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A Single Amino Acid Change in the Cytoplasmic Domain of the Simian Immunodeficiency Virus Transmembrane Molecule Increases Envelope Glycoprotein Expression on Infected Cells

CELIA C. LABRANCHE,[1] MONICA M. SAUTER,[1] BETH S. HAGGARTY,[1] PATRICIA J. VANCE,[1] JOSEPHINE ROMANO,[1] TIMOTHY K. HART,[2] PETER J. BUGELSKI,[2] MARK MARSH,[3] AND JAMES A. HOXIE[1]*

Hematology-Oncology Division, Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania 19104[1]; Department of Toxicology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406[2]; and Medical Research Council Laboratory for Molecular Cell Biology and Department of Biology, University College London, London WCIE 6BT, England[3]

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We have described a virus termed CP-MAC, derived from the BK28 molecular clone of simian immunodeficiency virus, that was remarkable for its ability to infect Sup-T1 cells with rapid kinetics, cell fusion, and CD4 down-modulation (C. C. LaBranche, M. M. Sauter, B. S. Haggarty, P. J. Vance, J. Romano, T. K. Hart, P. J. Bugelski, and J. A. Hoxie, J. Virol. 68:5509–5522, 1994 [Erratum 68:7665–7667]). Compared with BK28, CP-MAC exhibited a number of changes in its envelope glycoproteins, including a highly stable association between the external (SU) and transmembrane (TM) molecules, a more rapid electrophoretic mobility of TM, and, of particular interest, a marked increase in the level of envelope protein expression on the surface of infected cells. These changes were shown to be associated with 11 coding mutations in the *env* gene (5 in SU and 6 in TM). In this report, we demonstrate that a single amino acid mutation of a Tyr to a Cys at position 723 (Y723C) in the TM cytoplasmic domain of CP-MAC is the principal determinant for the increased expression of envelope glycoproteins on the cell surface. When introduced into the *env* gene of BK28, the Y723C mutation produced up to a 25-fold increase in the levels of SU and TM on chronically infected cells, as determined by fluorescence-activated cell sorter analysis with monoclonal and polyclonal antibodies. A similar effect was observed when a Tyr-to-Cys change was introduced at the analogous position (amino acid 721) in the SIVmac239 molecular clone, which, unlike BK28 does not contain a premature stop codon in its TM cytoplasmic tail. Substituting other amino acids, including Ala, Ile, and Ser, at this position produced increases in surface envelope glycoproteins that were similar to that observed for the Cys substitution, while a Tyr-to-Phe mutation produced a smaller increase. These results could not be accounted for by differences in the kinetics or efficiency of envelope glycoprotein processing or by shedding of SU from infected cells. However, immunoelectron microscopy demonstrated that the Y723C mutation in BK28 produced a striking redistribution of cell surface envelope molecules from localized patches to a diffuse pattern that covered the entire plasma membrane. This finding suggests that mutation of a Tyr residue in the simian immunodeficiency virus TM cytoplasmic domain may disrupt a structural element that can modulate envelope glycoprotein expression on the surface of infected cells.

In contrast to other retroviruses, the transmembrane envelope glycoprotein (TM) of lentiviruses contains a long cytoplasmic domain of approximately 150 to 200 amino acids. For the human and simian immunodeficiency viruses (HIV and SIV), mutations in this region have been shown to alter a number of viral properties, including tropism (31, 34, 78, 80), infection kinetics (9, 15, 31, 38, 78), syncytium formation (1, 13, 19, 46, 51, 59, 80), cell killing (15, 38), and infectivity (9, 12, 19, 31, 61, 63, 78). The biological effects of these mutations are highly variable and have been shown to be dependent on the strain of virus, the cell producing the virus, and the target cell (9, 19, 31, 38, 59, 78). However, the specific mechanisms responsible for each of these complex phenomena are unclear. Unlike the external envelope glycoprotein (SU) and the ectodomain of TM, where several functional regions have been mapped (3, 8, 17, 18, 26, 33, 35, 36, 50, 64), specific functional domains within the TM cytoplasmic tail have not as yet been identified.

For a number of viruses, the cytoplasmic domains of their envelope glycoproteins have been implicated in playing a role in virion assembly by directing the intracellular trafficking of envelope molecules, (55, 57, 60, 77), modulating their expression on the cell surface (20, 57, 60, 65, 76), and affecting their subsequent incorporation into virions (2, 4, 5, 20, 21, 23, 24, 41, 43, 48, 76, 79). There is also evidence that the cytoplasmic domains of vesicular stomatitis virus G protein (62), influenza virus hemagglutinin, and parainfluenza virus type 3 F protein (40) may interact with cellular molecules within the plasma membrane that affect their lateral mobility on the cell surface. However, as shown for Rous sarcoma virus (53), Moloney murine leukemia virus (56), and influenza virus hemagglutinin (29), the cytoplasmic domains of envelope glycoproteins may

* Corresponding author. Mailing address: Hematology-Oncology Division, Rm. 664, Clinical Research Building, University of Pennsylvania, 415 Curie Blvd., Philadelphia, PA 19104. Phone: (215) 898-0261. Fax: (215) 573-2028. Electronic mail address: hoxie@mail.med.upenn.edu.

not be required for transport of envelope glycoproteins to the cell surface, incorporation into virions, or, in some instances, infectivity.

For HIV and SIV, the role of the TM cytoplasmic tail in viral assembly is controversial. HIV type 1 (HIV-1) TM molecules with truncated cytoplasmic tails are processed, transported to the plasma membrane, and incorporated into virions (12, 13, 19, 51). Remarkably, even a glycosylphosphatidylinositol-linked envelope molecule lacking both membrane spanning and cytoplasmic domains can be transported to the cell surface (61, 75) and incorporated into virions (61). Indeed, because HIV-1 virions containing deletions within the TM cytoplasmic domain are generally reduced in infectivity (12, 19, 61), it has been argued that this region may be involved primarily in events associated with viral entry rather than in virion assembly (19). However, in other studies of HIV-1, deletions of the C-terminal region of the TM tail were shown to reduce the incorporation of envelope glycoproteins into virions (12, 78) and to prevent the polarized budding of virions in MDCK cells (39). For SIV, in which premature stop codons in the TM cytoplasmic tail are selected for during propagation in several human cell lines (34), truncations of this region have been shown to increase the level of envelope glycoproteins on virions and to enhance fusion and infectivity for some T-cell lines (9, 31, 59, 67, 80). For both HIV-1 (6, 11, 14, 16, 39, 74, 78) and SIV (22), there is increasing evidence that an interaction between the TM cytoplasmic domain and the viral matrix protein may modulate envelope glycoprotein incorporation into virions, as has been suggested for a number of other retroviruses (4, 5, 21, 23, 52, 69). Nonetheless, specific determinants within the HIV or SIV TM cytoplasmic tails that positively or negatively affect envelope glycoprotein expression on the cell surface or direct their incorporation into virions have not been identified.

We have described a variant of SIV, termed CP-MAC, derived in vitro from the BK28 clone of SIVmac (37). Compared with BK28, and an early passage of BK28 in Sup-T1 cells termed NC-MAC, CP-MAC exhibited a number of differences, including (i) an altered host range that largely restricted its replication to Sup-T1 cells, (ii) an increased stability in the association of SU and TM, (iii) an increased electrophoretic mobility of TM, and (iv) the ability to downregulate CD4. Of particular interest, CP-MAC-infected cells showed a marked increase in the expression of envelope glycoproteins on the cell surface. A limited number of amino acid substitutions in the envelope molecules (five in SU and six in TM) were shown to confer all properties of the CP-MAC phenotype to BK28. We now show that one of these mutations, a Tyr-to-Cys substitution at position 723 (Y723C) in the CP-MAC TM cytoplasmic domain, is the principal determinant for the increased surface expression of the envelope glycoproteins. Moreover, we demonstrate that a similar Tyr-to-Cys mutation, as well as a number of other nonconservative substitutions at the corresponding position in the SIVmac239 molecular clone, produces a similar effect. This finding suggests the presence of a structural element in a lentivirus TM cytoplasmic domain that modulates the level of envelope glycoproteins on infected cells.

MATERIALS AND METHODS

Viruses and cell lines. The SIV termed NC-MAC was derived from BK28-infected Sup-T1 cells and had been shown previously to infect Sup-T1 cells with slow kinetics and without cell fusion or CD4 down-modulation. CP-MAC arose spontaneously during long-term culture of a clone of NC-MAC-infected Sup-T1 cells and was shown to infect this cell type with rapid kinetics, cell fusion, and CD4 down-modulation (37). The infectious molecular clone of SIVmac, BK28 (provided by James Mullins), was obtained from the AIDS Reagent Repository. Plasmids pVP-1 and pVP-2, containing 5′ and 3′ halves, respectively, of the SIVmac239 genome, were obtained from Paul Luciw (73, 80). The $CD4^+$ T-cell lines Sup-T1, CEM, CEMx174, and HUT-78 and the rhabdomyosarcoma cell line RD have been described previously (37). All lines were maintained in RPMI–10% fetal calf serum.

MAbs and antisera. Murine monoclonal antibodies (MAbs) used to detect SIV proteins included 3A8, reactive with SIV $p27^{gag}$ (provided by J. McClure), 1H8 (58) and 43.1, each reactive with SIVmac TM (provided by J. McClure and C. Thiriart, respectively), and 101.1, reactive with SIVmac SU (provided by C. Thiriart). MAbs 7D3 and 5B11, derived in our laboratory, react with conformational and distinct epitopes on BK28 and CP-MAC SU that are highly expressed on the surface of infected cells (unpublished observations). The murine MAb P3X63 was used as a negative control. Antiserum from an SIVmac-infected rhesus macaque was provided by P. Fultz.

Construction of recombinant viruses. Mutants of BK28 were generated by using an internal 3.5-kb *Sac*I fragment (nucleotides [nt] 5735 to 9212) that contained desired mutations in *env* (37). For the virus termed BK/CP-env, this *Sac*I fragment was constructed by using cloned DNA amplified from infected cells and contained all of the *env* mutations present in CP-MAC. Restriction sites used included a *Cla*I site (nt 8055), a *Pst*I site (nt 8947), and two *Hin*dIII sites (nt 6215 and 6798) (37) (Fig. 1). A similar approach was used to produce BK/NC-723Y, which contained the consensus envelope mutations of NC-MAC. BK/NC-723C and BK/723C were constructed by inserting an *Nhe*I (nt 8724)-to-*Pst*I (nt 8947) fragment derived from the BK/CP-env *env* gene into the *Sac*I fragments of BK/NC-723Y and BK28, respectively. Mutants of SIVmac239 that contained substitutions at Tyr-721 in the TM cytoplasmic domain were produced by using plasmids pVP-1 and pVP-2. An *Nhe*I (nt 8998)-to-*Nde*I (nt 10007) fragment of pVP-2 was cloned into a modified pBluescript plasmid that contained a deletion within the f1 origin that removed a *Bgl*I site. Mutations were created by using the site-directed mutagenesis protocol of Deng and Nickoloff (10). The selection primer used to eliminate a unique *Bgl*I site in the ampicillin resistance gene of the plasmid was 5′-GCTCGGCCCTTCCGGCTGGCTGGTTT-3′, and mutagenesis primers were 5′-TTAAGGCAGGGGTGTAGGCCAGTGTTCC-3′, for a Y721C mutation, 5′-TTAAGGCAGGGGATTAGGCCAGTGTTCC-3′, for a Y721I mutation, 5′-TTAAGGCAGGGGGCTAGGCCAGTGTTCC-3′, for a Y721A mutation, 5′-TTAAGGCAGGGGTTTAGGCCAGTGTTCC-3′, for a Y721F mutation, and 5′-TTAAGGCAGGGGAGTAGGCCAGTGTTCC-3′, for a Y721S mutation. Following primer extension and ligation, plasmids were transformed into *Escherichia coli* BMH 71-18 *mutS*, which is deficient in DNA mismatch repair (Clontech Laboratories, Inc., Palo Alto, Calif.). Selection for mutagenized plasmids was performed by digestion with *Bgl*I and subsequent transformation into *E. coli* XL1 Blue. An *Nhe*I-to-*Bgl*II (nt 9374) fragment of the mutagenized insert was sequenced and cloned into pVP-2 for transfection protocols.

DNA transfection protocols. For mutants of BK28, 5 μg of the appropriate *Sac*I fragment was ethanol precipitated with 5 μg of a pBK28 plasmid from which the corresponding *Sac*I fragment had been deleted, resuspended in Tris-EDTA buffer, and transfected into RD cells by the calcium phosphate method (73). Following a glycerol shock, RD cells were cocultured with T-cell lines, and the nonadherent lymphoid cells were passaged. For production of SIVmac239-derived viruses, plasmids containing pVP-1 and mutagenized pVP-2 were codigested with *Sph*I, ligated with T4 DNA ligase, linearized with *Apa*I, and electroporated into CEMx174 cells (960 μF, 250 V). Cultures were monitored for virus production by serial determinations of reverse transcriptase (RT) activity, and the percentage of cells expressing $p27^{gag}$ was determined by immunofluorescence microscopy (28).

Analysis of mutations in infected cell lines. SU and TM coding regions were amplified by PCR from genomic DNA of chronically infected cell lines as previously described (37). Primers for SU were 5′-GAGCAGTCACGAAAG (forward primer) and 5′-ACCTGCCGTTGCGA (reverse primer), and those for TM were 5′-GTGGCAGAACTGTATCGATTGGAGT (forward primer) and 5′-GCCTCTGCAGTATTGGTTGGA (reverse primer). Sequencing of uncloned amplified DNA was performed by the dideoxy-chain termination method (Sequenase; U.S. Biochemicals).

Fluorescence activated cell sorting (FACS) analysis of surface envelope glycoproteins. Surface expression of envelope glycoproteins on infected cells was determined by flow cytometry using a FACScan analyzer (Becton Dickinson) as described previously (28). Living cells were labeled on ice in staining buffer (phosphate-buffered saline [PBS] supplemented with 0.1% bovine serum albumin and 0.02% sodium azide), using saturating concentrations of MAbs or sera, and then incubated with a 1/40 dilution of fluorescein isothiocyanate-conjugated $F(ab')_2$ goat anti-mouse immunoglobulin G (IgG; Tago Laboratories, Burlingame, Calif.). Cells were fixed for 18 h at 4°C in 2% paraformaldehyde prior to analysis.

Radioimmunoprecipitation and pulse-chase protocols. Infected cells were preincubated in Cys- and Met-free RPMI–10% dialyzed fetal calf serum and labeled with 250 μCi of [^{35}S]Cys-Met per ml for 15 min at 37°C. At various times, cells, viral pellets and medium were prepared and processed for immunoprecipitation of viral proteins as described previously (28, 37). Cells were lysed on ice in lysing buffer (0.02 M Tris [pH 8.0], 0.12 M NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM EGTA, 0.2 mM NaF, 5 μg of aprotinin per ml, 0.2% sodium deoxycholate, 0.5% Nonidet P-40), and cell-free medium was centrifuged

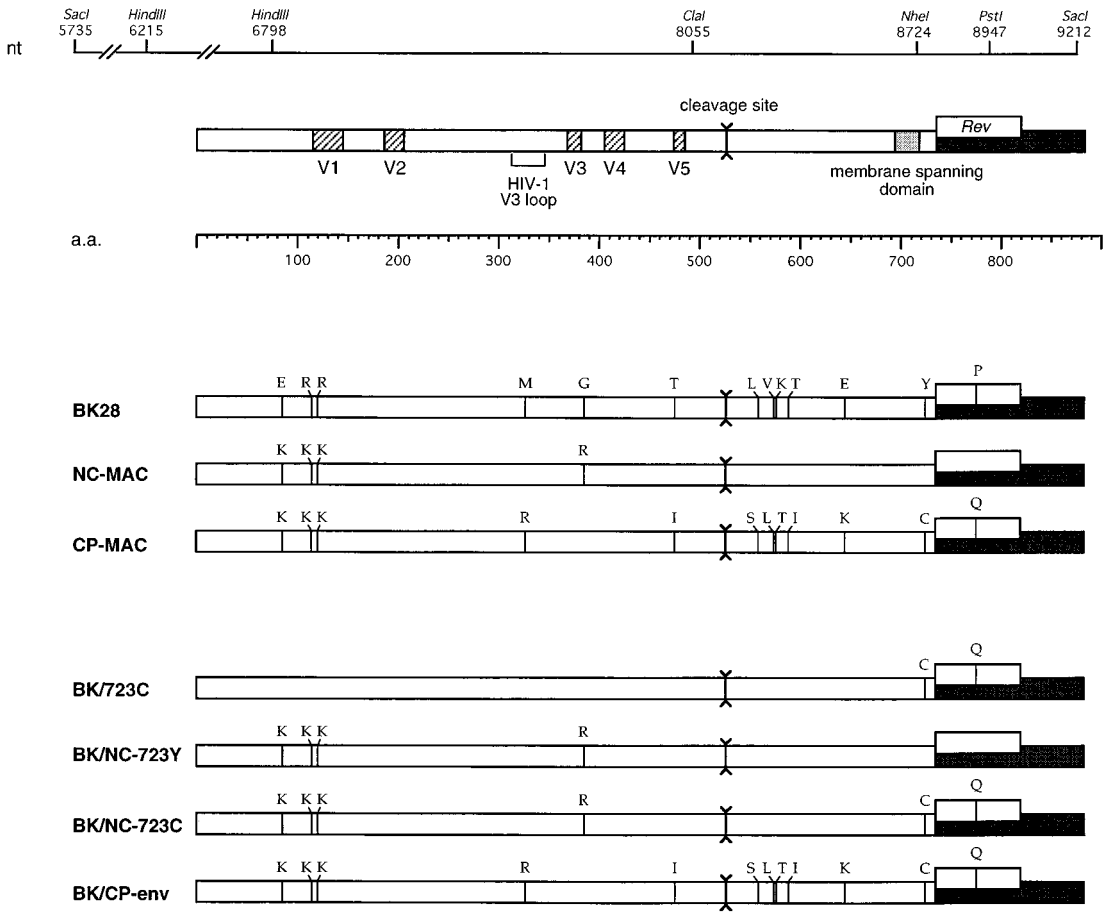

FIG. 1. Construction of BK28 mutants to evaluate effects of a Y723C mutation in the TM cytoplasmic domain. The BK28 *env* gene (nt 6580 to 9225) is represented with restriction sites that were used to construct recombinant viruses (see Materials and Methods). The second exon of *rev*, which overlaps the *env* gene in an alternate reading frame, is also shown. Relative amino acid (a.a.) positions are indicated. Amino acids in BK28 that were found to be mutated in either NC-MAC or CP-MAC are shown in the BK28 diagram, and the specific amino acid changes present in NC-MAC, CP-MAC, and the four recombinant viruses (BK/723C, BK/NC-723Y, BK/NC-723C, and BK/CP-env) are shown. V1, V2, V3, V4, and V5 refer to variable regions identified from in vivo studies of SIVmac239 (7). The locations of the corresponding HIV-1 V3 loop, the envelope cleavage site, and the membrane spanning domain are indicated. The area of *env* past the premature stop at codon 736 is represented by dark shading.

(100,000 × *g* 30 min). Pellets containing virions were lysed in lysing buffer for 30 min on ice, and the supernatant containing free SU was treated with a 1/10 volume of 10× lysing buffer. All lysates were preadsorbed with a 1/10 volume of protein A-agarose (Boehringer Mannheim Biochemicals) prior to immunoprecipitation. Viral proteins were immunoprecipitated with serum from an SIVmac-infected rhesus macaque and quantified by autoradiography and densitometry.

Immunoelectron microscopy analysis of surface envelope glycoproteins. Suspensions of infected cells were incubated with an anti-SU (101.1) or anti-TM (43.1) MAb for 30 min at 37°C, or alternatively, cells were first fixed at 4°C for 30 min in 2% paraformaldehyde and then stained with MAbs. Cells were washed twice in ice-cold PBS, fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 60 min at room temperature, and incubated with goat anti-mouse IgG-biotin followed by streptavidin-horseradish peroxidase. Cells were subsequently treated with diaminobenzidine followed by osmium tetroxide and then dehydrated in ethanol and embedded in epoxy resin. Thin sections were cut and stained with lead citrate. For controls, incubations were performed in the absence of primary antibodies.

RESULTS

Construction of SIVs with TM tail mutations. As previously described, CP-MAC exhibited a number of differences from NC-MAC in its envelope glycoproteins, including (i) a more rapid migration of TM on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), (ii) a more stable association of SU and TM, and (iii) an increased expression of envelope glycoproteins on the surface of infected cells (37). As summarized in Fig. 1, compared with BK28, NC-MAC contained four amino acid mutations in SU (E84K, R112K, R120K, and G385R), while CP-MAC contained five mutations, three that were shared with NC-MAC (E84K, R112K, and R120K) and two that were unique to CP-MAC (M327R and T4751). CP-MAC also contained six unique mutations in TM, five in the extracellular domain (L559S, V573L, K575T, T588I, and E643K) and a Y723C substitution in the TM cytoplasmic tail. Incorporation of the *env* mutations of CP-MAC into BK28, producing a virus termed BK/CP-env, conferred all properties of the CP-MAC phenotype to BK28, including its increased expression of envelope glycoproteins on the cell surface (37).

Mutations in cytoplasmic domains have been shown to affect envelope glycoprotein expression on infected cells and/or virions for a number of other enveloped viruses (20, 54, 57, 60, 65). Thus, in considering which of the mutations in the CP-MAC *env* gene could be responsible for the increased surface expression of envelope glycoproteins, the Y723C mutation was of particular interest. In addition, a Tyr analogous to that present at position 723 for BK28 is conserved six or seven amino acids from the predicted TM membrane spanning domain for all isolates of HIV-1, HIV-2, and SIV sequenced to

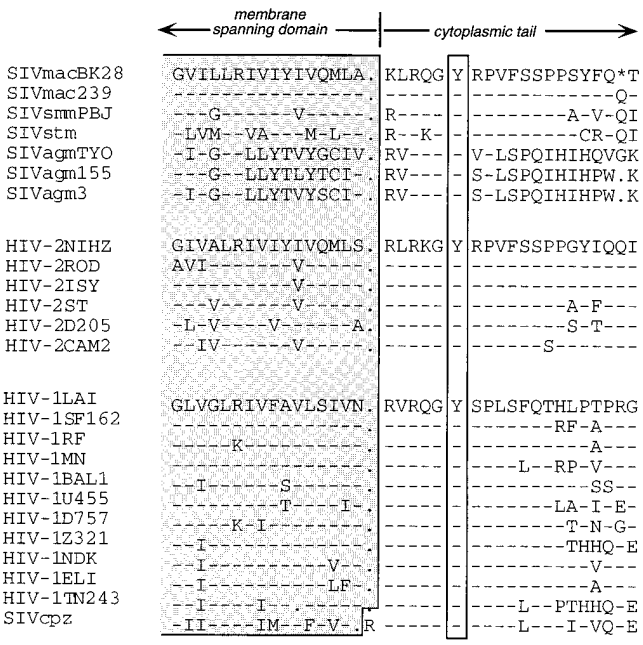

FIG. 2. Conservation of a Tyr residue in the cytoplasmic domains of HIV and SIV. Partial sequences for genetically divergent isolates of HIV and SIV are shown for the TM molecule at the junction of the predicted membrane spanning domain and the cytoplasmic tail (47). Sequences from SIV, HIV-2, and HIV-1 isolates are grouped separately, with SIVcpz included within the HIV-1 group. As shown, a Tyr in the cytoplasmic domain is located six or seven amino acids from the membrane spanning domain for all isolates. Amino acid identity within each group is indicated by a dash; periods are added to facilitate alignment. The premature stop codon of BK28 is indicated by an asterisk. The amino acid sequence for the BK28 *env* gene used in this study (27) is identical to that reported for SIVmac251 (32, 37, 47).

date (Fig. 2) (47). Therefore, the Y723C mutation of CP-MAC also represented a novel change in a highly conserved region of TM.

Recombinant viruses were made to determine the effects of the Y723C mutation independent of other changes in *env*. One virus, termed BK/723C, was constructed by using the *Nhe*I-to-*Pst*I fragment from CP-MAC, which within *env* differed from BK28 only by the Y723C mutation. As shown in Fig. 1, BK/723C also contained a transversion at nt 8908 (cytosine to adenosine) that produced a P65Q mutation in the second exon of *rev* and a noncoding mutation in the second exon of *tat*. A similar strategy was used to produce a second pair of viruses to evaluate the effects of the Y723C mutation on an envelope that contained the SU mutations present in NC-MAC. These viruses, designated BK/NC-723Y and BK/NC-723C, had a Tyr and a Cys, respectively, at position 723. As with BK/723C, BK/NC-723C also contained a P65Q mutation in *rev*. *Sac*I fragments shown in Fig. 1 containing these envelope genes were transfected into RD cells with a BK28 clone from which the corresponding *Sac*I fragment had been deleted, and virus was isolated following coculture with CEMx174 cells (see Materials and Methods). As a control, BK28 was produced by an identical protocol. Replication of the recombinant viruses was detectable between 2 to 3 weeks after transfection, and lines of chronically infected CEMx174 cells were established for all viruses. BK/CP-env, which contained all of the CP-MAC *env* mutations, was produced by coculturing transfected RD cells with Sup-T1 cells (37).

To evaluate the effect of the Y723C mutation in different cell types, viral supernatants from the infected CEMx174 cells were passaged onto HUT-78 or Sup-T1 cells, and chronically infected lines were established. Cells in these cultures were all productively infected, as determined by p27^{gag} expression on permeabilized cells by using immunofluorescence microscopy (not shown). Although Sup-T1 cells could be infected by BK/NC-723Y or BK/NC-723C, repeated attempts to infect this cell type with BK/723C were unsuccessful (see below). Sequence analysis of uncloned DNA amplified by PCR confirmed the presence of the expected mutations in all infected cell lines and failed to show any additional mutations in either SU or TM (not shown).

Host range of TM tail mutants. As described previously, mutations in the CP-MAC *env* gene were shown to alter the tropism of this virus for T-cell lines when compared with NC-MAC or BK28. Although CP-MAC was highly infectious for Sup-T1, it was unable to infect either HUT-78 or CEMx174, cell lines that are highly susceptible to NC-MAC and BK28 infection (37). To determine if the Y723C mutation affected tropism, supernatants containing viruses shown in Fig. 1 were passaged onto a variety of T-cell lines, and the cultures were monitored for p27^{gag} expression. As shown in Table 1, BK/723C exhibited a host range similar to that of BK28, although this virus was not able to infect Sup-T1 cells. Similarly, no changes were observed in tropism between BK/NC-723C or BK/NC-723Y, and, as described for NC-MAC, both viruses were able to infect Sup-T1 as well as CEMx174 and HUT-78 cells. BK/CP-env infected Sup-T1 cells with markedly accelerated kinetics compared with BK/NC-723C or BK/NC-723Y and was unable to productively infect either CEMx174 or HUT-78 cells (37). None of the viruses were able to infect CEM cells, as previously reported for both BK28 and CP-MAC (28, 37). Thus, the Y723C mutation by itself did not alter viral tropism, indicating that this difference between NC- and CP-MAC was determined by changes in the SU and/or TM ectodomains.

Analysis of surface envelope glycoprotein expression on infected cells. Envelope glycoprotein expression on the surface of chronically infected cells was quantitated by FACS analysis using MAbs to SIVmac TM (43.1) and SU (101.1) and serum from an SIV-infected rhesus macaque (anti-SIV). Mean channel fluorescence intensity values for infected and uninfected CEMx174, HUT-78, and Sup-T1 cells are shown in Fig. 3. On all cell types, the Y723C mutation markedly increased the levels of surface envelope glycoproteins. CEMx174 cells infected by BK/723C exhibited approximately 25-, 4-, and 10-fold

TABLE 1. Host ranges of SIV envelope mutants among CD4$^+$ T-cell lines[a]

Virus	% of cells positive for viral antigen at day 30[b]		
	Sup-T1	HUT-78	CEMx174
BK28	+	++++	++++
NC-MAC	++	++++	++++
CP-MAC	++++	−	−
BK/723C	−	++++	++++
BK/NC-723Y	++	++++	++++
BK/NC-723C	++	++++	++++
BK/CP-env	++++	−	−

[a] Viruses shown in Fig. 1 were normalized for RT activity and used to inoculate the panel of CD4$^+$ cell lines shown. Infection was monitored weekly for 30 days by immunofluorescence assay on fixed cells, using an anti-p27^{gag} MAb. Cells used to produce the viral supernatants were HUT-78 (for BK28), CEMx174 (for BK/723C, BK/NC-723Y, and BK/NC-723C), and Sup-T1 (for BK/CP-env, NC-MAC, and CP-MAC).

[b] ++++, 100%; ++, 5 to 25%; +, <5%; −, 0%. Results for CEM were negative in all cases.

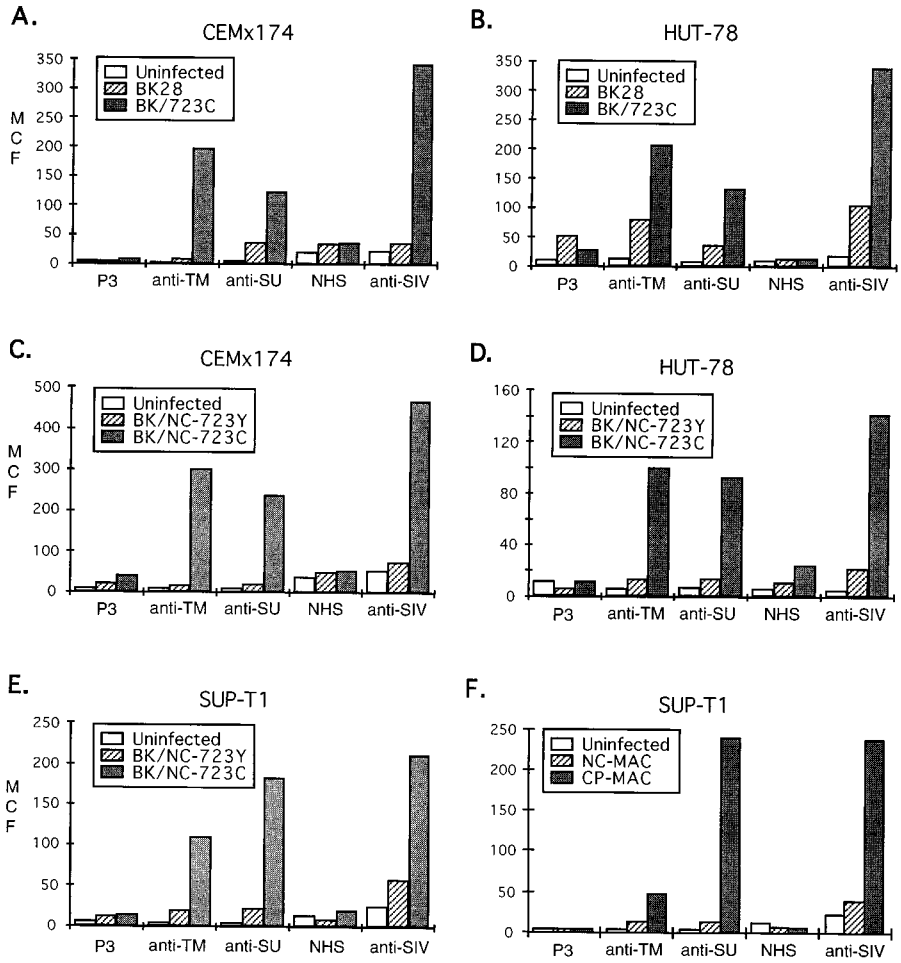

FIG. 3. FACS analysis of surface envelope glycoproteins of BK28-derived viruses in chronically infected cell lines. Cell lines infected by the viruses indicated were stained for FACS analysis with a MAb to SIVmac TM (43.1) or SU (101.1) or with serum from an SIV-infected macaque (anti-SIV). Control antibodies included the murine MAb P3X63 (P3) and normal human serum (NHS). The mean channel of fluorescence intensity (MCF) is shown as an indication of surface antigen expression. Panels A to E compare values obtained for cells infected by viruses whose *env* genes differed only by the presence of a Tyr or Cys at position 723 in the TM cytoplasmic tail; panel F shows values for a similar stain of NC-MAC- and CP-MAC-infected Sup-T1 cells. The data shown are from one representative experiment of three performed with each set of viruses. RT activity, as an indication of the relative amount of virus production, is shown for each of the infected cell lines in Table 2. Cell populations analyzed were 100% infected, as determined by $p27^{gag}$ expression on permeabilized cells by using immunofluorescence microscopy.

increases in reactivity to 43.1, 101.1, and the anti-SIV serum, respectively, compared with CEMx174 cells infected by BK28 (Fig. 3A). Smaller but reproducible increases (three- to four-fold) were also seen for HUT-78 cells infected by these same viruses (Fig. 3B). A marked increase was also seen in CEMx174, HUT-78, and Sup-T1 cells infected by BK/NC-723C compared with BK/NC-723Y, in which the Y723C mutation increased surface levels of envelope glycoprotein from 6- to 17-fold (Fig. 3C to E). This striking effect was similar to that observed for the uncloned CP-MAC virus on infected Sup-T1 cells compared with NC-MAC-infected cells (Fig. 3F). Similar results were seen when cells were stained with a 10-fold increase in MAb concentration (not shown), indicating that these differences were not caused by conformational changes in SU or TM that affected antibody affinity. As shown in Table 2, this increase could also not be explained by a higher level of virus production for viruses with the Cys mutation, since levels of RT activity among viruses were comparable. In fact, for the infected cells compared in Fig. 3B to D, RT activity was actually higher for viruses with a Tyr-723. Therefore, among the CP-MAC *env* mutations, the Y723C mutation was clearly shown to be the principal determinant for the increased expression of envelope glycoproteins on infected cells, and this effect could be demonstrated on a variety of lymphoid cell lines.

Effects of Tyr mutations in the SIVmac239 cytoplasmic tail. BK28 and the BK28-derived viruses shown in Fig. 1 all contained a premature stop codon in their TM cytoplasmic tails (27, 34). To determine if a Tyr-to-Cys mutation would increase expression of surface envelope glycoproteins for a virus with a full-length TM protein, site-directed mutagenesis was performed to produce this mutation at the analogous position (amino acid 721) in the SIVmac239 molecular clone. A number of other amino acid changes, including nonconservative (Ala, Ile, or Ser) or conservative (Phe) substitutions, were introduced at this position. Mutations were introduced into a cloned 3′ half of the SIVmac239 genome and electroporated into CEMx174 cells with the corresponding SIVmac239 5′ genomic fragment (see Materials and Methods). Mutant viruses produced from these cultures were termed SIVmac239C, SIVmac239F, SIVmac239A, SIVmac239I, and SIVmac239S for viruses with Cys, Phe, Ala, Ile, and Ser substitutions, re-

TABLE 2. RT values for cell lines infected by viruses with Tyr mutations in the TM cytoplasmic tail[a]

Cell line	Virus	RT (cpm)
CEMx174	BK28	9,449
	BK/723C	21,034
	BK/NC-723Y	181,014
	BK/NC-723C	115,507
	SIVmac239	23,140
	SIVmac239 C	18,202
	SIVmac239 F	34,570
	SIVmac239 A	14,130
	SIVmac239 S	24,857
	SIVmac239 I	33,573
HUT-78	BK28	78,946
	BK/723C	26,430
	BK/NC-723Y	23,034
	BK/NC-723C	10,821
SUP-T1	BK/NC-723Y	494,992
	BK/NC-723C	691,328
	NC-MAC	457,177
	CP-MAC	85,889

[a] Viruses derived from BK28 (Fig. 1) and site-directed mutants of SIVmac239 with Cys (C), Phe (F), Ala (A), Ser (S), and Ile (I) substitutions were produced following electroporation of CEMx174 cells, and chronically infected lines were established as described in the text. RT levels of viral pellets are shown. Background RT levels for uninfected cells were <200 cpm.

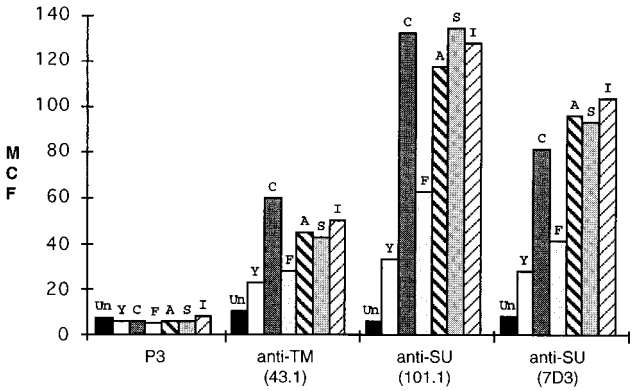

FIG. 4. FACS analysis of surface envelope glycoproteins on cells infected by SIVmac239 with and without mutations at Tyr-721. FACS analysis is shown for CEMx174 cells uninfected (Un) or chronically infected with wild-type SIVmac239 containing a Tyr at position 721 (Y) or SIVmac239-derived viruses with different mutations, including Cys (C), Phe (F), Ala (A), Ser (S), or Ile (I) substitutions, at this position. Staining was performed with anti-TM (43.1) and anti-SU (101.1 and 7D3) MAbs. RT levels of infected cell lines are shown in Table 2. MCF, mean channel fluorescence.

spectively. Wild-type SIVmac239 with a Tyr at position 721 was produced by the same protocol. Viral replication was detectable within 1 to 2 weeks, and chronically infected lines were established for all transfections. Cells in these cultures were 100% infected, as determined by cytoplasmic $p27^{gag}$ expression, and produced comparable levels of RT activity (Table 2). Sequence analysis of uncloned PCR-amplified DNA from all cultures confirmed that the mutations at position 721 had remained stable and that no additional changes in the TM cytoplasmic tail had occurred (not shown). Importantly, no premature stop codons were found in TM during the propogation of these viruses in CEMx174 cells.

FACS analysis of chronically infected CEMx174 cells was performed with anti-TM (43.1) and anti-SU (101.1 and 7D3) MAbs. As shown in Fig. 4, cells infected by SIVmac239C exhibited a two- to fourfold increase in surface reactivity for all MAbs. Similar results were observed with a third anti-SU MAb, 5B11, produced in our laboratory, that recognizes an epitope distinct from 7D3 and 101.1 (not shown). These results demonstrated that a Tyr-to-Cys mutation could elevate envelope glycoprotein expression on cells infected by SIVs with long as well as truncated TM cytoplasmic tails. Cells infected by SIVmac239A, SIVmac239I, and SIVmac239S exhibited a similar increase, indicating that the effect of this mutation resulted from the loss of a Tyr rather than the acquisition of a Cys. Interestingly, the Y721F mutation in SIVmac239F produced a significantly smaller effect. These results were highly reproducible. FACS analyses for five experiments are summarized in Fig. 5 and expressed as the ratios of fluorescence intensity values for viruses with a mutation at position 721 compared with SIVmac239. As shown, SIVmac239C, SIVmac239A, SIVmac239I, and SIVmac239S exhibited a twofold increase in anti-TM reactivity and approximately a three- to fourfold increase in anti-SU reactivity. In contrast, a Phe-721 substitution in SIVmac239F produced no change in TM and a <2-fold increase in SU. These differences could not be explained by different levels of virus production, since the amount of pelletable RT activity in these cultures differed by <2-fold (Table 2).

These results demonstrated that mutations of a Tyr six amino acids from the putative membrane spanning domain could increase the surface expression of envelope glycoproteins, whereas substitution of another aromatic amino acid produced a much smaller effect. In addition, because SIVmac239 mutants did not contain any change in the second exon of *rev*, these findings also showed that the P65Q rev mutation present in CP-MAC and the other BK28-derived viruses shown in Fig. 1 was not required for this effect.

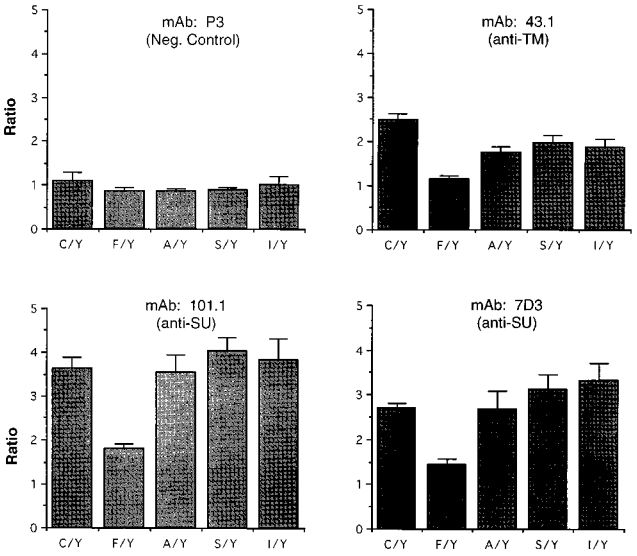

FIG. 5. Summary of the effects of Tyr-721 mutations in SIVmac239 on surface levels of envelope glycoproteins. Shown are the ratios of FACS fluorescence intensity values for SIVmac239 mutants with Cys (C), Phe (F), Ala (A), Ser (S), and Ile (I) mutations relative to values for SIVmac239, containing a Tyr (Y) at position 721, for each of the MAbs indicated (i.e., C/Y denotes the ratio of the value for SIVmac239C over that for SIVmac239). For each antibody, the average of ratios from five experiments is shown. Error bars indicate standard error of the mean.

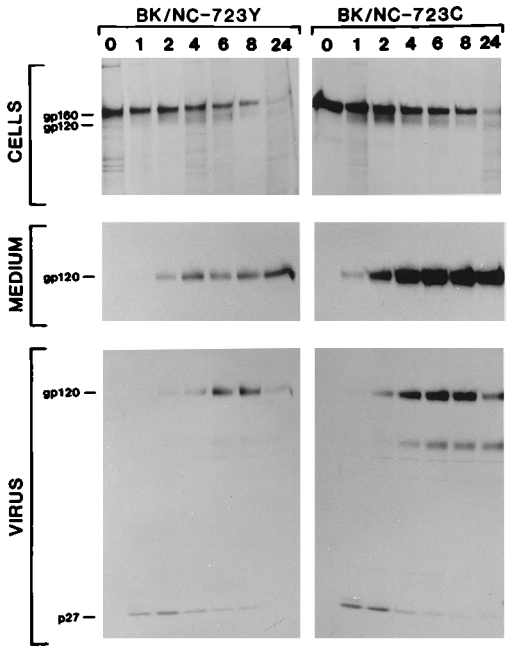

FIG. 6. Pulse-chase analysis of Sup-T1 cells infected by BK/NC-723Y and BK/NC-723C viruses. Sup-T1 cells chronically infected by BK/NC-723Y or BK/NC-723C virus were metabolically labeled for 15 min with [^{35}S]cysteine and methionine and chased for 0, 1, 2, 4, 6, 8, or 24 h. Viral proteins were immunoprecipitated from cell lysates, viral pellets, and the medium with an antiserum from an infected rhesus macaque and analyzed by SDS-PAGE and fluorography. Envelope glycoproteins (gp160 and gp120) and p27^{gag} are indicated. Exposure times for autoradiographs shown were 1 day for cell lysates, 4 days for medium, and 7 days for viral fractions.

Pulse-chase analysis of envelope glycoprotein processing. Pulse-chase analysis was performed to determine if the effects of the Y723C mutation were associated with differences in envelope glycoprotein processing or in the amount of SU shed from cells and/or virions. Sup-T1 cells chronically infected with BK/NC-723Y or BK/NC-723C were pulse-labeled for 15 min, and samples were harvested at various time points for analysis of cell lysates, viral pellets, and free viral proteins in the medium (37). For three different experiments, no consistent differences were seen in total amounts of envelope glycoproteins labeled (not shown). As shown in Fig. 6, no significant differences were observed in the kinetics or the efficiency of cleavage of the gp160 envelope precursor molecule to gp120. For both viruses, free gp120 was first detectable in the medium at approximately 1 to 2 h, after which point an increase was seen for BK/NC-723C to a level approximately fivefold greater than for BK/NC-723Y. This increase is consistent with the higher amount of envelope glycoproteins present on the surface of BK/NC-723C-infected cells. In viral pellets, comparable amounts of p27^{gag} were found at each of the time points, indicating that the number of viral particles produced was similar for each of the viruses. Densitometric analysis of the amount of gp120 relative to p27^{gag} in viral pellets revealed a slight increase (<2-fold) for BK/NC-723C, similar to that seen previously in a pulse-chase analysis of NC-MAC- and CP-MAC-infected cells (37). However, in contrast to the striking effect of this mutation on envelope glycoprotein levels on the cell surface, the quantitative effect on SU in virions was relatively minor. Thus, the increased surface expression of envelope glycoproteins for viruses with a Tyr mutation could not be explained by differences in efficiency or kinetics of envelope processing or by differences in the amount of gp120 shed from cells or virions.

Evaluation of cell surface envelope glycoproteins by immunoelectron microscopy. To evaluate further the effects of the Y723C mutation on cell surface envelope glycoproteins, immunoelectron microscopy was performed. HUT-78 cells infected by BK28 or BK/723C and Sup-T1 cells infected by BK/NC-723Y or BK/NC-723C were stained with an anti-SU MAb (101.1) and processed for immunoelectron microscopy using horseradish peroxidase-conjugated anti-mouse IgG (see Materials and Methods). Corresponding stains of Sup-T1 cells infected by NC-MAC or CP-MAC were also performed. For both HUT-78 cells infected by BK28 (Fig. 7a) and Sup-T1 cells infected by either BK/NC-723Y (Fig. 7c) or NC-MAC (Fig. 7e), SU was sparsely distributed on the cell surface and, when present, was localized in patches and in areas adjacent to extracellular aggregates of viral particles. Particularly on Sup-T1 cells, the majority of sections showed no detectable surface staining despite the fact that all cells exhibited cytoplasmic p27^{gag} by immunofluorescence microscopy (see above). In contrast, cells infected by viruses with a Y723C mutation, including HUT-78 cells infected by BK/723C (Fig. 7b), Sup-T1 cells infected by BK/NC-723C (Fig. 7d), and Sup-T1 infected by CP-MAC (Fig. 7f), all exhibited intense labeling over the entire plasma membrane. Remarkably, unlabeled areas of the plasma membrane could not be detected on these cells. Identical results were obtained when comparisons were made of CEMx174 cells infected by BK28 or BK/723C and CEMx174 cells infected by BK/NC-723Y or BK/NC-723C (not shown) or when cells were stained with the anti-TM MAb 43.1 (not shown). Similar results were also obtained for cells that were fixed in 2% paraformaldehyde prior to staining, indicating that these surface patterns were not affected by antibody binding (not shown). Therefore, in addition to producing an increase in the amount of envelope glycoproteins on the cell surface, the Y723C mutation also produced a striking redistribution of these molecules on the plasma membrane in a pattern identical to that observed for CP-MAC.

DISCUSSION

The envelope glycoproteins of membrane-containing viruses are essential to the viral life cycle. Although many aspects of these activities involve the extracellular portions of these proteins, the cytoplasmic domains also play important roles. For HIV-1, HIV-2, and SIV, the TM cytoplasmic tail can modulate a number of biological properties, including tropism (9, 31, 34, 78), infection kinetics (9, 15, 31, 38, 78), and cytopathic effects (1, 15, 19, 38, 46, 51, 59, 63, 66, 80). However, the mechanisms that underlie these effects are unclear, and little is known about the specific functional domains involved. The carboxy-terminal portion of TM has been suggested to form an amphipathic alpha helix that may interact with cellular membranes, bind calmodulin (45, 68), and mediate cytolysis (44). A region near the C terminus has also been implicated in directing viral budding to the basolateral surface in polarized cells (39). However, for HIV and SIV, mutational analyses have largely been limited to introducing premature termination codons that produce TM molecules with truncated or absent cytoplasmic tails (9, 12, 19, 31, 34, 78, 80). Although in some studies these mutations have affected the expression of envelope glycoproteins on the cell surface (75), the conformation of the TM ectodomain (67), and the incorporation of glycoproteins into virions (12, 16, 31, 78, 80), no clear functional domains within the TM cytoplasmic tail that mediate these effects have been defined.

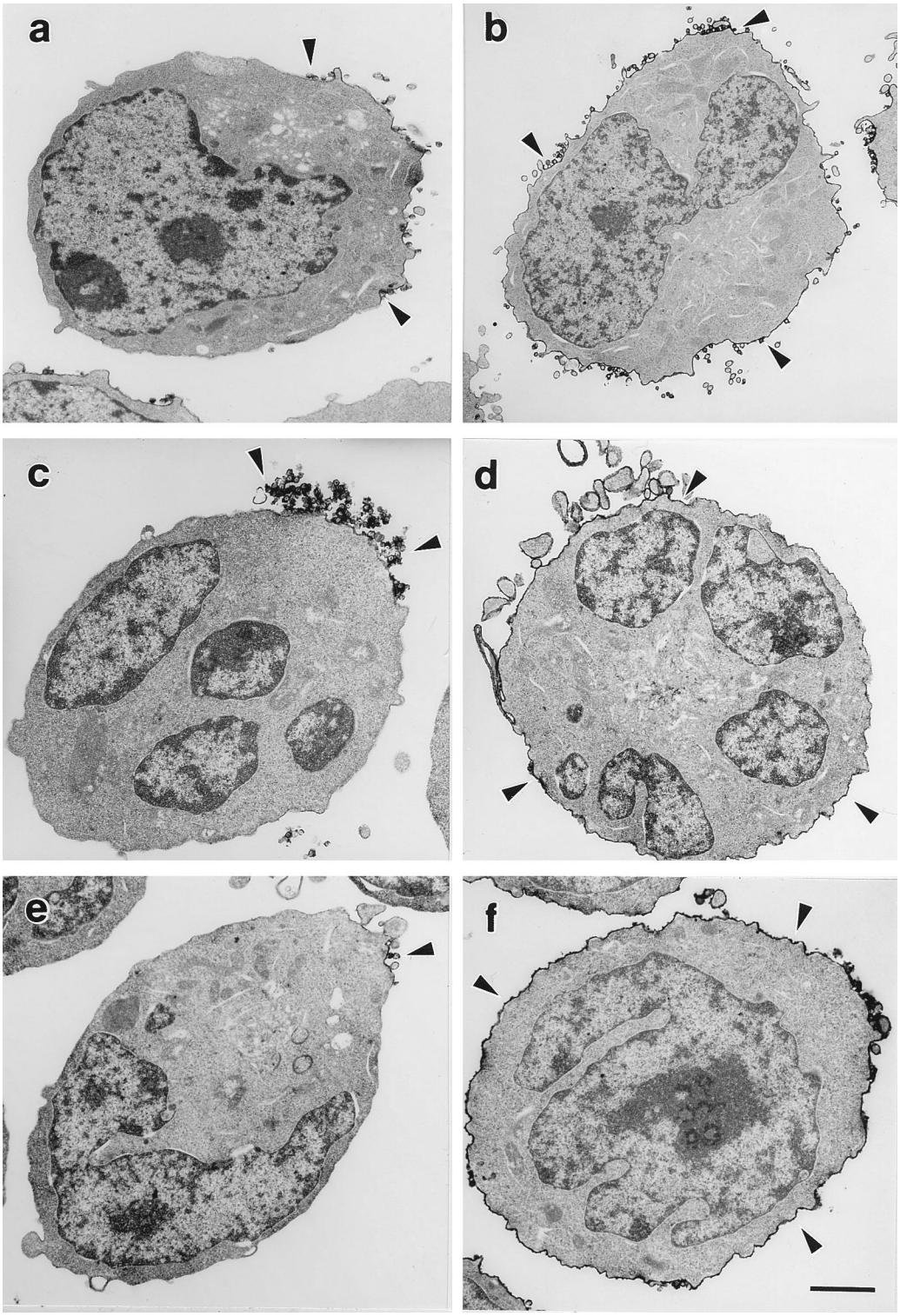

FIG. 7. Immunoelectron microscopy of SIV-infected cells. Chronically infected cells were labeled with an anti-SU MAb (101.1) and stained for immunoelectron microscopy with horseradish peroxidase-conjugated anti-mouse IgG. (a, c, and e) Viruses containing a Tyr at position 723. (a) BK28 in HUT-78 cells; (c) BK/NC-723Y in Sup-T1 cells; (e) NC-MAC in Sup-T1 cells. Staining is present only in limited regions of the cell surface (arrowheads). Note the cluster of extracellular viral particles in panel c that are darkly stained. (b, d, and f) Viruses containing a Cys at position 723. (b) BK/723C in HUT-78 cells; (d) BK/NC-723C in Sup-T1 cells; (f) CP-MAC in Sup-T1 cells. Staining is detectable over the entire plasma membrane (arrowheads). Bar = 2 μm.

In this report, we demonstrate that a single Tyr-to-Cys change in the TM cytoplasmic tail of SIVmac, six amino acids from the predicted membrane spanning domain, increases the level of envelope glycoproteins on the cell surface. This effect was shown to occur for viruses grown in three different cell lines and for SIVs with either truncated or full-length cytoplasmic tails. A similar effect was observed when other nonconservative amino acid changes, including Ile, Ala, and Ser substitutions, were introduced at this position in the SIVmac239 molecular clone. However, only a minimal increase was seen following a Phe substitution, suggesting that the reduced surface expression of envelope glycoproteins for Tyr-containing envelopes is mediated by a structural determinant that is dependent on the presence of an aromatic amino acid at this position. The effect of this mutation was clearly shown to increase the amount of envelope protein on the plasma membrane rather than to induce a conformational change in the TM or SU ectodomain, since similar results were found with several monospecific and polyspecific antibodies.

The Y723C mutation had initially been recognized as 1 of 11 mutations in the CP-MAC *env* gene that produced several phenotypic differences compared with BK28 or the closely related NC-MAC. In addition to a high level of surface envelope glycoproteins expressed on cells, CP-MAC exhibited (i) an altered host range that largely restricted its infectivity to Sup-T1 cells, (ii) a highly stable association of SU and TM, and (iii) a more rapid electrophoretic mobility of TM on SDS-PAGE (37). While the present study indicates that the Y723C mutation is sufficient to account for CP-MAC's increased expression of surface envelope molecules, it is apparent that other changes in SU and/or TM are required for the full phenotype. Although the E84K, R112K, R120K, and G385R mutations in SU that were used to construct BK/NC-723Y and BK/NC-723C were able to facilitate infection of Sup-T1 cells, the kinetics of this infection were markedly delayed compared with the kinetics of BK/CP-env or CP-MAC (37). Therefore, mutations in addition to Y723C are apparently required to confer the rapid infection kinetics and restricted tropism of CP-MAC for this cell type. We have also shown that the Y723C mutation in BK/NC-723C does not increase the stability of the SU-TM interaction, nor does it affect the migration of TM on SDS-PAGE (61a). Studies are now in progress to identify the mutations that are responsible for these other biological and biochemical properties of CP-MAC.

It is of interest that the increased surface expression of envelope glycoproteins caused by a Tyr mutation was generally greater for SIVs with truncated rather than long cytoplasmic tails. With the exception of HUT-78 cells infected by BK/723C, in which a 3- to 4-fold increase in SU and TM reactivity was seen relative to BK28, the Y723C mutation produced increases ranging from 5- to 25-fold (Fig. 3). In contrast, Cys, Ala, Ile, and Ser mutations in SIVmac239 produced increases from twofold for the anti-TM antibody to three- to fourfold for the anti-SU antibody. Truncations of the TM cytoplasmic domain of SIV have been reported to increase the expression of envelope glycoprotein on the cell surface and/or virions (31, 80). Therefore, it is possible that determinants distal to the premature stop codon in SIV can modulate the effects of a Tyr-721 mutation in SIVmac239. Nonetheless, these findings clearly indicate that for viruses with long or prematurely truncated cytoplasmic tails, the expression of surface envelope glycoproteins can be altered by a single amino acid change in the cytoplasmic tail.

The mechanism by which a Tyr mutation in the TM cytoplasmic domain increases surface glycoprotein expression on infected cells is unclear. It appears unlikely that these effects are the result of differences in envelope glycoprotein processing. Pulse-chase analyses indicated that the synthesis and proteolytic cleavage of envelope precursor molecules were indistinguishable in BK/NC-723Y- and BK/NC-723C-infected cells and that labeled virus appeared in the medium with similar kinetics (Fig. 6). Identical results have been found in a similar analysis of NC-MAC- and CP-MAC-infected cells (37). In addition, the increase in cell surface glycoprotein expression could not be explained by increased shedding of BK/NC-723Y SU molecules from infected cells. Despite the fact that comparable amounts of virus were being produced (Fig. 6 and Table 2), more free gp120 was actually found in the medium for BK/NC-723C-infected cells, consistent with the increased amount of surface SU present on these cells. Finally, it is apparent that these differences in cell surface expression did not result from alterations in the incorporation of envelope molecules into virions, as has been described for mutations in the Sindbis virus E2 cytoplasmic domain (20), since viral pellets exhibited comparable amounts of gp120 relative to $p27^{gag}$. Interestingly, a Tyr in the cytoplasmic tail of the Semliki Forest virus E2 transmembrane glycoprotein has recently been implicated in the incorporation of spike glycoproteins into budding virions (79). Although our findings do not rule out the possibility that a Y723C mutation alters the efficiency of glycoprotein incorporation into virions, it is clear from our study and others (31) that this residue is not absolutely required in SIV.

Taken together, these findings suggest that the increased surface expression of envelope glycoproteins for SIVs with a Tyr-723 mutation is due to cellular processes other than those involved in biosynthesis or viral assembly. Multiple factors are known to influence the transport to and incorporation of proteins into the plasma membrane. For example, there is increasing evidence that specific interactions occur between the cytoplasmic domains of viral envelope molecules and the cytoskeleton (40, 62). Interestingly, our immunoelectron microscopy studies demonstrated that a Y723C mutation in viruses derived from BK28 caused a striking redistribution of envelope glycoproteins on the cell surface. For cells infected by viruses with a Tyr at this position, envelope glycoprotein was present in localized patches, leaving the majority of the cell surface unlabeled (Fig. 7). In contrast, envelope molecules containing the Y723C mutation were evenly distributed over the entire plasma membrane. It is possible that this redistribution reflects the loss of a structure that normally mediates an interaction of the TM tail with the cytoskeleton and directs envelope molecules to particular regions on the plasma membrane.

For a number of cellular membrane proteins, structural motifs in their cytoplasmic domains that frequently involve a Tyr or another aromatic amino acid have been shown to play a role in modulating surface expression (70, 71). These motifs appear to function as signals for constitutive endocytosis or sorting events that occur in the endocytic and/or exocytic pathways (42, 70, 71). Molecular modeling of the SIV TM cytoplasmic tail has predicted that amino acids around Tyr-723 have a high probability of forming a tight turn (61a). This turn resembles a structural motif that directs the clathrin-dependent endocytosis of a number of cellular molecules, including the low-density lipoprotein, transferrin, and polymeric Ig receptors and lysosomal acid phosphatase (71). For the low-density lipoprotein receptor, a Tyr-to-Cys mutation at position 807 in the cytoplasmic tail disrupts this motif and increases expression of the receptor on the cell surface by reducing its rate of constitutive internalization. Endocytosis of viral glycoproteins has been shown to occur for simian virus 5, Rous sarcoma virus, vesicular stomatitis virus, and Semliki Forest virus (25, 30, 49, 72),

though only in the case of the vesicular stomatis virus G protein has a Tyr-containing motif been implicated in endocytosis (70). Efforts are in progress in our laboratories to determine if Tyr-723 contributes to an endocytosis motif for the SIV TM molecule and, if so, whether differences in endocytosis rates could explain the effects of mutations at this position on surface envelope glycoprotein levels.

It is of interest that the Tyr that we have implicated as playing a role in the surface expression of SIVmac envelope molecules is highly conserved for all HIV-1, HIV-2, and SIV isolates (Fig. 2). This finding suggests that a Tyr present six to seven amino acids from the membrane spanning domain plays an important role in the viral life cycle. As we have shown, viruses that contain a variety of mutations at this position are clearly replication competent, although further studies will be needed to evaluate more subtle effects on their infection kinetics. Nonetheless, it is likely that a mutation that can upregulate the expression of envelope glycoproteins on the cell surface would be strongly selected against in vivo, since infected cells would be more susceptible to humorally mediated immune responses. The ability to produce viral mutants with increased surface expression of envelope molecules could be important for the design of viruses with altered immunogenic and/or pathogenic properties in vivo. Preliminary results have indicated that the BK/723C and SIVmac239C mutants described in this report are able to replicate in rhesus macaque peripheral blood lymphocytes (18a). Future studies should be able to address the in vivo consequences of this mutation on viral pathogenesis as well as the host immune response. Finally, it will also be of interest to determine if a mutation at the analogous position in the HIV-1 TM cytoplasmic tail has a similar effect.

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C.C.L. and M.M.S. contributed equally to this study.

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