63), and values for the mean channel of fluorescence intensity were determined by FACS. Cells in the infected cultures were 100% infected as determined by viral p27^{gag} expression.

^b Serial assays for a clone of NC-MAC-infected Sup-T1 cells (NC-MAC.3) as it lost CD4 expression over 4 months. Day 0 refers to the initial analysis 3 months after infection with BK28.

^c Virus from NC-MAC.3 day 110 cells was passed onto uninfected Sup-T1 cells, and a new infected line, designated CP-MAC, was derived. CD4 expression is shown for uninfected Sup-T1 cells, CP-MAC-infected cells, and an NC-MAC-infected clone that maintained a high level of CD4 expression (NC-MAC.1).

^d ND, not determined.

^e Direct comparison of CD4 expression on uninfected Sup-T1 cells, NC-MAC- and CP-MAC-infected cellular clones, and Sup-T1 cells infected by the recombinant virus, BK/CP-env, which contained the *env* mutations of CP-MAC.

munoprecipitation of viral proteins was carried out as previously described (31, 33). Infected cells were pelleted and resuspended in Cys- and Met-free RPMI-10% dialyzed fetal calf serum containing [³⁵S]Cys and [³⁵S]Met (200 μ Ci/ml [Tran³⁵S-label; ICN Biochemicals]), and cultures were incubated at 37°C for 15 h. Labeled virus was pelleted and lysed, as described above. For detection of free SU glycoprotein, culture supernatant was treated with a 1/10 volume of 10 $\times$ lysing buffer. All lysates were preadsorbed with a 1/10 volume of protein-A agarose (Boehringer Mannheim Biochemicals) prior to immunoprecipitation. For pulse-chase experiments, infected cells were preincubated in Cys- and Met-free RPMI-10% dialyzed fetal calf serum, as described above, and labeled with 250 μ Ci of [³⁵S]Cys and [³⁵S]Met per ml for 15 min at 37°C, after which time excess unlabeled Cys and Met were added to a final concentration of 30 mg/liter. At various times, the cultures were processed for immunoprecipitation as described above. Viral proteins were immunoprecipitated with either 1H8 (anti-TM), soluble recombinant CD4 (provided by R. Sweet), macaque anti-SIV serum, or rabbit anti-SIV SU serum; separated by SDS-PAGE; and quantified by autoradiography and densitometry.

Electron microscopy and quantitative morphometric analysis of virions. Medium containing SIV-infected cells was added to an equal volume of 5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.3) at 4°C and postfixed in 1% OsO₄, and envelope glycoproteins were stained with 1% tannic acid and then with aqueous 1% uranyl acetate. The linear surface densities of spikes on virions (quantitative morphometric analysis) were determined for 67 to 72 virions of each virus and were analyzed by Student's *t* test, as previously described (27).

FIG. 1. Infection kinetics of NC-MAC and CP-MAC on Sup-T1 cells. Viral supernatants containing NC-MAC (closed circles) or CP-MAC (open squares) were normalized for RT activity (approximately 250,000 cpm) and were used to inoculate uninfected Sup-T1 cells. This inoculum corresponded to a 50% tissue culture infective dose of 10^{-2} for NC-MAC and 10^{-4} for CP-MAC. In addition, an inoculum of CP-MAC diluted 100-fold was used (closed squares). The cultures were monitored at various times after infection by IFA for the percentage of cells expressing p27^{gag} (top panel) and RT activity in the culture supernatant (bottom panel).

RESULTS

Derivation of SIVmac variants from BK28. Cell-free virus from BK28-infected HUT-78 cells was passed onto Sup-T1 cells, and a chronically infected line was established. In a manner similar to that observed for an uncloned SIVmac (33), infection occurred in the complete absence of cell fusion or cell killing and with a less than twofold reduction in CD4 expression (data not shown). Cell clones were obtained by limiting dilution, and the virus produced from these clones was termed NC-MAC. Like BK28, NC-MAC failed to induce cell fusion or killing during infection of Sup-T1 cells (data not shown). The NC-MAC-infected cellular clones were all productively infected and produced high levels of RT (>200,000 cpm) for several months. Remarkably, over a period of 4 to 6 months, one of four clones exhibited a gradual decline in the level of surface CD4 (clone NC-MAC.3 in Table 1). This decrease was seen with MAbs that both compete (OKT4A) and do not compete (OKT4) with SU for binding to CD4, indicating that CD4 was being down-modulated from the cell surface. To determine whether this difference resulted from a change in the virus or from the cells producing the virus, supernatant from the CD4-negative cells shown in Table 1 (NC-MAC.3 on day 110) was passed onto uninfected Sup-T1 cells, and a new

FIG. 2. Amino acid differences of the NC-MAC and CP-MAC *env* clones compared with BK28. Predicted amino acid sequences are shown for selected regions of *env* for PCR-generated clones of NC-MAC-infected cells and two cellular clones of CP-MAC-infected cells and are compared with the parental BK28 sequence. Dot, amino acid identity; asterisk, in-frame stop codon; dash, a single nucleotide deletion. Regions

B (cont.)

corresponding to variable domains in SU (39) are shown. (A) Sequences of SU from amino acids 40 to 150; (B) sequences including the carboxy-terminal half of SU (amino acids 301 to 527) and the entire TM coding region. Regions of *env* omitted from the figure contained only sporadic point mutations and are described in Results.

chronically infected line was established. As shown in Table 1, surviving cells exhibited a nearly complete loss of surface CD4, indicating that this effect was clearly due to a change in the virus. We termed this virus CP-MAC (for cytopathic SIV_{mac}). Cells from the CP-MAC-infected culture were cloned by limiting dilution, and two cellular clones, designated CP-MAC.1 and CP-MAC.2, were used as a source of virus for subsequent studies.

To further evaluate the biological properties of CP-MAC, viral supernatant from CP-MAC-infected cells was compared with virus from a clone of NC-MAC-infected cells that had remained CD4 positive (clone NC-MAC.1; Table 1). Viruses were normalized for RT activity and were inoculated onto uninfected Sup-T1 cells. Cultures were serially monitored for RT activity and p27^{gag} expression. As shown in Fig. 1 (top panel), CP-MAC infection occurred with markedly accelerated kinetics compared with NC-MAC infection. By day 7, CP-MAC had infected 100% of cells, while only 30% of cells inoculated with NC-MAC were antigen positive even after 56 days. A 100-fold reduction in the CP-MAC inoculum produced a delay in the onset of infection; however, following the initial

detection of viral replication at day 22, a rapid spread of infection occurred, with 100% of cells expressing p27^{gag} by day 30. Corresponding increases in RT activity were also seen (Fig. 1, bottom panel). As initially observed, CP-MAC infection induced nearly complete down-modulation of CD4 (data not shown). Moreover, in striking contrast to NC-MAC, CP-MAC also induced syncytium formation during the onset of infection (data not shown). Thus, CP-MAC was biologically distinguishable from NC-MAC with respect to its ability to rapidly infect Sup-T1 cells and to induce CD4 down-modulation and cell fusion.

Molecular analysis of envelope genes from NC-MAC and CP-MAC variants. To evaluate the envelope genes of CP-MAC, SU and TM regions were amplified by PCR from CP-MAC.1, CP-MAC.2, and NC-MAC.1 cells, and 10 clones were sequenced for each of the three viruses. Compared with BK28, the average nucleotide substitution frequencies were 0.26% for NC-MAC, 0.65% for CP-MAC.1, and 0.67% for CP-MAC.2. As described for the genetic diversity of SIV_{mac} in vivo (5, 39, 58), a majority of nucleotide substitutions resulted in amino acid changes (91.2% for NC-MAC, 86.0%

FIG. 3. Incorporation of CP-MAC consensus mutations into BK28 *env*. Mutations found in all PCR clones of the CP-MAC *env* gene shown in Fig. 2 were incorporated into a 3.5-kb *SacI* fragment of BK28, as described in Materials and Methods. The shaded region denotes the *env* open reading frame. Amino acid positions for mutations in SU are 84E→K, 112R→K, 120R→K, 327M→R, and 475T→I. Amino acid positions for mutations in TM are 559L→S, 573V→L, 575K→T, 588T→I, 643E→K, and 723Y→C. Relative nucleotide positions of restriction sites used to construct the fragment are indicated. Also noted downstream of the premature TM stop codon (*) is a 65P→Q mutation in *Rev* (see Results).

for CP-MAC.1, and 86.6% for CP-MAC.2). Similarly to in vivo models, a high proportion of mutations involved either G→A or A→G transitions (77.2% for NC-MAC, 47.6% for CP-MAC.1, and 45.6% for CP-MAC.2). Direct sequencing of uncloned PCR-amplified DNA from the pBK28 plasmid showed no differences from the published sequence, indicating that the changes observed for NC-MAC and CP-MAC were not the result of errors introduced by PCR.

Figure 2 shows the predicted amino acid changes relative to BK28 in selected regions of the envelope glycoproteins for NC-MAC and the two cellular clones of CP-MAC. Although sporadic mutations were noted, distinct patterns of substitutions were apparent. Mutations in SU are shown in Fig. 2A and B. For NC-MAC, all 10 clones showed four coding mutations (numbers refer to positions; one-letter amino acid codes are used): 84E→K, 112R→K, 120R→K, and 385G→R. For the two CP-MAC lines, all 20 clones shared with NC-MAC the 84E→K and 112R→K mutations, and 16 of 20 shared the 120R→K mutation. However, the 385G→R mutation present in NC-MAC was absent from all CP-MAC clones. Interestingly, 20 of 20 CP-MAC clones also exhibited in SU a 327M→R change in the region analogous to the V3 loop of HIV-1 (amino acids 313 to 346), and 19 of 20 contained a 475T→I mutation near the C terminus of SU. A 49R→G mutation was also present in 6 of 10 of the clones from CP-MAC.1 but not in any of 10 clones from CP-MAC.2. Because CP-MAC.1 and CP-MAC.2 were biologically indistinguishable (data not shown), we considered it unlikely that the 49R→G mutation was relevant to the CP-MAC phenotype.

No consistent changes in TM were observed for NC-MAC, while six mutations were found in all 20 of the CP-MAC clones: 559L→S, 573V→L, 575K→T, 588T→I, 643E→K, and 723Y→C (Fig. 2B). Five of these changes were located in the extracellular domain of TM, while a single Tyr-to-Cys substitution was located in the TM cytoplasmic tail six amino acids from the predicted membrane-spanning domain. The premature stop codon of BK28 at position 736 was conserved in all clones, although a single clone from CP-MAC.1 also exhibited a new stop codon at position 735. Thus, this analysis of PCR-derived envelope clones showed consensus mutations for CP-MAC that were distinct from NC-MAC and the parental BK28 virus (Fig. 3).

Although all TM clones were not sequenced past the TM premature stop codon, six of six CP-MAC clones (two from CP-MAC.1 and four from CP-MAC.2) contained a C-to-A transversion at nt 8908 that encoded a Gln-to-Lys substitution

at position 777 in the untranslated region of the TM reading frame. This mutation also produced a noncoding mutation in the second exon of *tat* and a Pro-to-Gln substitution at position 65 in the second exon of *rev* (data not shown). No changes were observed in five of five NC-MAC clones sequenced in this region.

Construction and biological characterization of a recombinant virus with the CP-MAC envelope mutations. To determine if the amino acid changes found in the CP-MAC envelope were responsible for its altered biological phenotype, a chimeric virus that incorporated these mutations onto the BK28 background was constructed. Recombinant viruses were produced by cotransfecting RD cells with an internal *SacI* fragment containing the consensus envelope mutations of CP-MAC and the pBK28 plasmid from which the corresponding *SacI* fragment had been deleted (see Materials and Methods and Fig. 3). Infectious progeny virus was rescued from the RD cells by cocultivation with Sup-T1 cells. Fourteen days after transfection, p27-positive cells were first detectable by IFA, and by day 17 large syncytia were observed (data not shown). After 20 days, 100% of the cells were infected, and a chronically infected line was established. We termed the virus derived from this culture BK/CP-env. Incorporation of the CP-MAC mutations into BK/CP-env was confirmed by sequencing the entire envelope open reading frame from uncloned PCR-amplified genomic DNA. No other mutations were identified.

The biological effects of the CP-MAC envelope mutations were evaluated in a time course infection assay. Viral supernatants from NC-MAC-, CP-MAC-, and BK/CP-env-infected cells were normalized for RT activity and were inoculated onto uninfected Sup-T1 cells. Additional cultures received either a 10-fold increase in the NC-MAC inoculum or a 10- or 100-fold decrease in the BK-CP-env inoculum. For identical input levels of virus, the kinetics of CP-MAC infection was more rapid than that for NC-MAC (Fig. 4), similar to that previously observed (Fig. 1). NC-MAC infection was detectable by day 9 and spread slowly, with only 20% of cells positive for p27^{gag} by day 30. As shown, a 10-fold increase in the NC-MAC inoculum had little effect on these kinetics. In contrast, CP-MAC infection was again rapid, with 100% of cells becoming infected within 8 days. Importantly, BK/CP-env infection was also rapid, with kinetics similar to that of CP-MAC. Reductions in the BK/CP-env inocula produced a lag in the onset of infection, but once initiated, infection spread rapidly throughout the culture. As with CP-MAC, syncytia were observed during the onset of

FIG. 4. Infection kinetics of NC-MAC, CP-MAC, and BK/CP-env on Sup-T1 cells. Viral supernatants of NC-MAC, CP-MAC, and BK/CP-env were normalized to an RT level of 20,000 cpm (closed squares) and were used to inoculate Sup-T1 cells. In addition, inocula containing RT values that were 10-fold higher (open squares) for NC-MAC and 10-fold (open circles) and 100-fold (open diamonds) lower for BK/CP-env were used. Cultures were monitored by IFA for infection for p27^{gag} antigen expression on fixed cells (A) and for RT activity in culture supernatant (B) at various time points after inoculation.

BK/CP-env infection, while no syncytia were apparent at any time during the course of NC-MAC infection (Fig. 5).

The ability of BK/CP-env to down-modulate CD4 was also evaluated using cells derived from the experiment shown in Fig. 4. Both CP-MAC- and BK/CP-env-infected Sup-T1 cells showed a marked reduction in surface CD4 compared with uninfected Sup-T1 cells and compared with a clone of NC-MAC-infected cells (Table 1). A small amount of CD4 on CP-MAC- and BK/CP-env-infected cells was detectable with

OKT4 but not with OKT4A, indicating that any CD4 expressed on the cell surface was completely blocked by viral envelope glycoproteins (33).

The similarity between BK/CP-env and CP-MAC with respect to the rapid infection of Sup-T1 and the ability to down-modulate CD4 demonstrated that these biological characteristics were due to mutations in the envelope gene. Although the strategy used to produce the BK/CP-env virus also introduced the 65P→Q mutation in Rev, this mutation is not in

FIG. 5. Syncytium induction by BK/CP-env. Sup-T1 cells from the experiment shown in Fig. 4 were photographed during infection by NC-MAC on day 20 (A) and by BK/CP-env on day 5 (B). No cytopathic effects were apparent during NC-MAC infection, while large degenerating syncytia were evident for BK/CP-env.

TABLE 2. Infectivity of SIV variants on CD4-positive T-cell lines^a

Virus	Result with the following target cells:				
	Sup-T1	HUT-78	CEMx174	CEM	Molt4-clone 8
BK28	+	++++	++++	—	++++
NC-MAC	++	++++	++++	—	++++
CP-MAC	++++	—	—	—	++
BK/CP-env	++++	—	—	—	++

^a Viral supernatants from BK28-infected HUT cells (designated BK28) and NC-MAC-, CP-MAC-, or BK/CP-env-infected Sup-T1 cells were filtered, normalized for RT activity, and used to inoculate a panel of T-cell lines. Infection was monitored weekly for 30 days by IFA on fixed cells by using the 3A8 anti-p27^{gag} MAb. Symbols indicate the percentages of cells positive for viral antigen at day 30 as follows: +++++, 100%; ++, 25 to 50%; +, <25%; —, no cells positive.

any previously described functional domain for HIV-1 Rev (30, 48). Because CP-MAC and BK/CP-env were found to produce comparable levels of envelope glycoproteins (see below), Rev function appeared to be intact, and we considered it unlikely that this mutation contributed to the cytopathic properties of CP-MAC. As described below, additional studies were performed to evaluate other biological, biochemical, and morphological effects of the CP-MAC envelope mutations.

Alterations in host ranges of CP-MAC and BK/CP-env compared with those of NC-MAC and BK28. Cell-free supernatants of BK28 (from infected HUT-78 cells) and NC-MAC, CP-MAC, and BK/CP-env (from infected Sup-T1 cells) were normalized for RT activity and used to inoculate a panel of T-cell lines (Table 2). Both BK28 and NC-MAC infected Sup-T1 cells with retarded kinetics, similar to the data shown in Fig. 1 for NC-MAC. However, both of these viruses were infectious for HUT-78, CEMx174, and Molt4-clone 8, indicating that the four coding mutations in the NC-MAC SU glycoprotein (Fig. 2) had not altered the host range of this

virus. In contrast, although CP-MAC and BK/CP-env were highly infectious for Sup-T1 cells, these viruses were unable to infect HUT-78 or CEMx174. This finding was particularly striking in view of the marked susceptibility of HUT-78 and CEMx174 to SIVmac infection (33). Both CP-MAC and BK/CP-env infected Molt4-clone 8 cells more slowly than BK28 or NC-MAC. Similarly to previous reports for SIVmac, all viruses were unable to infect CEM cells (33, 56). Thus, the *env* mutations of CP-MAC appeared to reflect the adaptation of this virus to Sup-T1 cells. Remarkably, while these changes conferred the ability to infect Sup-T1 cells with high efficiency, they apparently abrogated infectivity for other CD4⁺ cells that were initially susceptible to the parental virus, BK28.

Differences in the envelope glycoproteins of NC-MAC and CP-MAC. Envelope glycoproteins from NC-MAC, CP-MAC, and BK/CP-env viral lysates were analyzed by Western blotting with the anti-TM MAb 1H8. Figure 6A shows the TM of NC-MAC to be approximately 32 kDa, consistent with the presence of a premature stop codon in the TM cytoplasmic tail

FIG. 6. Evaluation of viral glycoproteins by SDS-PAGE and Western blotting. (A) Size difference of TM glycoproteins of NC-MAC versus CP-MAC and BK/CP-env. NC-MAC (lane 1), CP-MAC (lane 2), and BK/CP-env (lane 3) viruses were pelleted from culture supernatant and lysed, and viral proteins were separated by SDS-PAGE. Proteins were transferred to nitrocellulose, and TM was detected using 1H8, as described in Materials and Methods. (B) Coprecipitation of TM and SU for CP-MAC and BK/CP-env. The viruses indicated were metabolically labeled with [³⁵S]Cys and [³⁵S]Met, pelleted, lysed, and immunoprecipitated with 1H8 (lanes 1, 4, and 7), a polyclonal rabbit anti-SU antiserum (lanes 2, 5, and 8), or recombinant sCD4 (lanes 3, 6, and 9). Coprecipitation of TM and SU is evident for CP-MAC and BK/CP-env but not for NC-MAC. (C) Disruption of the SU-TM complex of CP-MAC and BK/CP-env. Prior to immunoprecipitation, CP-MAC and BK/CP-env lysates were boiled for 10 min in lysis buffer and were then immunoprecipitated with 1H8 (lanes 1 and 3) or rabbit anti-SU (lanes 2 and 4). As shown, the SU-TM complex (shown in panel B) is dissociated by boiling.

FIG. 7. Envelope glycoprotein expression on the surfaces of infected cells. Sup-T1 cells either uninfected or infected by the viruses indicated were surface stained as described in Materials and Methods with MAb 43.1 (anti-TM), 101.1 (anti-SU), with a negative control MAb (P3), or with antiserum from an infected rhesus macaque (MAC) or normal human serum (NHS). Cells were fixed in 4% paraformaldehyde and were analyzed by FACS. Numbers on the y axis indicate the mean channel of fluorescence intensity. Cells in the infected samples were 100% positive for p27^{gag} expression on permeabilized cells. RT levels (in counts per minute) in the culture supernatants were 402,103 for NC-MAC, 186,925 for CP-MAC, and 383,687 for BK/CP-env.

(Fig. 2). Interestingly, the TMs of CP-MAC and BK/CP-env were found to be even smaller, migrating at 28 kDa. The basis for this slight decrease in R_f remains unclear but could be due to differences in glycosylation (although no changes in N-linked glycosylation sites were found for CP-MAC) or to changes in migration caused by one or more amino acid substitutions. In addition, the anti-TM MAb also reacted with a prominent 70-kDa band for NC-MAC but not CP-MAC or BK/CP-env. This band could be dissociated to a single 32-kDa band after treatment at pH 4.8 (data not shown), indicating that the TM of NC-MAC was forming a stable homodimer.

Immunoprecipitation of ³⁵S-labeled viral lysates using either 1H8, rabbit anti-SU serum, or soluble CD4 showed a remarkably stable interaction for the SU and TM molecules of CP-MAC and BK/CP-env, a characteristic described previously for SIV_{agm}(tyo-1) (3) and for HIV-2 and SIV_{mac} following adaptation to human H9 cells (63). As shown in Fig. 6B, the TM-specific MAb precipitated SU with TM for both CP-MAC (lane 4) and BK/CP-env (lane 7) but precipitated only TM and its 70-kDa dimer from NC-MAC lysates (lane 1). Similarly, the anti-SU serum and soluble CD4 precipitated TM associated with SU from CP-MAC and BK/CP-env, but only SU from NC-MAC lysates (Fig. 6B). Although it was stable in lysis buffer, this SU-TM complex could be dissociated by boiling. As shown in Fig. 6C, when CP-MAC and BK/CP-env lysates were first boiled for 10 min, the anti-TM MAb (lanes 1 and 3) and the anti-SU serum (lanes 2 and 4) precipitated only TM and SU, respectively. Thus, one or more of the amino acid changes in SU and/or TM envelope molecule of CP-MAC produced a striking increase in the stability of the SU-TM association.

Expression of envelope glycoproteins on the surface of infected Sup-T1 cells. To evaluate envelope glycoprotein expression on the cell surface, Sup-T1 cells infected by NC-MAC, CP-MAC, or BK/CP-env were stained in suspension with anti-SU (101.1) or anti-TM (43.1) MAb or with serum from an SIV-infected rhesus macaque and were analyzed by flow cytometry. SU expression on CP-MAC- and BK/CP-env-infected cells, as determined by 101.1 reactivity, was markedly

increased (approximately 10-fold) compared with that of NC-MAC-infected cells (Fig. 7). Smaller but reproducible increases (three- to fivefold) were also noted with the anti-TM MAb. These differences in envelope glycoprotein expression could not be explained by quantitative differences in the amount of virus produced, since comparable levels of RT were observed for the three infected cell lines (see the legend to Fig. 7). In addition, the increase in surface envelope glycoproteins seen with MAbs was not likely the result of a conformational change altering the exposure of particular epitopes, since an increase of six- to ninefold was also observed with the polyspecific macaque serum.

Differences in envelope glycoprotein distribution on virions.

In view of the increased level of envelope glycoproteins present on the surfaces of CP-MAC- and BK/CP-env-infected cells, we then determined if differences were also present in the number of envelope spikes on mature virions. Freshly fed cultures were incubated overnight and fixed in glutaraldehyde, and envelope spikes were stained for analysis by electron microscopy (see Materials and Methods). As shown in Fig. 8a, b, and c, CP-MAC and BK/CP-env virions exhibited a higher density of envelope spikes on virions than NC-MAC virions. This difference was quantified by counting the number of spikes per unit length of viral envelope for each virus. Figure 8 (bottom panel) shows the cumulative frequency distributions for envelope spikes per nanometer of viral membrane (27). CP-MAC and BK/CP-env virions possessed nearly twofold more envelope spikes than NC-MAC virions (mean numbers per micrometer of viral membrane $\pm$ standard deviations were 55.8 ± 16.0 for CP-MAC, 53.6 ± 11.3 for BK/CP-env, and 29.2 ± 16.0 for NC-MAC; Student's *t* test [$P < 0.05$]). Thus, mutations in the *env* gene of CP-MAC increased the expression of envelope glycoproteins both on the cell surface and on virions.

Evaluation of SU shedding for CP-MAC and NC-MAC. The increased amount of SU envelope glycoproteins on CP-MAC- and BK/CP-env-infected cells and virions raised the possibility that more SU was shed from NC-MAC-infected cells and/or virions. This possibility was suggested by a more stable interaction between the CP-MAC SU and TM glycoproteins, which remained intact even in the presence of mild detergent (Fig. 6B). To evaluate this possibility directly, the relative amounts of SU shedding were determined by pulse-chase analysis. NC-MAC-, CP-MAC-, and BK/CP-env-infected cells were labeled with [³⁵S]Cys and [³⁵S]Met for 15 min and then chased for 1, 2, 4, and 6 h. Cell-free medium was harvested, and the free (presumably shed) SU was separated from virus-associated SU by ultracentrifugation. Cell lysates were also prepared for each of the time points, and viral proteins were immunoprecipitated and quantified (see Materials and Methods). The amounts of SU in the supernatant, the viral pellet, and the cell lysate and the amounts of virus-associated p27^{gag} are shown for the 6-h time point (Table 3). For each virus, the ratio of free SU to pelletable p27^{gag} was used as an indication of the degree of SU shedding relative to the amount of virus produced. In addition, the percentage of the total amount of SU produced that was shed was determined.

NC-MAC showed a ratio of free SU to virus-associated p27^{gag} that was actually 12- to 18-fold lower than that of CP-MAC or BK/CP-env. Assuming that the amounts of p27 incorporated into viral particles were the same for each virus, this result indicated that NC-MAC actually exhibited less SU shedding than either CP-MAC or BK/CP-env. Differences were also apparent in the percentages of total SU that were shed into the medium (2% for NC-MAC, compared with 5 and 16% for CP-MAC and BK/CP-env, respectively). The pulse-chase analysis also showed no differences in the rate of gp160

FIG. 8. Expression of envelope glycoprotein spikes on virions. Transmission electron micrographs of NC-MAC (a), CP-MAC (b), and BK/CP-env (c) are shown. Envelope glycoprotein spikes (arrows) are visible as electron-dense structures on the surfaces of virions following processing and staining with the osmium-tannic acid-uracyl acetate-lead citrate method (bar, 100 nm). The graph depicts the results of quantitative morphometric analysis of the envelope spike distribution on each virus. The curves represent the cumulative frequency distributions of the spike densities (27). The mean spike density (spikes per micrometer $\pm$ 1 standard deviation) for NC-MAC (29.2 ± 16.0) was significantly lower ($P < 0.05$ by Student's t test) than those for CP-MAC (55.8 ± 16.0) and BK/CP-env (53.6 ± 11.3). There was no difference between CP-MAC and BK/CP-env.

cleavage or the appearance of labeled SU in the medium (data not shown). These results indicated that the reduced amounts of envelope on the surfaces of NC-MAC-infected cells and virions were not the result of either more SU shedding or changes in glycoprotein processing.

TABLE 3. Comparison of SU shedding from infected cells^a

Virus	Densitometry value				SU (free)/p27 ^b	% SU shed ^c
	SU (free)	SU (virus)	SU (cells)	p27 (virus)		
NC-MAC	101	1,842	2,651	5,904	0.02	2
CP-MAC	403	590	7,173	1,078	0.37	5
BK/CP-env	3,828	7,368	12,650	15,092	0.25	16

^a Sup-T1 cells producing NC-MAC, CP-MAC, or BK/CP-env were pulse-labeled with [³⁵S]cysteine and [³⁵S]methionine for 15 min and were chased for 6 h (see Materials and Methods). Culture medium was harvested, and intact virus was separated from free SU by ultracentrifugation. Viral proteins were immunoprecipitated with anti-SIV serum, separated by SDS-PAGE, and quantified by autoradiography and densitometry. SU (free) indicates the amount of SU in the supernatant and is used as an indication of shed SU. SU (virus) and p27 (virus) indicate the amounts of each protein in the pellet as an indication of virus-associated SU and p27^{gag}. SU (cells) indicates the amount of SU immunoprecipitated from cell lysates.

^b The amount of SU shedding relative to virus production is indicated by the ratio of free SU to p27^{gag} in the pellet.

^c % SU shed = {SU (free)/[SU (free) + SU (virus) + SU (cells)]} $\times$ 100.

DISCUSSION

In this report, we have described the characterization of CP-MAC, a SIVmac variant derived from the BK28 molecular clone. Compared with BK28 and the closely related NC-MAC virus, CP-MAC exhibited a number of differences, including (i) the ability to infect Sup-T1 cells with rapid kinetics, cell fusion, and CD4 down-modulation; (ii) an alteration in cell tropism that largely restricted its host range to Sup-T1 cells; (iii) a more stable interaction between its SU and TM envelope molecules; and (iv) increased levels of envelope glycoproteins expressed on the surfaces of infected cells and virions. We have shown that a limited number of amino acid changes compared with the sequence for BK28 (five in SU and six in TM) could completely account for these effects. Differences in tropism, infection kinetics, and cytopathic effects are well recognized for HIV and SIV and likely have relevance to in vivo pathogenesis. However, even when molecular determinants for these effects have been identified, the underlying mechanisms are unknown. The striking biological and structural changes exhibited by CP-MAC should be useful to more precisely map the molecular determinants and to understand their effects on envelope structure and function.

The altered cell tropism exhibited by CP-MAC appears to reflect a highly efficient adaptation for infection of Sup-T1 cells. NC-MAC infected Sup-T1 cells with slow kinetics and without cytopathic effects, whereas CP-MAC infected this cell

type rapidly and with syncytium formation. Moreover, the *env* mutations that facilitated infection of Sup-T1 apparently precluded the ability of CP-MAC to infect CEMx174 and HUT-78 cells, two cell types that are characteristically very susceptible to SIV_{mac} infection (see Table 2 and reference 33). This finding is in contrast to those for some isolates of HIV-1 (4, 9) and SIV (40) that have been shown to broaden their host range following propagation in T-cell lines. Infection by both NC-MAC and CP-MAC was inhibited by anti-CD4 MAbs (data not shown), indicating that despite differences in host range, these viruses still utilized CD4 for infection. The basis for the restricted infectivity of CP-MAC to Sup-T1 cells is unclear but is not the result of differences in CD4 expression, since all T-cell lines exhibited CD4 levels that were within two- to threefold of one another by FACS analysis (data not shown). There is abundant evidence that HIV and SIV entry require molecules in addition to CD4 (33, 47, 68). The presence or absence of these putative coreceptors has been implicated in the failure of HIV to infect murine cells expressing human CD4 (47) and in differences in tropism of HIV and SIV for T cells or macrophages (29, 34, 57). The extraordinary specificity of CP-MAC for a single cell type suggests that if CD4-associated molecules are playing a role, there may well be multiple or polymorphic determinants that vary, even among different T-cell lines.

Among the mutations in the CP-MAC envelope glycoprotein that could alter its tropism, the 327M→R change located in a region analogous to the V3 loop of HIV-1 is of particular interest. For HIV-1, combinations of amino acid changes in V3 have conferred macrophage tropism to T-cell-tropic isolates (34, 57, 66), and even single amino acid changes in V3 have been shown to alter tropism for T- and non-T-cell lines (29, 35, 67, 69). Early studies of SIV indicated that an analogous role for this disulfide loop was questionable, since amino acid mutations outside this region were required for macrophage tropism (53). However, recent studies of SIV have mapped determinants for infection of macrophages (29) and CEMx174 cells (38, 54) to either V3 itself or regions of SU that include V3, suggesting that this domain is functionally similar for both HIV-1 and SIV. Envelope mutations outside V3 have also been shown to alter HIV-1 tropism (13, 66, 75), including a point mutation in V1, which conferred infectivity for Sup-T1 and Molt4-clone 8 to HIV-1_{JRCSF} (4). In addition, the 327M→R change of CP-MAC is of interest, since for HIV-1 the acquisition of basic residues in V3 has been associated with the transition from nonsyncytium-inducing to more cytopathic syncytium-inducing viruses (15, 49). It remains to be determined if the 327M→R change alone or in combination with other envelope mutations is responsible for the restricted tropism and/or increased cytopathicity of CP-MAC for Sup-T1 cells.

CP-MAC was initially distinguishable from NC-MAC by its ability to down-modulate CD4 from the cell surface. While NC-MAC reduced CD4 expression less than twofold, CP-MAC produced nearly complete down-modulation (Table 1). HIV- and/or SIV-induced down-modulation of CD4 has been shown to result from (i) intracellular complexing of CD4 with envelope precursor molecules (14, 31, 33, 36) and retention in the endoplasmic reticulum (14, 36), (ii) effects of the viral Nef protein (1, 25), and (iii) degradation of newly synthesized CD4 induced by Vpu (73). Nef and Vpu reduce CD4 expression by a specific interaction with the CD4 cytoplasmic tail (1, 25, 45, 73), whereas envelope-induced down-modulation results from the binding of gp160 and/or gp120 to the CD4 ectodomain (14, 36). The ability of CP-MAC to down-modulate CD4 could be attributed entirely to changes in envelope, since this property

was conferred on BK28 by the CP-MAC envelope gene. Moreover, both NC-MAC and CP-MAC possess premature stop codons within the *nef* open reading frame, indicating that Nef does not play a role in the biological phenotype of either virus (data not shown). It is possible that the effects of NC-MAC and CP-MAC on CD4 expression result from a difference in SU-CD4-binding affinity. As shown in Table 1 and as previously described for an uncloned SIV_{mac} in Sup-T1 cells (33), CD4 was detectable on NC-MAC-infected cells even with MAbs such as OKT4A that compete with SU for binding to CD4. OKT4A binding occurred despite the presence of virus and/or free SU in the culture medium. This finding indicates either that the NC-MAC envelope glycoprotein complex is not binding to CD4 or that it is binding with an affinity lower than that of the MAb. Because NC-MAC infection of Sup-T1 cells can be inhibited by anti-CD4 MAbs (data not shown) and because the NC-MAC SU can be precipitated by recombinant CD4 (Fig. 6B), it is clear that this glycoprotein binds to CD4. Thus, we favor the hypothesis that an increase in CD4-binding affinity for CP-MAC SU may explain the ability of this virus to down-modulate CD4. Recent studies using a fluoresceinated CD4-binding assay (32) have indicated that the native CP-MAC envelope glycoprotein complex expressed on the surfaces of infected cells has a high CD4-binding affinity, comparable to that determined for T-cell-adapted isolates of HIV-1 (44). Efforts are in progress to identify the specific amino acid residues in SU and/or TM that produce this high CD4-binding affinity.

The transition from NC-MAC to CP-MAC resulted in a marked increase in the stability of the SU-TM interaction. Similarly to laboratory-adapted isolates of HIV-1, the NC-MAC SU and TM were readily dissociable upon solubilization in detergent (28, 41, 63). In contrast, these molecules remained associated for CP-MAC, as evidenced by their coprecipitation using anti-SU- or TM-specific MAbs or recombinant CD4 (Fig. 6B). A similarly stable association has been reported for SIV_{agm}(tyo-1) propagated in Molt-4 cells (3) and for HIV-2 and SIV_{mac} grown in H9 cells (63). The determinants responsible for this effect in these genetically divergent viruses are unknown. Interestingly, several of the amino acid changes in CP-MAC (573V→L, 575K→T, 588T→I, and 643E→K) occurred in areas of TM that have been implicated in reducing the stability of the HIV-1 SU-TM complex (7, 8, 23). NC-MAC also differed from CP-MAC by the presence of stable TM dimers. Of note, three of the CP-MAC mutations in TM (573V→L, 575K→T, and 588T→I) are in a region analogous to a leucine zipper-like motif, which has been suggested to contribute to envelope oligomerization (18). Further analysis should identify mutations that mediate these structural changes and help to determine their biological significance.

Finally, one of the most interesting changes exhibited by CP-MAC was the high level of SU envelope glycoproteins expressed on infected cells and virions. FACS analysis demonstrated increases in cell surface expression of approximately 10- and 5-fold for SU and TM, respectively (Fig. 7), which were reflected in a 2-fold increase in the number of spikes on virions (Fig. 8). A number of viral gene products have been implicated in modulating envelope expression on the cell surface and/or virions including Nef (64), Vpu (73), and the p18 matrix protein (17, 21, 77). However, our finding that the recombinant BK/CP-*env* virus containing only the CP-MAC *env* mutations exhibited the same increase clearly mapped this effect to the envelope gene. Several amino acid mutations in SU and in the TM ectodomain have been shown to reduce envelope glycoprotein expression by disrupting processing or reducing the stability of the SU-TM association (7, 28, 41). However, very

few of these mutations increased envelope expression (7, 74). In contrast, deletions in the HIV-1 (24) and SIV (37, 60, 78) TM cytoplasmic tails have been reported to increase envelope glycoprotein expression on cells and/or virions, although for HIV-1 a reduced incorporation of envelope glycoproteins into virions has also been reported (19). Recent studies in which the HIV-1 TM membrane-spanning domain and cytoplasmic tail were replaced by a glycopospholipid linkage showed a higher level of envelope glycoproteins on the cell surface (61, 72) and on virions (61), indicating the potential for determinants in the membrane-spanning domain and/or the cytoplasmic tail to affect envelope expression. Interestingly, CP-MAC did contain a single amino acid substitution (723Y→C) in the TM cytoplasmic tail. Ongoing studies in our laboratory have determined that this change is the principal determinant for the increased envelope expression on cells and virions for CP-MAC (44). Efforts to determine the mechanism for this effect are in progress.

These studies indicate the remarkable extent to which divergent biological properties of SIV can be generated by a limited number of mutations in the envelope glycoproteins. Interestingly, while the nucleotide substitution frequencies for NC-MAC and CP-MAC compared with that for BK28 were only 0.3 and 0.6%, respectively, a high proportion of these mutations were found to encode amino acid changes. Similar findings have been observed for in vivo models of SIV infection and have been presumed to reflect selection pressures generated by the host immune response (5, 39, 58). However, for rhesus macaques infected with SIVmac239, Kodama et al. recently reported distinct patterns of amino acid changes in different tissues, suggesting that genetic variation could also occur during viral adaptation to different cell types (39). The present findings with our in vitro model support this view and indicate that selection pressures generated by the host cell can be an important determinant for genetic variation even in the absence of an immune response. Given the limited number of amino acid changes involved, future studies should be able to identify the molecular determinants that are responsible for these effects and provide insight into the mechanisms through which changes in envelope structure contribute to the diverse biological phenotypes of SIV and HIV.

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