An Internalization Signal in the Simian Immunodeficiency Virus Transmembrane Protein Cytoplasmic Domain Modulates Expression of Envelope Glycoproteins on the Cell Surface

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Abstract. A Tyr to Cys mutation at amino acid position 723 in the cytoplasmic domain of the simian immunodeficiency virus (SIV) transmembrane (TM) molecule has been shown to increase expression of envelope glycoproteins on the surface of infected cells. Here we show that Tyr-723 contributes to a sorting signal that directs the rapid endocytosis of viral glycoproteins from the plasma membrane via coated pits. On cells infected by SIVs with a Tyr at position 723, envelope glycoproteins were transiently expressed on the cell surface and then rapidly endocytosed. Similar findings were noted for envelope molecules expressed in the absence of other viral proteins. Immunoelectron microscopy demonstrated that these molecules were localized in patches on the cell surface and were frequently associated with coated pits. In contrast, envelope glycoproteins containing a Y723C mutation were diffusely distributed over the entire plasma membrane. To determine if an internalization signal was present in the SIV TM, chimeric molecules were constructed that contained the CD4 external and membrane spanning domains and a SIV TM cytoplasmic tail with a Tyr or other amino acids at SIV position 723. In Hela cells stably expressing these molecules, chimeras with a Tyr-723 were rapidly endocytosed, while chimeras containing other amino acids at position 723, including a Phe, were internalized at rates only slightly faster than a CD4 molecule that lacked a cytoplasmic domain. In addition, the biological effects of the internalization signal were evaluated on infectious viruses. A mutation that disrupted the signal and as a result, increased the level of viral envelope glycoprotein on infected cells, was associated with accelerated infection kinetics and increased cell fusion during viral replication. These results demonstrate that a Tyr-dependent motif in the SIV TM cytoplasmic domain can function as an internalization signal that can modulate expression of the viral envelope molecules on the cell surface and affect the biological properties of infectious viruses. The conservation of an analogous Tyr in all human and simian immunodeficiency viruses suggests that this signal may be present in other primate lentiviruses and could be important in the pathogenesis of these viruses in vivo.

THE glycoproteins of enveloped viruses play an important role in the viral life cycle. They perform critical functions during viral entry and assembly and serve as principle targets of humoral and cellular immune responses (Hunter and Swanstrom, 1990). These proteins are synthesized in the rough endoplasmic reticulum of the infected cell, processed during trafficking along the exocytic pathway, and selectively incorporated into budding virions. During viral assembly, interactions between the cytoplasmic domains of these envelope glycoproteins and internal proteins of the virion likely influence the site of viral budding (Rhee and Hunter, 1990; Owens et al., 1991; Lodge et al., 1994), the incorporation of envelope proteins into virions (Whitt et al., 1989; Gaedigk-Nitschko and Schlesinger, 1991; Lyles et al., 1992; Bilsel et al., 1993; Gray and Roth, 1993; Naim and Roth, 1993; Zhao et al., 1994), and the infectivity of the viral particle (Granowitz et al., 1991; Dubay et al., 1992; Johnston et al., 1993; Yu et al., 1992, 1993; Owens and Rose, 1993; Brody

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et al., 1994; Zhao et al., 1994). There is also increasing evidence that envelope glycoproteins interact with cellular proteins that affect their transport to, mobility and expression on the cell surface (Doyle et al., 1985; Lazarovits et al., 1990; Lydy and Compans, 1993). However, the specific cellular factors and viral determinants involved in these interactions are poorly understood, as is their role in viral pathogenesis.

For the human and simian lentiviruses, human immunodeficiency virus (HIV)[1]-1, HIV-2, and simian immunodeficiency virus (SIV), the surface (SU) and transmembrane (TM) glycoproteins are transported as complex oligomers to the cell surface where they are incorporated into budding virions (Allan et al., 1985; Kowalski et al., 1987; Willey et al., 1988; Chakrabarti et al., 1990; Earl et al., 1990; Stein and Engleman, 1990; Doms et al., 1993). Although the TM cytoplasmic domains of these glycoproteins are not required for processing or transport to the cell surface (Dubay et al., 1992; Gabuzda et al., 1992; Johnston et al., 1993), these domains can affect the site of budding in polarized cells (Owens et al., 1991; Lodge et al., 1994), the incorporation of envelope glycoproteins into virions (Dubay et al., 1992; Yu et al., 1993; Brody et al., 1994), the conformation of the envelope glycoprotein ectodomain (Spies et al., 1994), the ability of the envelope to induce cell fusion (Mulligan et al., 1992; Ritter et al., 1993; Spies and Compans, 1994), and the infectivity of the viral particle (Dubay et al., 1992; Gabuzda et al., 1992; Johnston et al., 1993; Salzwedel et al., 1993; Yu et al., 1993). Interestingly, for SIV the TM cytoplasmic domain has also been implicated in modulating the expression of envelope molecules on the cell surface, since SIVs with prematurely truncated or absent cytoplasmic tails express increased levels of envelope proteins on the plasma membrane (Johnston et al., 1993; Zingler and Littman, 1993). These findings could be relevant to biological changes associated with premature termination codons that spontaneously occur when SIVs are propagated in human cells (Kodama et al., 1989; Johnston et al., 1993; Ritter et al., 1993). Given the importance of cell-associated viral glycoproteins in mediating cytopathic effects and as targets of humoral and cellular immune responses, it is likely that the extent to which envelope glycoproteins are expressed on the plasma membrane could influence the pathogenesis of these viruses in vivo. Nonetheless, viral and cellular mechanisms that modulate the expression of envelope molecules on the cell surface remain to be determined.

We have previously described a variant of SIVmac derived from the BK28 molecular clone, termed CP-MAC, that exhibits a remarkably high level of surface envelope glycoprotein on infected cells (LaBranche et al., 1994). The determinant for this effect was shown to be a single amino acid mutation of a Tyr to a Cys at position 723 in the TM cytoplasmic domain (LaBranche et al., 1995). However, no differences were found in the kinetics or efficiency of envelope glycoprotein processing or in the transport of these molecules to the cell surface. We now show that Tyr-723 contributes to a signal that directs the rapid and efficient endocytosis of envelope molecules from the cell surface via clathrin-coated pits. This effect could be shown on virus-infected cells as well as on cells expressing envelope glycoproteins in the absence of other viral molecules. The SIV TM cytoplasmic tail could mediate efficient endocytosis of a heterologous molecule, CD4, when it was used to replace the CD4 cytoplasmic domain, and this activity was shown to depend on Tyr-723. In addition, by modulating the level of viral glycoproteins on infected cells, this signal was shown to effect the infectivity as well as the cytopathic properties of the virus during replication in vitro. Together these results indicate that SIV has adopted a cellular membrane protein sorting signal to regulate the expression of its envelope proteins on the surface of infected cells, and suggest that this signal may contribute to viral pathogenesis in vivo.

Materials and Methods

Cell Lines

The $CD4^+$ T-cell lines Sup-T1, HUT-78, and CEMx174 have been described previously (LaBranche et al., 1994). HeLa cells were obtained from M. Malim (University of Pennsylvania, Philadelphia, PA). BHK-21 cells and human epidermal carcinoma (HEp2) cells were obtained from the European Molecular Biology Laboratory (Heidelberg, Germany) and C.R. Hopkins (MRC Laboratory of Molecular Cell Biology, London, UK), respectively. PA317, a murine fibroblast packaging cell line was obtained from the American Type Culture Collection (Rockville, MD) (CRL 9078). Human T lymphoid cell lines were maintained in RPMI supplemented with 10% fetal calf serum. BHK-21 cells were maintained in Glasgow's modified Eagle's medium supplemented with 10% tryptose phosphate broth, 5% FCS, penicillin, and streptomycin (G-MEM). All other lines were maintained in DME supplemented with 10% FCS.

Viruses

The infectious molecular clone of SIVmac, BK28, was obtained from James Mullins (University of Washington, Seattle, WA) through the National Institutes of Health AIDS Research and Reference Reagent Program (Kornfeld et al., 1987). The laboratory-derived variants of BK28, designated NC-MAC and CP-MAC, have been described (LaBranche et al., 1994, 1995). Relative to BK28, CP-MAC contained five amino acid substitutions in SU (E84K, R112K, R120K, M327R, T475I), five in the TM ectodomain (L559S, V573L, K757T, T588I, E643K), and a single amino acid mutation (Y723C) in the TM cytoplasmic tail. NC-MAC contained four amino acid mutations in SU (E84K, R112K, R120K, G385R) and no mutations in TM. BK/NC-723Y was constructed using a 3.5-kb SacI fragment (nucleotides [nts] 5735–9212) containing the entire *env* gene of NC-MAC and a molecular clone of BK28 from which this corresponding SacI fragment had been deleted. BK/NC-723C was constructed by inserting an NheI-PstI (nts 8724-8947) fragment from the CP-MAC *env* gene into the BK/NC-723Y *env*. The *env* of BK/NC-723C was identical to that of BK/NC-723Y except for the presence of the Y723C mutation. BK/723C was produced by inserting this same NheI-PstI fragment from CP-MAC into the BK28 *env* gene.

Monoclonal Antibodies

Murine mAbs used for detection of SIV proteins included 43.1 and 3A8, reactive with SIVmac TM and $p27^{gag}$, respectively (provided by J. McClure, Bristol-Myers Squibb, Seattle, WA) and 101.1, reactive with SIVmac SU (provided by C. Thiriart, SmithKline Biologicals, Rixensart, Belgium). The anti-CD4 mAb Q4120 was obtained from Q. Sattentau, Centre d'Immunology de Marseille-Luminy, France through the Medical Research Council AIDS Directed Programme Reagents Programme. The anti-CD4 mAb, No. 21 was developed in our laboratory from BALB/c mice immunized with Snp-T1 and is an IgG_1 that has been shown to react

1. *Abbreviations used in this paper*: HEp, human epidermal cells; HIV, human immunodeficiency virus; LDL, low density lipoprotein; LDLr, low density lipoprotein receptor; MA, matrix; nt, nucleotide; RT, reverse transcriptase; SFV, Semliki Forest virus; SIV, simian immunodeficiency virus; SU, surface; TM, transmembrane.

with CD4 by Western blot and immunoprecipitation assays and which completely inhibits CP-MAC–induced fusion of Sup-T1 cells. Fab fragments of mAb 101.1 were prepared using the ImmunoPure Fab preparation Kit (Pierce Chemical Co., Rockford, IL). Negative control mAbs included P3x63 (IgG_{2b}) or nonreactive IgG mAbs produced in our laboratory.

Expression of *env* Constructs

Expression of the viral envelope proteins, was performed using the Semliki Forest virus (SFV) expression system (Liljeström and Garoff, 1991). To express the CP-MAC *env*, 3.5-kb SacI fragments containing the entire *env* gene of CP-MAC with a BamHI site close to the 3′ end were ligated and cut with BamHI to generate a fragment containing one complete copy of the *env* gene with BamHI ends. This fragment was ligated into the BamHI site of the eukaryotic expression vector pGW_1, provided by S. Moss (MRC-LMCB, London, UK) (Moss et al., 1990). A novel BamHI site was engineered 17–bases upstream of the *env* start codon to remove most of the 5′ untranslated sequence using an (Muta-Gene M13; Bio-Rad Laboratories, Cambridge, MA) in vitro mutagenesis kit and the mutagenesis primer 3′-CATACTTACTTGTTGGATCCAGAAGATGTATTA-5′. The mutant *env* gene was cut from pGW1 with BamHI and cloned into $pSFV_1$ to generate the $pSFV_1$:CP-MAC/723C expression vector. A similar strategy was used to generate a $pSFV_1$ vector expressing the BK28 *env* gene, designated $pSFV_1$:BK28/723Y. The $pSFV_1$:CP-MAC/723Y vector was constructed by inserting a 376-bp NheI-BglII fragment from BK28 into the CP-MAC *env* cloned into pGW_1. The *env* gene was then cut with BamHI and cloned into $pSFV_1$.

Recombinant SFV viruses were generated essentially as described (Liljeström and Garoff, 1991). $pSFV_1$ and $pSFV_{helper}$ constructs were linearized with NruI and SpeI, respectively, and transcribed in vitro using SP6 polymerase (Pharmacia Fine Chemicals). RNA was co-electroporated into BHK-21 cells and the transfected cells were plated in G-MEM and grown at 37°C/5% CO_2. After 36 h, the virus-containing medium was collected, clarified by centrifugation (3,000 rpm, 10 min), aliquoted, and stored at −70°C. Viral stocks were titered on BHK-21 or HEp2 cells.

For analysis of the *env* proteins, BHK-21 or HEp2 cells were grown on glass coverslips to approximately 50% confluence in 16-mm-diam wells. Virus stocks were diluted in binding medium, BM (RPMI-1640 without bicarbonate, 10 mM Hepes, 0.2% BSA, pH 6.8). Cells were washed twice and following addition of virus (200 μl/well) were incubated for 1.5 h at 37°C. 1 ml G-MEM or DME was added per well of BHK-21 or HEp2 cells, respectively, and the cells incubated at 37°C for 16 h. Cells were then washed with cold PBS, fixed with 3% paraformaldehyde in PBS for 10 min at room temperature, and quenched with 50 mM NH_4Cl in PBS and processed for immunofluorescence microscopy as described below.

Construction and Expression of CD4/SIV-TM Chimeric Proteins

Chimeric proteins containing the CD4 ecto- and membrane spanning domains and SIV TM cytoplasmic tails were constructed by introducing unique HindIII sites into CD4 (at either nt 1336 or 1351) and into the BK28 *env* gene (at nt 8731). Mutagenesis was performed by PCR on the pT4b molecular clone of CD4 (provided by D. Littman, New York University, New York), cloned into the EcoR1 site of sp65 (Promega Biotec, Madison, WI). Primer pairs used to generate CD4 chimeras in which the cytoplasmic domain was entirely replaced by SIV sequences were, forward primer, 5′-GGAAGTGTCTGTAAAACGGG-3′ (nts 852–871) and reverse primer, 5′-GCCGAAGCTTGACACAGAAG-3′ (nts 1345–1325). For CD4 chimeras containing four amino acids of the predicted CD4 cytoplasmic domain, the reverse primer 5′-GGCGAGCTTTCGGTGCCGGCACCTGAC-3′ (nts 1356–1333) was used (see Fig. 6). The resulting fragments were digested with BstE2 and HindIII and cloned into pT4b. SIV TM constructs were generated by PCR on BK28 with the forward primer, 5′-GCTAGCTAAGCTTAGGCAGG-3′ (nts 8724–8743) and the reverse primer, 5′-AATAGCGAATTCAAGAGGCGT-3′ (nts 8936–8916). In addition to creating a 5′ HindIII site, these primers created a 3′ EcoRI cloning site. TM fragments were also generated that incorporated Cys, Phe, Ala, Ile, or Ser mutations at Tyr-723. TM fragments were digested with HindIII and EcoRI and cloned into sp64 (Promega Biotec). The CD4-containing sp65 and SIV TM-containing sp64 plasmids were then digested with PvuI and HindIII and ligated to generate an EcoRI fragment encoding the CD4/SIV chimeric proteins. The CD4/LDLr chimera was generated using a similar strategy in which an LDLr molecular clone (provided by N. Landau, Aaron Diamond AIDS Research Center, New York) was amplified (forward primer, 5′-TGGAAGCTTTGGCGGCTTAAGAACATC-3′; reverse primer, 5′-GCAGAATTCGGCGAGGTCTCAGGAAGG-3′) to generate a 5′ HindIII junction site at the beginning of the cytoplasmic domain and a 3′ EcoRI cloning site after the native stop codon. This fragment was cloned into sp64 and ligated as above to generate the construct shown in Fig. 6. A CD4/tail-minus construct with a premature termination codon at Arg-400 of CD4 was generated from the CD4 cDNA cloned into pGW_1 by site directed mutagenesis using the BioRad Muta-Gene M13 in vitro mutagenesis kit and the mutagenesis primer, 3′-CACGGCCGTGACTTCCGCGGTTC-5′. All constructs were cloned into the EcoRI site of the Moloney murine leukemia virus expression vector pMV-6 (Kirschmeier et al., 1988).

To generate HeLa clones stably transfected with the chimeric molecules, the amphotropic packaging line, PA317, was transfected with 30 μg of plasmid DNA by the calcium phosphate method. After 24 h, the cells were washed and incubated in DME/10% FCS for 24 h. The virus-containing supernatant was then filtered (0.45 μm) and supplemented with polybrene (8 μg/ml; Sigma Chemical Co., St. Louis, MO) before infecting HeLa cells for 24 h. After infection, the HeLa cells were trypsinized and diluted into DME/10% FCS containing 400 μg/ml G418 for selection of resistant colonies. Clones were screened for expression of the chimeric protein by immunofluorescence microscopy and flow cytometry with an anti-CD4 mAb.

Immunofluorescence Microscopy

For detection of viral proteins on SIV-infected Sup-T1 cells, or on HeLa cells expressing chimeric proteins, cells were fixed with methanol and acetone (1:1) at room temperature for 10 min and stained with anti-SIV SU, TM, or $p27^{gag}$ mAbs or an anti-CD4 mAb followed by FITC-conjugated $F(ab')_2$ goat anti–mouse IgG (Biosource International or Pierce). For detection of *env* proteins expressed by $pSFV_1$, surface staining was determined by fixing cells in 3% paraformaldehyde prior to staining. For experiments in which access to intracellular antigens was required, cells were permeabilized by incubation in 0.1% Triton X-100. Stained coverslips or slides were mounted in Moviol and viewed using a Zeiss Axioskop fluorescence microscope.

Endocytosis Assays

Endocytosis of SIV envelope glycoproteins was evaluated using immunofluorescence microscopy. For cells chronically infected by SIVs, cells were incubated at 37°C with a 1/1,000 dilution of anti-SU mAb ascites (101.1), and at various times, aliquots of cells were chilled to 4°C and surface antibody removed with acid elution buffer (RPMI/0.2% BSA/10 mM MES, pH 2.5). Cells were then permeabilized with methanol/acetone (1:1) and stained with FITC-conjugated $F(ab')_2$ goat anti–mouse IgG. For assays performed on *env*-expressing BHK-21 or HEp2 cells, cells grown on glass coverslips were incubated for 14 h at 37°C with 10 pfu/cell of $pSFV_1$. Medium was then supplemented with a 1/1,000 final dilution of anti-SU (101.1) or anti-TM mAb (43.1) ascites for an additional 2 h. Cells were washed, fixed in 3% paraformaldehyde, and stained with a rhodamine-conjugated goat anti–mouse IgG (Pierce Chemical Co.), either before or after permeabilization with 0.1% Triton X-100. Where indicated surface antibody was removed before fixation with acid elution buffer.

A quantitative assay for endocytosis of CD4-based constructs was performed as described previously (Pelchen-Matthews et al., 1989). Briefly, HeLa cells stably expressing various CD4/SIV constructs were seeded at 30–50,000 per well in 24 × 16 mm^2 well plates and grown for 2 d (final density ~1–2 × 10^5 cells/well). For analysis the cells were cooled on ice, washed with BM and incubated with 0.3–0.4 nM ^{125}I-Q4120 for 2 h on ice. Unbound mAb was removed by washing with cold BM and the cells were warmed to 37°C for the indicated times to allow endocytosis to occur. Cells were chilled on ice and for each time point the total cell-associated and internalized ^{125}I-Q4120 determined by either harvesting the cells directly or following removal of the remaining cell surface mAb by washing in acid medium. ^{125}I-Q4120 was determined by γ counting. The proportion of internalized activity for each time point was determined by dividing the acid-resistant activity by the total cell-associated activity and endocytic rates were calculated by analysis of data from the first 5 min of warm-up.

Flow Cytometry

FACS® analysis of cell surface molecules was performed as previously described using a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA) (LaBranche et al., 1994). Sup-T1 cells chronically in-

fected by SIVs or HeLa cells stably expressing CD4 constructs were incubated in staining buffer (PBS supplemented with 0.1% BSA and 0.02% sodium azide) with saturating concentrations of mAbs (30 min for 4°C) followed by FITC-conjugated secondary antibody (30 min for 4°C). Cells were washed and fixed in 4% paraformaldehyde before analysis with Lysis-II software (Becton Dickinson & Co.).

Western Blotting and Immunoprecipitation

HeLa lines expressing CD4-based chimeric proteins were removed with cold EDTA and lysed at 5×10^6 cells/ml in lysis buffer (0.02 M Tris base, pH 8, 0.12 M NaCl, 0.2 mM EGTA, 0.2 mM NaFl, 0.2% DOC, 0.5% NP-40, 0.2 mM PMSF, 5 μg/ml aprotinin). Lysates were boiled with an equal volume of sample buffer (50 mM Tris, pH 6.8, 2% SDS, 30% glycerol) and run on an 8–16% Tris-glycine mini gel (Novex, San Diego, CA) under nonreducing conditions. After transfer to nitrocellulose, CD4 was detected with the anti-CD4 mAb No. 21 followed by HRP-conjugated goat anti–mouse IgG (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Blots were developed using enhanced chemoluminescence (ECL; Amersham Corp.) and the intensity of CD4 bands quantitated by densitometry. Immunoprecipitation of ^{35}S-cysteine and methionine-labeled viral proteins was performed and previously described (LaBranche et al., 1994).

Immunoelectron Microscopy

Immunoelectron microscopy of infected cells was performed as described previously (LaBranche et al., 1995). Cell suspensions in RPMI/10% FCS were incubated with anti-SU mAb (101.1) for 30 min at 37°C. Cells were washed twice in ice-cold PBS and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 60 min at room temperature. Fixed cells were washed twice in PBS and incubated with goat anti–mouse IgG-biotin followed by streptavidin-HRP. Cells were incubated in diaminobenzidine medium to develop the HRP reaction product, postfixed with osmium tetroxide, and embedded in epoxy resin. Thin sections were cut and stained with lead.

Viral Infectivity Assays

Recombinant SIVs containing the NC-MAC envelope gene with either a Tyr or Cys at position 723 were produced in CEMx174 cells, as described (LaBranche et al., 1994). For infectivity assays, viruses were normalized for reverse transcriptase (RT) activity and inoculated onto Sup-T1 cells. The percentage of cells expressing viral $p27^{gag}$ was evaluated by immunofluorescence microscopy on methanol and acetone fixed cells using the 3A8 anti-$p27^{gag}$ mAb, and RT levels were determined on virions pelleted from culture supernatants (LaBranche et al., 1994). The stability of mutations in viruses during in vitro infection assays was determined by sequence analysis of uncloned viral DNA amplified by PCR from genomic DNA, as described previously (LaBranche et al., 1994).

Results

Effects of a Tyr-723 Mutation on the Distribution of Envelope Glycoproteins in SIV-infected Cells

As previously described, a number of amino acid substitutions of Tyr-723 in viruses derived from the BK28 molecular clone of SIV, or the analogous tyrosine in SIVmac239 were shown to up-regulate the expression of envelope molecules on the cell surface without affecting the rate or efficiency of envelope protein synthesis (LaBranche et al., 1995). To determine if mutations at this position altered the distribution of envelope glycoproteins in infected cells, Sup-T1 cells chronically infected by BK/NC-723Y or BK/NC-723C were permeabilized and stained with the anti-TM mAb, 43.1. Within *env*, these viruses differed only by the presence of a Tyr or a Cys codon, respectively, at the position corresponding to amino acid 723 (LaBranche et al., 1995). On cells infected by BK/NC-723Y, the envelope glycoproteins were primarily detected in clustered intracellular structures and were minimally expressed on the cell surface (Fig. 1, *A*). In contrast, cells infected by BK/NC-723C showed detectable but considerably reduced intracellular staining and prominent fluorescence at the cell surface (Fig. 1, *B*). FACS® analysis of nonpermeabilized cells using mAbs specific for either TM or SU envelope glycoproteins confirmed the marked increase in surface envelope glycoproteins on BK/NC-723C–infected cells (Fig. 1, *C*). These findings indicated that on BK/NC-723Y–infected cells, envelope glycoproteins were expressed predominantly in intracellular compartments, and that the Y723C mutation caused these molecules to be redistributed to the plasma membrane. Similar findings were noted when these same viruses infected two other CD4-positive lymphoid cell lines, HUT-78 and CEMx174 (not shown).

Effects of the Y723C Mutation on Envelope Glycoprotein Distribution Using an env Expression Vector

To determine if the difference in the distribution of envelope glycoproteins on infected cells could also be demonstrated in the absence of other viral proteins, *env* expression was evaluated in BHK-21 or HEp2 cells using the SFV replicon expression system (Liljeström and Garoff, 1991; Paul et al., 1993; Olkkonen et al., 1994). In these experiments the CP-MAC *env* gene, which contained the Y723C mutation (CP-MAC/723C), was compared to an identical construct that lacked this mutation, termed CP-MAC/723Y. Experiments were also performed using the *env* gene of the parental SIV molecular clone, BK28 termed BK28/723Y, which expressed a Tyr at position 723. The $pSFV_1$ vector containing the cloned *env* genes was used to generate recombinant SFV virions to infect target cells and drive expression of the relevant SIV envelope protein. Expression of a functional SIV envelope protein at the cell surface was demonstrated by the ability of these cells to form syncytia when cocultured with appropriate $CD4^+$ T-cell lines, Sup-T1 for CP-MAC-, and HUT-78 for BK28-derived envelope proteins (LaBranche et al., 1994, 1995) (data not shown). SDS-PAGE analysis of [^{35}S]methionine-labeled cell lysates indicated that equivalent amounts of CP-MAC/723C, CP-MAC/723Y and BK28/723Y envelope protein were made in these SFV-infected cells (data not shown).

The distribution of envelope glycoproteins was evaluated on intact and Triton X-100 permeabilized cells using an anti-SU mAb (101.1). As shown in Fig. 2, CP-MAC/723C glycoproteins were expressed at high levels on the cell surface, similar to that seen for BK/NC-723C virus-infected cells (see above), and for Sup-T1 cells infected by the CP-MAC virus (LaBranche et al., 1994). In contrast, minimal surface staining was seen for the CP-MAC/723Y and the BK28/723Y envelope proteins, although intense intracellular staining was observed for all three viral envelope proteins after permeabilization. This intracellular staining appeared to be associated with the endoplasmic reticulum and nuclear envelope and with cytoplasmic vesicular structures. Similar patterns were observed when these constructs were expressed in HEp2 cells (not shown). Thus, in cells expressing *env* in the absence of other viral proteins, envelope glycoproteins containing a

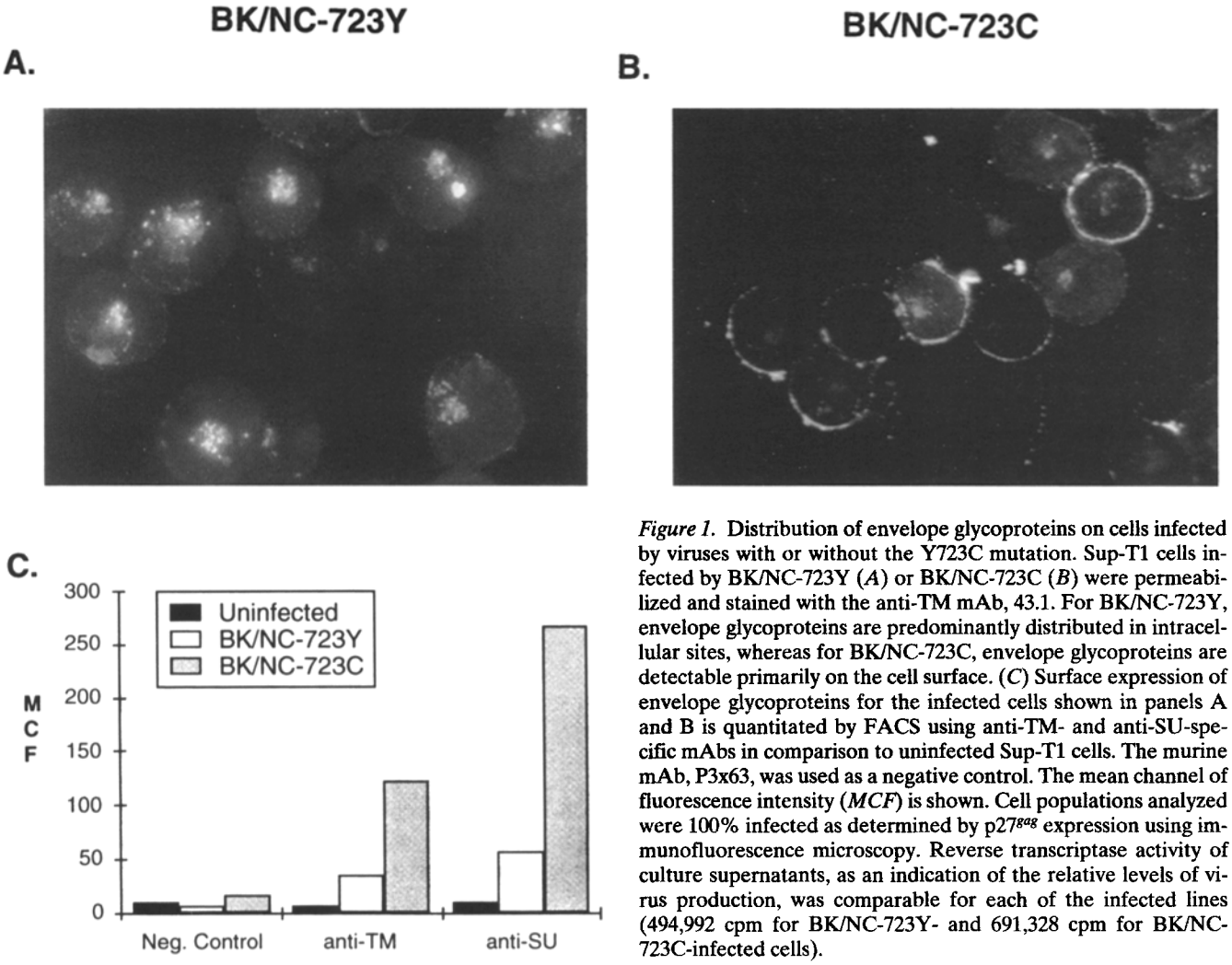

Figure 1. Distribution of envelope glycoproteins on cells infected by viruses with or without the Y723C mutation. Sup-T1 cells infected by BK/NC-723Y (*A*) or BK/NC-723C (*B*) were permeabilized and stained with the anti-TM mAb, 43.1. For BK/NC-723Y, envelope glycoproteins are predominantly distributed in intracellular sites, whereas for BK/NC-723C, envelope glycoproteins are detectable primarily on the cell surface. (*C*) Surface expression of envelope glycoproteins for the infected cells shown in panels A and B is quantitated by FACS using anti-TM- and anti-SU-specific mAbs in comparison to uninfected Sup-T1 cells. The murine mAb, P3x63, was used as a negative control. The mean channel of fluorescence intensity (*MCF*) is shown. Cell populations analyzed were 100% infected as determined by $p27^{gag}$ expression using immunofluorescence microscopy. Reverse transcriptase activity of culture supernatants, as an indication of the relative levels of virus production, was comparable for each of the infected lines (494,992 cpm for BK/NC-723Y- and 691,328 cpm for BK/NC-723C-infected cells).

Tyr at position 723 are minimally expressed on the cell surface and are distributed predominantly in intracellular compartments. As with the SIV-infected cells, a Y723C mutation caused the envelope proteins to be partially redistributed to the plasma membrane.

Envelope Glycoproteins on Infected or env-expressing Cells Are Actively Endocytosed from the Cell Surface

We had previously shown that the increased surface expression of envelope glycoproteins caused by the Y723C mutation could not be explained by alterations in transport to the plasma membrane, shedding of envelope from infected cells, or differences in the incorporation of envelope molecules into virions (LaBranche et al., 1994, 1995). To determine if the low surface and high intracellular levels of envelope molecules on BK/NC-723Y–infected cells resulted from glycoproteins that were transiently expressed on the cell surface and then endocytosed, cells were incubated in the presence of an anti-SU mAb (101.1) at 37°C for varying periods of time and then fixed and permeabilized to detect internalized antibody. As shown in Fig. 3, despite the low steady state level of surface envelope glycoproteins (*A*), intracellular anti-SU mAb could clearly be shown as early as 15 min (*B*), and by 60 min (*D*) was densely clustered in intracellular vesicles. Similar findings were observed when these experiments were performed with 15 μg/ml of a Fab fragment of the 101.1 mAb, indicating that the effect was not due to crosslinking of envelope molecules on the cell surface (not shown). No uptake was seen for an isotype matched control mAb (not shown). Endocytosis of envelope molecules was also observed when identical experiments were performed on Sup-T1 cells infected by BK/NC-723C (not shown), although these experiments could not determine if quantitative differences in endocytosis existed between these two viruses (see below).

To determine if envelope glycoproteins expressed in the absence of other viral proteins were also internalized, similar experiments were performed using the SFV constructs described above. BHK-21 cells expressing the CP-MAC/723C, CP-MAC/723Y or BK28/723Y envelope proteins were incubated for 2 h at 37°C with mAb 101.1, and then fixed and stained for immunofluorescence microscopy. On cells expressing CP-MAC/723C, cell surface staining was seen on >90% of cells, while on cells expressing CP-MAC/723Y or BK28/723Y little if any cell surface staining was observed (Fig. 4 *A*, *top*). However, when cells were permeabilized before staining, prominent intracellular fluorescence of internalized antibody was seen for CP-MAC/

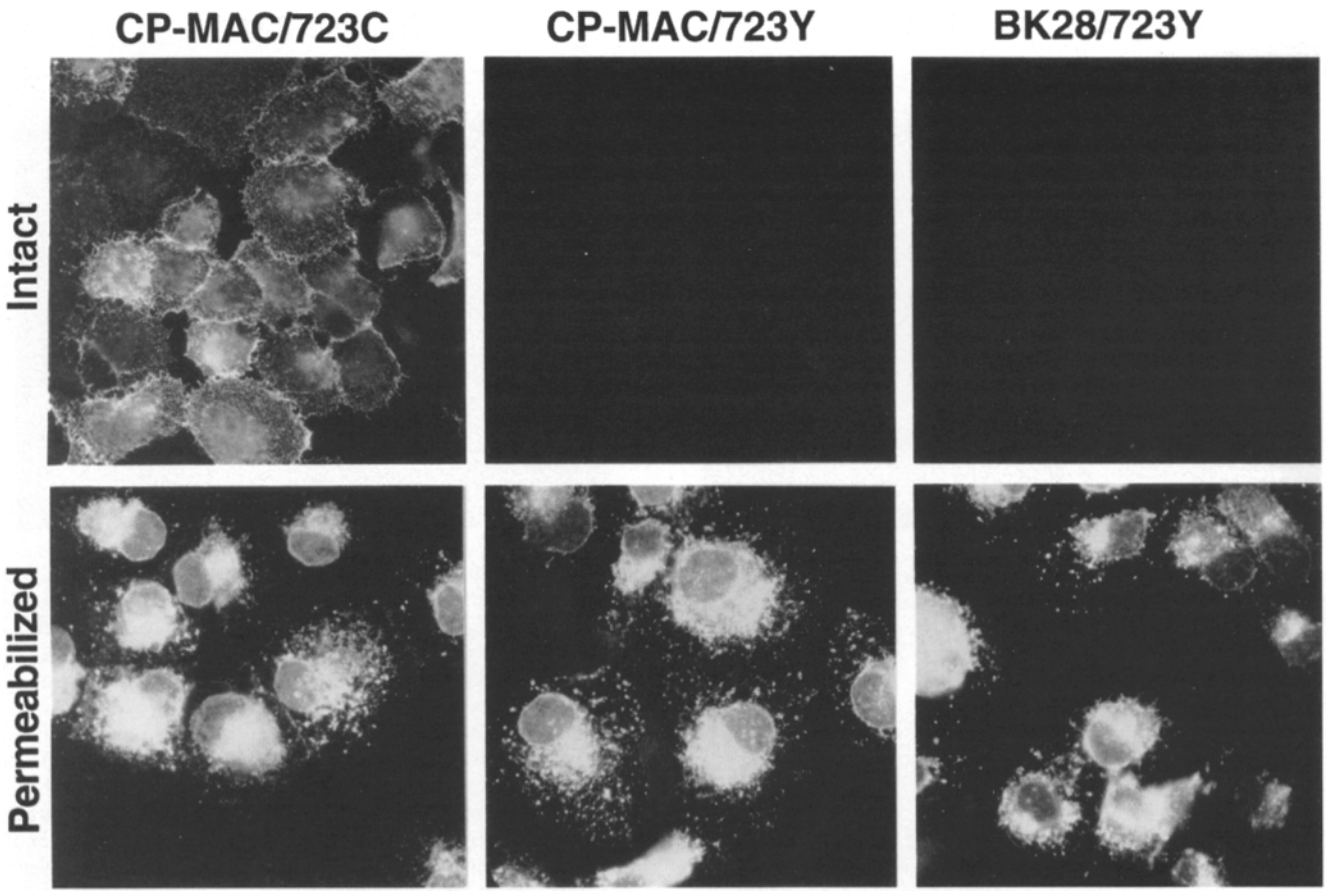

Figure 2. Effects of the Y723C mutation on the distribution of envelope glycoproteins in cells expressing *env*. BHK-21 cells expressing SIV envelope glycoproteins from recombinant SFV vectors were stained with anti-SU mAb (101.1), as described in Materials and Methods, either intact (*top*) or after permeabilization (*bottom*) with Triton X-100. *Env* genes included CP-MAC, which contained a Y723C mutation (CP-MAC/723C), CP-MAC in which the 723-Cys was changed to a Tyr (CP-MAC/723Y), and BK28, which also contained a Tyr at position 723 (BK28/723Y). Although all *env* proteins could be detected intracellularly (*Bottom*) only CP-MAC/723C *env* was expressed at high levels on the cell surface.

723Y- and BK28/723Y-*env* expressing cells (Fig. 4 *A*, *bottom*). Intracellular staining was also evident for CP-MAC/723C–expressing cells, although this pattern was somewhat masked by the bright surface staining that was also present. When identical experiments were performed in HEp2 cells and the anti-SU mAb was stripped from the cell surface with acid treatment prior to staining, a less prominent intracellular fluorescence pattern was seen for CP-MAC/723C-compared to CP-MAC/723Y-expressing cells (Fig. 4 *B*). Identical results were obtained when ex-

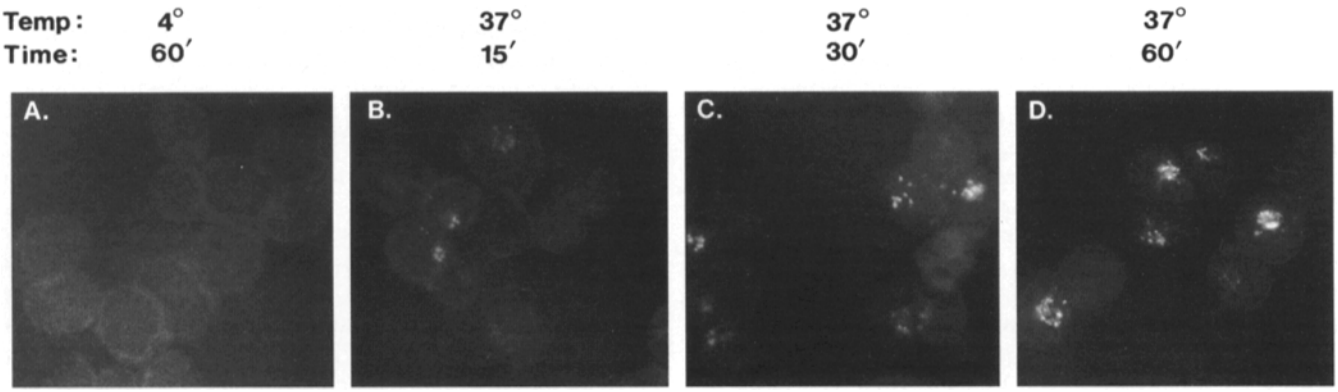

Figure 3. Endocytosis of envelope glycoproteins on SIV-infected cells. Sup-T1 cells chronically infected with BK/NC-723Y were incubated with anti-SU mAb for the indicated times at 37°C and fixed in methanol and acetone. Internalized antibody was detected with a FITC-labeled secondary antibody. As shown, intracellular antibody could be detected at 15 min in a vesicular pattern, which increased in intensity at the 30- and 60-min time points.

periments were performed using a mAb reactive with the TM component of the envelope protein (43.1) (not shown). These results indicated that the CP-MAC/723Y and BK/28 envelope proteins, both of which contain a tyrosine at position 723, are transported to the cell surface and are then rapidly internalized resulting in a low steady state level of expression on the plasma membrane. In contrast, the CP-MAC envelope protein is transported to the cell surface where it accumulates. Together, these findings suggested that differences in surface expression of envelope glycoproteins caused by the Y723C mutation on SIV infected as well as *env*-expressing cells could have resulted from differences in the kinetics and/or efficiency of endocytosis of these molecules from the plasma membrane.

Endocytosis of SIV Envelope Glycoproteins on Infected Cells Is Mediated by Coated Pits

The above findings demonstrated that in addition to being incorporated into viral particles, envelope glycoproteins expressed on the cell surface can also be actively endocytosed. Similar findings have been noted for the simian virus-5 (Ng et al., 1989) and Rous sarcoma virus (Johnston et al., 1995) envelope glycoproteins. To determine the mechanism by which the SIV proteins were internalized, immunoelectron microscopy was performed. Sup-T1 cells chronically infected with SIVs that contained either a Tyr or a Cys at position 723 were incubated for 30 min at 37°C with anti-SU mAb (101.1) and processed for immunoelectron microscopy using an HRP-labeled anti–mouse Ig, as described previously (LaBranche et al., 1995). For cells infected by SIVs with a 723-Tyr, low-magnification views showed that envelope glycoproteins were minimally expressed on the cell surface (Fig. 5, *a*), a result consistent with data shown in Fig. 1 and our previous observations (LaBranche et al., 1995). However, although the vast majority of the plasma membrane on these cells was unlabeled, a careful analysis of multiple fields at higher magnifications revealed an association of envelope molecules with invaginations on the plasma membrane that resembled clathrin-coated pits (Fig. 5, *b–e*). Given the reduced amount of envelope glycoprotein expressed on the surface of these cells and the fact that coated pits typically make up 1–2% of the plasma membrane (Anderson et al., 1977; Pelchen-Matthews et al., 1991), this association was striking. Similar findings were seen on cells infected by NC-MAC, BK/NC-723Y, and BK28, all of which contained a Tyr at position 723.

Immunoelectron microscopy was also performed on cells infected with viruses that contained a Y723C mutation. In marked contrast to cells infected by viruses with a 723-Tyr, an analysis of numerous cells showed labeling over the entire plasma membrane, as previously reported (LaBranche et al., 1995) (Fig. 5, *f*). At higher magnifications, labeled pits could also be demonstrated on these cells (Fig. 5, *g–i*). However, this finding was not surprising in view of the diffuse distribution of envelope glycoproteins on the cell surface. Similar findings were seen for all viruses that contained the Y723C mutation including CP-MAC, BK/NC-723C, and BK/723C, and identical results were observed in chronically infected HUT-78 and CEMx174 cells (not shown).

These results indicated that SIV envelope glycoproteins containing a 723-Tyr are endocytosed, at least in part, by coated pits. This finding raised the possibility that the envelope protein could contain a Tyr-dependent internalization signal similar to those identified in the cytoplasmic domains of a number of cellular membrane proteins, including the low density lipoprotein receptor (LDLr), the transferrin receptor, and lysosomal acid phosphatase (Vaux, 1992; Trowbridge et al., 1993).

The Cytoplasmic Domain of SIV TM Mediates Endocytosis When Transferred to a Heterologous Molecule

To determine if a functional internalization signal was present in the SIV TM, chimeric molecules were constructed that contained the CD4 external and membrane spanning domains and the TM cytoplasmic tail from BK28 (Fig. 6). As with other CD4-based chimeric molecules, Val-395/Arg-396 of the CD4 sequence was used as the junction site between the membrane spanning and the cytoplasmic domains (Littman et al., 1988; Bedinger et al., 1988; Aiken et al., 1994; Anderson et al., 1994). Chimeras were also generated in which Tyr-723 in the SIV TM tail was changed to a Cys, Ala, Ser, Ile, or Phe. Control constructs included a chimeric molecule containing the CD4 external and membrane spanning domains and the entire LDLr cytoplasmic domain (designated CD4/LDLr) and a CD4 molecule containing only a 4-amino acid cytoplasmic domain (designated CD4/tail-minus) (Fig. 6). Constructs were cloned into the pMV-6 retroviral vector and stable lines of HeLa cells expressing these molecules were derived (see Materials and Methods). All constructs were expressed at the cell surface as detected using an ^{125}I-labeled anti–CD4 binding assay and FACS® analysis (Table I, see below).

Endocytosis of CD4/SIV TM chimeric molecules was evaluated using an ^{125}I-labeled anti–CD4 mAb in an acid elution protocol, (see Materials and Methods). As shown (Fig. 7 *A* and Table II) the CD4/LDLr chimera was endocytosed rapidly with ~60% of the cell-associated mAb internalized within 2 min of warm-up and 81% intracellular after 60 min. The plateau in the curves shown in Fig. 7 has been noted in other studies of CD4 endocytosis and reflects the steady state equilibrium between internalization and recycling to the cell surface (Pelchen-Matthews et al., 1989). This result was in marked contrast to the CD4/tail-minus construct, which was internalized at 0.7%/min with only 11% intracellular at 60 min. These studies indicated that the internalization signal present in the LDLr cytoplasmic domain was able to function efficiently when transferred to CD4.

When identical assays were performed on the CD4/SIV TM chimeras, the CD4 construct containing the BK28 TM cytoplasmic domain (CD4/SIV 723-Tyr) was endocytosed at 4.2%/min with 51% intracellular at 60 min. Although slower than the CD4/LDLr chimera, the CD4/SIV 723-Tyr was internalized three to four times faster than constructs containing mutations at position 723, including Phe-723. Significantly, all these mutant constructs showed slightly elevated rates of endocytosis over the CD4-tail minus construct suggesting some residual endocytosis signaling in-

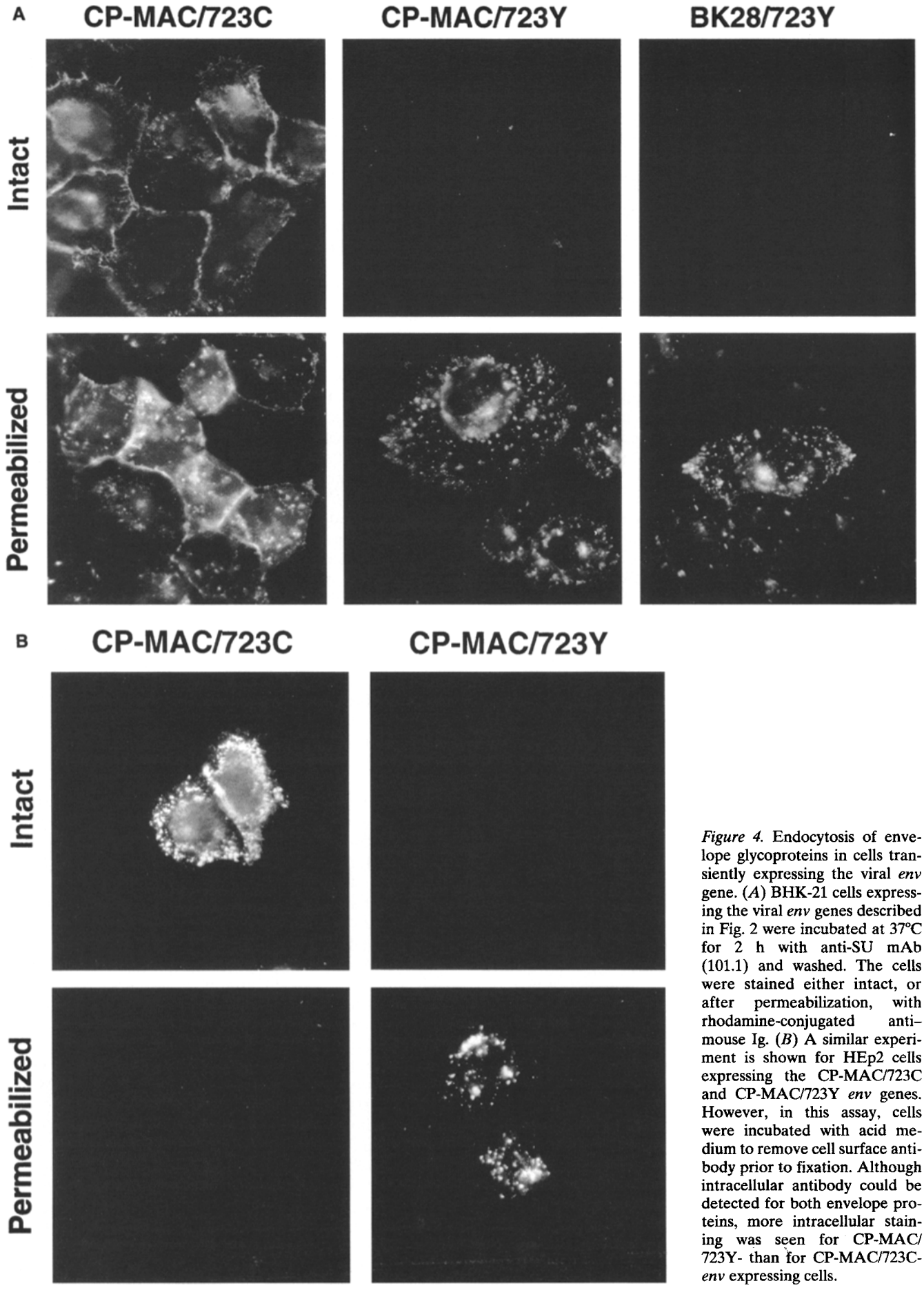

Figure 4. Endocytosis of envelope glycoproteins in cells transiently expressing the viral *env* gene. (*A*) BHK-21 cells expressing the viral *env* genes described in Fig. 2 were incubated at 37°C for 2 h with anti-SU mAb (101.1) and washed. The cells were stained either intact, or after permeabilization, with rhodamine-conjugated anti-mouse Ig. (*B*) A similar experiment is shown for HEp2 cells expressing the CP-MAC/723C and CP-MAC/723Y *env* genes. However, in this assay, cells were incubated with acid medium to remove cell surface antibody prior to fixation. Although intracellular antibody could be detected for both envelope proteins, more intracellular staining was seen for CP-MAC/723Y- than for CP-MAC/723C-*env* expressing cells.

formation in the mutant chimeras (Fig. 7 *A* and Table II). These studies demonstrated that the SIV TM cytoplasmic tail contained an internalization signal that could mediate endocytosis of a heterologous molecule and that the efficiency of this signal was dependent on the presence of a tyrosine at position 723.

Increasing the Distance of Tyr-723 from the Membrane Spanning Domain Improves the Efficiency of the SIV Endocytosis Signal

As described above, the BK28 TM cytoplasmic domain was able to direct endocytosis of CD4. However in these chimeric proteins, mutations that disrupted this endocytosis signal failed to produce differences in the expression of the chimeras on the cell surface (Table I). This result was in contrast to our findings for envelope proteins produced by infectious viruses or the SFV expression system. Studies of the transferrin receptor have indicated that the efficiency of the YTRF internalization signal is influenced by its distance from membrane spanning domain, with at least seven amino acids required for rapid endocytosis (Collawn et al., 1990). Because in the CD4/SIV-TM chimeras, Tyr-723 was predicted to be six amino acids from the membrane spanning domain we considered the possibility that the internalization signal at Tyr-723 might function inefficiently in this setting. Moreover, the junction between the SIV membrane spanning and cytoplasmic domains is not well-defined, raising the possibility that Tyr-723 could be located further than six amino acids from the membrane in the viral protein (see Discussion). We therefore constructed another set of chimeric molecules in which an additional four amino acids of the CD4 cytoplasmic tail were included (Fig. 6). Constructs were made that contained SIV TM tails with a Tyr or Cys at SIV position 723, designated CD4/spacer-723Y and CD4/spacer-723C, respectively. These molecules were introduced into pMV-6 and stably expressed in HeLa cells, as described above.

In contrast to the previous chimeras, and similar to the intact viral envelope protein, the CD4/spacer-723Y chimera was expressed at a low level on the cell surface (see below). Moreover, as shown in Fig. 7 *B*, this chimera was internalized three- to fivefold faster than the CD4/SIV-723Y chimera (18%/min with 77% internal at 60 min), demonstrating that the introduction of a 4-amino acid spacer between the transmembrane domain and Tyr-723 improved the efficiency of the endocytosis signal (Table II). Importantly, a Y723C mutation in the CD4/spacer-723C molecule markedly reduced endocytosis to a rate comparable to that of other chimeras with mutations at this position (1.4%/min). These results indicated that the internalization motif identified in the SIV TM cytoplasmic tail could function almost as efficiently as the LDLr signal provided it was positioned at a sufficient distance from the membrane spanning domain.

Surface and Intracellular Expression of CD4/SIV Chimeric Molecules

Additional studies were performed to determine if the differences in endocytosis rates of CD4/SIV-TM chimeric molecules expressed on HeLa cells correlated with differences in the distribution of these molecules on the cell surface and at intracellular sites. The expression of CD4/spacer-723Y, CD4/spacer-723C and CD4/LDLr chimeras was evaluated on permeabilized cells by immunofluorescence microscopy. As shown in Fig. 8 for both CD4/spacer-723Y (*A*) and the CD4/LDLr (*C*), intense intracellular staining was apparent with a prominent vesicular pattern and with little staining seen on the cell surface. In contrast, for CD4/spacer-723C (*B*) a diffuse pattern of surface fluorescence was seen. Similar results were observed for several clones of each cell line (not shown). When the surface expression on these chimeras was quantitated using an ^{125}I-labeled anti–CD4 mAb binding assay or FACS® analysis, a three- to fourfold increase was observed for CD4/spacer-723C (Table I). This result was particularly impressive in view of the finding that the total level of chimeric proteins in whole cell lysates was ~10-fold less for the CD4/spacer-723C compared to the CD4/spacer-723Y chimera (Table I). These findings demonstrated that, as with the intact viral envelope protein, a Y723C mutation caused this CD4/SIV-TM chimera to be redistributed from intracellular compartments to the cell surface.

Biological Effects of a Tyr-723 Mutation on Infectious Viruses

Because the expression of HIV and SIV envelope glycoproteins has been shown to correlate with virally induced cytopathic effects (Lifson et al., 1986; Bosch et al., 1989; Steffy et al., 1992), we evaluated the biological consequences of a mutation at Tyr-723 on viral infection of a human T cell line. For this experiment we used recombinant SIVs containing the envelope glycoprotein of NC-MAC, a variant SIV that has been shown to infect Sup-T1 cells (LaBranche et al., 1994). BK/NC-723Y and BK/NC-723C, which contained either a Tyr or Cys, respectively, at position 723 in the TM cytoplasmic domain (LaBranche et al., 1995) were inoculated onto Sup-T1 cells, and the kinetics of viral infection were evaluated by serially determining the percentage of cells expressing viral antigens and level of reverse transcriptase activity in culture supernatants. As shown in Fig. 9, the replication of BK/NC-723Y was markedly attenuated in Sup-T1 cells and only 15-20% of cells were positive for $p27^{gag}$ even after 70 d (*a, open squares, open diamonds*). This retarded infection is similar to that initally described for NC-MAC infection of Sup-T1 cells (LaBranche et al., 1994). In contrast, while BK/NC-723C exhibited a similar late onset of infection, once infection was initiated, virus spread through the culture was rapid with >90% of cells expressing viral antigens by day 60 (*a, closed squares*). A 10-fold dilution in the inoculum resulted in a further delay in the onset of infection, but was followed by a similar rapid spread once the infection was detectable (*a, closed diamonds*). This difference in infection kinetics was also reflected in RT levels with BK/NC-723C showing a more rapid increase as well as a five- to sixfold higher level compared to BK/NC-723Y (Fig. 9 *b*). Sequence analysis of uncloned viral DNA amplified by PCR from infected cultures demonstrated that no additional mutations in the viral *env* gene had occurred for either BK/NC-723Y or BK/NC-723C during the course of this infection (not shown).

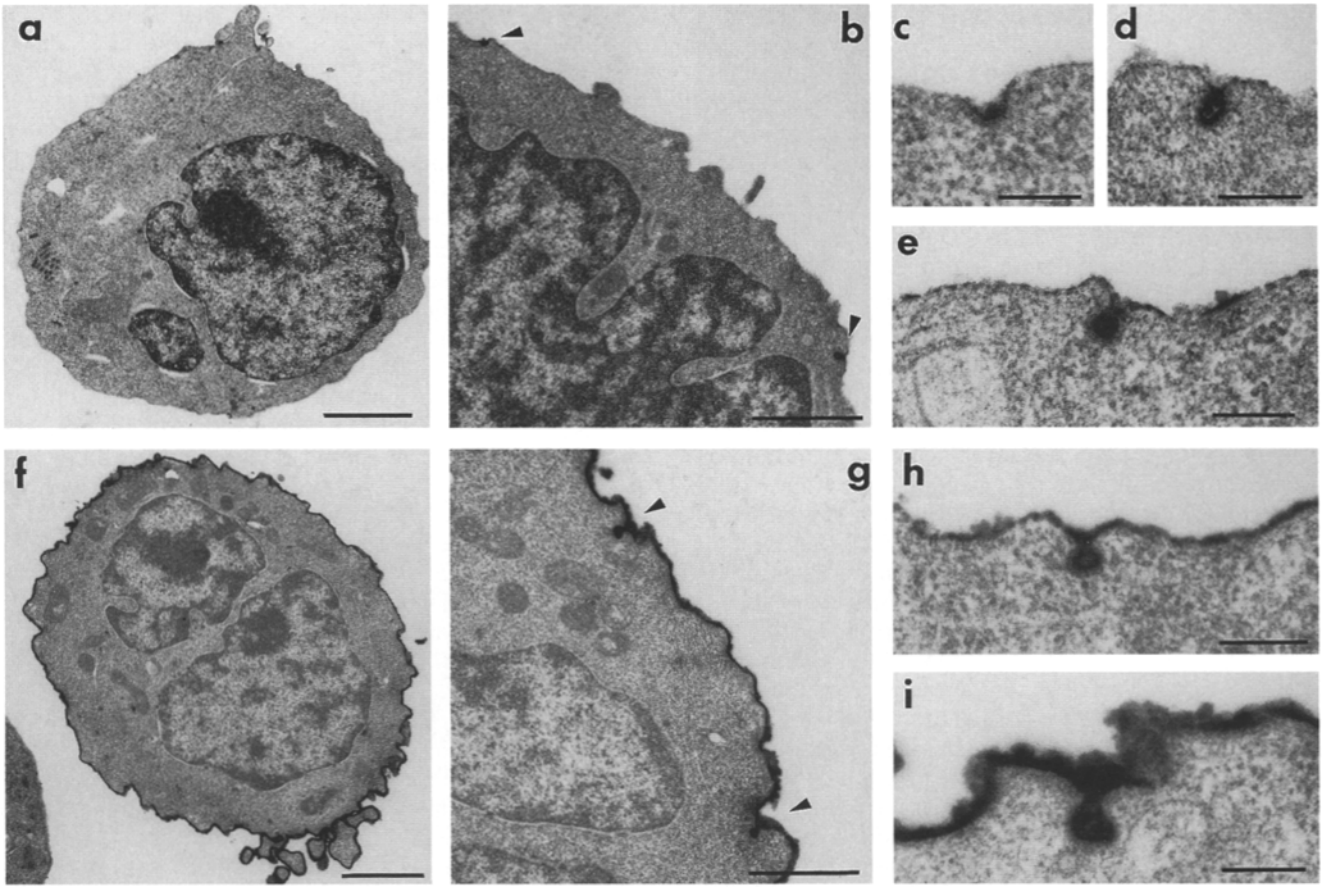

Figure 5. Immunoelectron microscopy of surface envelope glycoprotein distribution on SIV-infected Sup-T1 cells. Sup-T1 cells chronically infected by SIVs containing a Tyr (*a–e*) or Cys (*f–i*) at position 723 in the TM cytoplasmic tail were labeled with an anti-SU mAb (101.1) and stained for immunoelectron microscopy with HRP-conjugated anti–mouse Ig. Viruses were NC-MAC (*a–c*), BK/NC-723Y (*d* and *e*), CP-MAC (*f–h*) and BK/NC-723C (*i*). A low-magnification view of NC-MAC–infected cells (*a*) shows the paucity of envelope glycoprotein distributed on the cell surface, while intermediate (*b*) and high magnification views (*c–e*) show localization of labeled envelope molecules in coated pits. For viruses with a 723-Cys, a low-magnification view (*f*) shows envelope glycoproteins diffusely distributed over the entire plasma membrane. Intermediate and high magnification views of other cells also show the presence of detectable envelope molecules both in and around coated pits (*g–i*). Arrows in *b* and *g* show the presence of labeled envelope glycoproteins in pits at intermediate and low magnification views. Bars: (*a* and *f*) 2 μm; (*b* and *g*) 0.2 μm; (*c–e*, *h*, and *i*) 0.2 μm.

In addition to a more rapid spread of infection, cytopathic effects were also striking in BK/NC-723C-infected cultures, as seen by the presence of large syncytia (Fig. 10 *B*). In contrast, for BK/NC-723Y no syncytia were seen at any time during the course of the infection, despite the presence of multiple single antigen-positive cells (Fig. 10 *A*). These results demonstrate that the loss of the internalization signal at Tyr-723 for this SIV and the resulting increase in envelope glycoproteins on the surface of infected cells significantly altered the biological properties of the virus in vitro.

Discussion

The cytoplasmic domains of viral envelope glycoproteins have been shown to play critical roles in viral pathogenesis (Dubay et al., 1992; Gabuzda et al., 1992; Gray and Roth, 1993; Johnston et al., 1993; Yu et al., 1993; Zhao et al., 1994). These domains may interact with the core proteins to facilitate the incorporation of envelope proteins into virions during viral assembly and may also modulate functional properties of the envelope proteins (Gonzalez et al., 1993; Yu et al., 1993; Zhao et al., 1994; Bugelski et al., 1995). In addition, these domains likely interact with cellular factors that can affect envelope glycoprotein transport, sorting and mobility (Doyle et al., 1985; Haffar et al., 1990; Johnston et al., 1993; Lydy and Compans, 1993; Lodge et al., 1994). For HIV-1 and SIV, a number of studies have suggested that the TM cytoplasmic domain may also affect the level of envelope protein expression on the cell surface (Johnston et al., 1993; LaBranche et al., 1995; Salzwedel et al., 1993; Weiss and White, 1993; Zingler and Littman, 1993), although the mechanism for this effect remains unclear. We previously found that the presence of a Tyr at position 723 in the SIV TM cytoplasmic domain corresponds to a low level of surface envelope protein on infected cells (LaBranche et al., 1995). In the present study, we show that this residue is part of a signal that mediates rapid and efficient endocytosis of envelope glycoproteins from the plasma membrane and that contributes to the cytopathic potential of the virus. The activity of this internalization signal was shown on SIV-infected cells and on cells

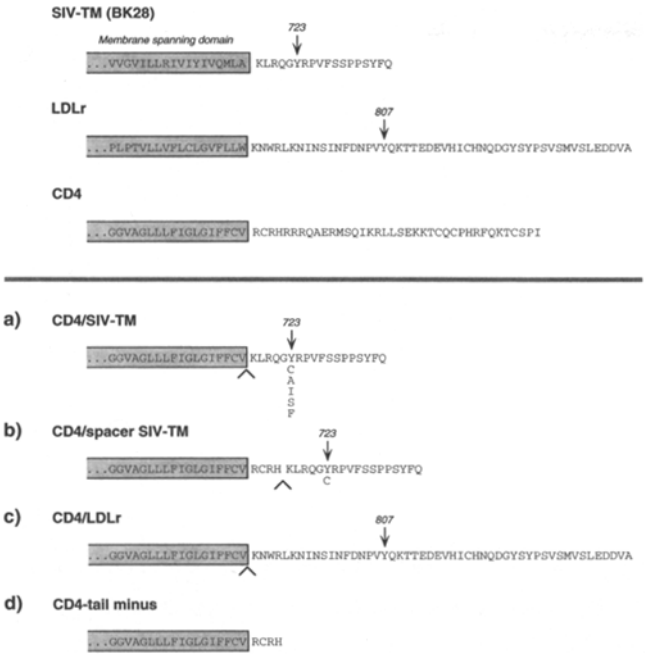

Figure 6. Construction of CD4-based chimeric molecules. Amino acid sequences are shown above the horizontal line for the putative junctions between the membrane spanning domains and cytoplasmic domains for the BK28 SIV TM, the LDLr, and CD4. Tyr residues implicated as contributing to endocytosis signals for SIV-TM and the LDLr (Davis et al., 1987) are indicated at positions 723 and 807, respectively. A series of chimeric molecules were constructed as described in Materials and Methods and are shown below the horizontal line. These include: (*a*) CD4/SIV-TM constructs in which the cytoplasmic domain consisted of only the 18-amino acid SIV-TM cytoplasmic tail, (*b*) CD4/spacer SIV-TM constructs in which the first four amino acids from the CD4 cytoplasmic tail were included, and (*c*) CD4/LDLr in which the entire cytoplasmic domain was derived from the LDLr. For the CD4/SIV-TM and CD4/spacer SIV-TM chimeras, constructs were produced as indicated that included single amino acid substitutions of Tyr-723. For each chimera, the junction between CD4 and the SIV-TM or LDLr cytoplasmic domain is indicated. A CD4-tail minus construct is also shown (*d*), which contained a prematurely truncated cytoplasmic domain.

expressing the viral *env* gene in the absence of other viral proteins. By immunoelectron microscopy, envelope glycoproteins containing the signal were preferentially associated with endocytic structures that resembled clathrin-coated pits, and mutations of Tyr-723 profoundly altered the distribution of the envelope protein, causing these molecules to be dispersed over the entire plasma membrane. The presence of an endocytosis motif was clearly demonstrated by the ability of the SIV TM cytoplasmic tail to mediate internalization of a heterologous molecule, CD4, when used to replace the CD4 cytoplasmic domain. A number of amino acid substitutions at Tyr-723 in these chimeric proteins disrupted the signal and reduced endocytosis to a level approaching that of a tail-less CD4 molecule. As seen for the intact envelope glycoprotein, a Y723C mutation in the CD4/spacer SIV-TM chimera also caused this molecule to be redistributed from intracellular sites to the plasma membrane. Finally, mutation of the putative internalization motif in an infectious virus had profound effects on the kinetics of viral infection and on cytopathic properties of the virus during replication in vitro. The demonstration of an endocytosis motif in this viral glycoprotein is highly reminiscent of early studies of the "JD" mutation in the human LDLr, where a Tyr to Cys mutation at position 807 in the cytoplasmic domain increased receptor expression on the cell surface by reducing its efficiency of internalization (Davis et al., 1986). These results demonstrate the first example of a similar internalization motif being used by a virus to regulate the level of envelope proteins on the cell surface.

For a number of cell surface transmembrane proteins, clathrin-dependent endocytosis can be mediated by a cytoplasmic domain structural motif that includes one or more aromatic amino acids, generally a tyrosine (Trowbridge et al., 1993; Vaux, 1992). For the LDLr, transferrin receptor, and lysosomal acid phosphatase, this motif has been suggested to resemble a reverse β turn in which the Tyr plays a critical role as an electron donor (Collawn et al., 1990; Bansal and Gierasch, 1991; Eberle et al., 1991). Moreover, mutations predicted to disrupt this turn structure reduce the endocytosis rates for these molecules and, as a consequence, increase their steady state expression on the cell surface (Davis et al., 1986; Lazarovits and Roth, 1988; Peters et al., 1990). Although internalization motifs on cellular molecules appear to share a common structure rather than sequence, broad consensus sequences have been proposed to involve either a 4-amino acid motif with an aromatic amino acid in the first position and an aromatic/large hydrophobic amino acid in the fourth position (Aro-X-X-Aro/Large hydrophobic) or a 6-amino acid motif with aromatic amino acids in the first and sixth positions (Aro-X-X-X-X-Aro) (Trowbridge et al., 1993). As shown in Fig. 6, the YRPV sequence in the SIV-TM fits the former of these patterns suggesting that amino acids on the carboxy terminal side of Tyr-723 could be involved in forming a turn. However, molecular modeling of the SIV TM cytoplasmic domain has indicated that the sequence, RQGY (amino acids 720–723) has a high potential to adopt a β turn conformation, suggesting that residues NH_2-terminal to Tyr-723 might be involved in producing the signal (Laurence Pearl, unpublished observations). Further mutagenesis will be required to define the structure of the SIV-TM endocytosis motif. Interestingly, all isolates of HIV-1 exhibit a striking similarity to SIVs in this region of TM, with an RQGY consensus sequence proximal to the analogous Tyr (Myers et al., 1993). Moreover, the following YSPL sequence on the carboxyl side of this Tyr is similar to the sequence YQPL, which has been shown to contribute to an internalization signal for the CD3 γ and δ chains (Letourneur and Klausner, 1992). Therefore, it is plausible that a Tyr-dependent endocytosis signal may also exist for HIV-1. Indeed, recent findings by Rowell et al. for HIV-1 envelope glycoproteins expressed by vaccinia have implicated the analogous Tyr at position 707 in directing loss of envelope glycoprotein from the cell surface, presumably by affecting its rate of internalization (Rowell et al., 1995).

Tyr-dependent internalization signals in cellular proteins are functional when transferred to heterologous molecules, where their activity has been shown to be largely independent of position and orientation (Collawn et al.,

Table I. Surface Expression and Total Cellular Levels of CD4-based Constructs in HeLa Cells

	Surface level*		Total levels§
	^{125}I-antibody binding	FACS®‡ (anti-CD4 mAb)	Densitometry units
	fmol bound/10^6 cells		
CD4/SIV-TM chimeras			
CD4/SIV-TM (723 Y)	167	317	3,657
CD4/SIV-TM (723 C)	214	412	5,042
CD4/SIV-TM (723 S)	168	517	4.454
CD4/SIV-TM (723 I)	222	357	3,906
CD4/SIV-TM (723 F)	338	320	3,692
CD4/SIV-TM (723 A)	317	838	4,034
CD4/spacer-TM chimeras			
CD4/spacer SIV-TM (723 Y)	35	16	2,129
CD4/spacer SIV-TM (723 C)	115	51	262
CD4/LDLr chimera	20	28	354
CD4/tail-minus	484	275	4,565
HeLa Control¶	ND**	1	ND

*HeLa cells stably expressing the CD4-based chimeric molecules indicated (see Fig. 6) were evaluated for surface expression using either an ^{125}I-labeled anti-CD4 mAb or FACS® analysis. For the iodinated antibody assay, numbers reflect surface binding at the kD concentration of antibody (0.3–0.4 mM) and therefore represent approximately half the number of CD4 chimeric molecules expressed.

‡Numbers for FACS® analysis indicated mean channel fluorescence intensity units using an anti-CD4 mAb. For each determination shown the background results for an isotype matched negative control mAb were subtracted.

§Total expression was evaluated in Western blots of whole cell lysates using an anti-CD4 mAb. Numbers indicate values obtained by densitometric scans of autoradiographs.

¶Uninfected HeLa cells were used as a control.

**Not determined.

1990; Collawn et al., 1991; Jadot et al., 1992). However, studies of the transferrin receptor YTRF signal have indicated that a critical distance of seven or more residues is required between the Tyr and the membrane spanning domain for optimal function (Collawn et al., 1990). As we demonstrated, CD4/SIV-TM chimeras in which Tyr-723 was located six amino acids from the predicted membrane spanning domain were endocytosed less efficiently than similar constructs that included the first four amino acids of the CD4 cytoplasmic tail (Fig. 7). The more efficient function of the viral endocytosis signal in the artificial setting of this chimeric protein raises some question concerning the assignment of the membrane spanning domain/cytoplasmic tail junction for the native SIV TM molecule. Hunter et al. have recently proposed that the beginning of the SIV TM cytoplasmic domain may be proximal to that shown in Fig. 6, since viral proteins containing premature termination codons at Gln-714 were still anchored in the plasma membrane (Hunter, E., personal communication). Thus, it is possible that Tyr-723 is located more than six amino acids from the membrane spanning domain in the viral glycoprotein, enabling the internalization motif to function optimally in its natural context.

*Table II. Endocytosis Rates of CD4/SIV-TM Chimeric Molecules**

Chimera type	Amino acid at SIV position No. 723	No. of assays	Rate‡	Percent internal§
			%/min	*60 min*
CD4/SIV-TM	Tyr	14	4.2 ± 1.8	51 ± 3
	Cys	8	1.1 ± 0.5	21 ± 2
	Phe	5	1.5 ± 0.4	22 ± 2
	Ala	4	1.5 ± 0.6	18 ± 3
	Ser	2	1.7 ± 1.1	21 ± 0.4
	Ile	2	1.5 ± 0.6	21 ± 0.3
CD4/spacer SIV-TM	Tyr	4	17.7 ± 6.9	77 ± 11
	Cys	4	1.4 ± 0.6	22 ± 1
CD4/LDLr	–	4	31.0 ± 5.0	81 ± 11
CD4/tail-minus	–	6	0.7 ± 0.2	11 ± 1

*Endocytosis of the CD4-based chimeras indicated and diagrammed in Fig. 6 was evaluated on HeLa cells stably expressing these molecules using an ^{125}I-labeled anti-CD4 mAb, as previously described (Pelchen-Matthews et al., 1989), and summarized in Materials and Methods. For constructs containing the SIV-TM cytoplasmic domain, the amino acid at SIV position 723 is indicated.

‡Endocytosis rates were determined by plotting the acid-resistant counts as a percentage of the total cell-associated counts for each time point and calculating the rate of uptake over the first 5 min of the assay (± SEM).

§Numbers indicate the percentage of anti-CD4 mAb initially bound that was internalized (i.e., resistant to acid elution) after 60 min (± SEM).

It is important to recognize that the SIV used in the present study contained a TM cytoplasmic domain that was prematurely truncated, a consequence of its initial propagation in human cells (Kodama et al., 1989). Whereas HIV and SIV in their natural host cells maintain cytoplasmic tails of approximately 150–165 amino acids, in human cell lines SIVs characteristically develop premature stop codons 18–24 amino acids from the predicted membrane spanning domain (Hirsch et al., 1989; Kodama et al., 1989). It will be of interest to evaluate the function of the endocytosis motif we have identified in the context of a full-length cytoplasmic domain. We have previously shown that mutations of the analogous Tyr in an SIV with a full-length TM up-regulated the level of surface envelope glycoprotein on infected cells, although, interestingly, this effect was appreciably smaller than that seen for SIVs with a prematurely truncated TM (LaBranche et al., 1995). This finding could be explained by determinants in the full-length tail that modulate the function of the endocytosis motif at Tyr-723 or by the presence of additional sorting signals distal to the premature stop codon. Indeed, the observation that SIVs with premature termination codons in their cytoplasmic tails express increased amounts of surface envelope glycoprotein (Johnston et al., 1993; Zingler

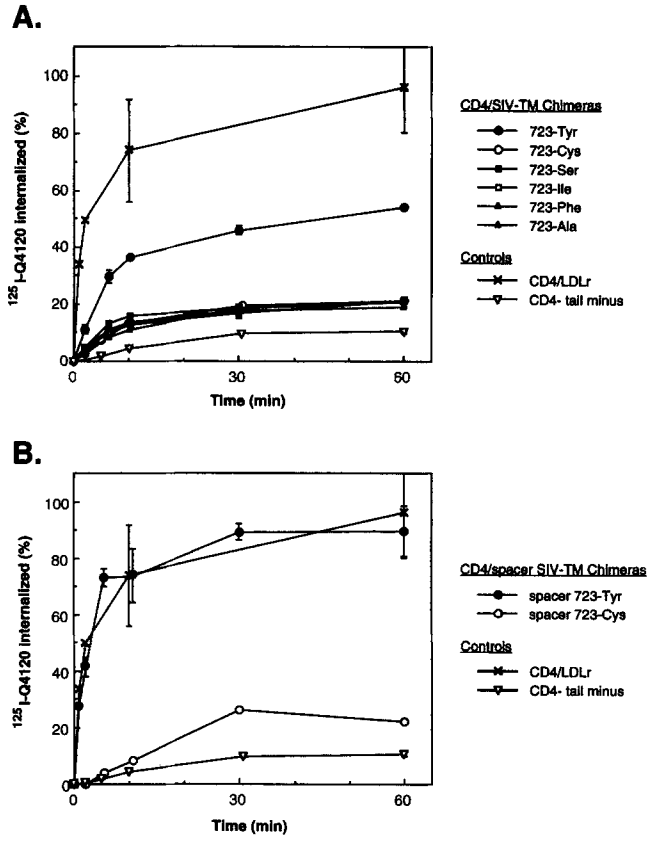

Figure 7. Endocytosis of CD4/SIV-TM chimeric molecules. Endocytosis rates of CD4-based constructs that were stably expressed in HeLa cells were quantitated using an acid-elution endocytosis assay previously described (Pelchen-Matthews et al., 1989). Each panel shows the acid-resistant (internal) ^{125}I counts as a percentage of the total cell-associated counts for each time point. *A* shows results for CD4/SIV-TM chimeras in which the cytoplasmic domain consisted entirely of the SIV TM tail (Fig. 6). Results are compared to the CD4/LDLr and CD4-tail minus constructs. As shown, the SIV-TM domain in the CD4/SIV-TM construct, which contains a 723-Tyr, is endocytosed rapidly, although at a rate considerably slower than that of a similar construct containing the LDLr tail. All amino acid substitutions at position 723 including a Phe, reduced endocytosis to rates approaching that of the tail-minus CD4. *B* shows a similar study for chimeras that included the first four amino acids of the CD4 cytoplasmic domain. As shown, the construct containing the SIV Tyr-723 was endocytosed at a rate similar to the CD4/LDLr chimera. Endocytosis of the chimera containing a Tyr to Cys substitution at position 723 was markedly impaired.

and Littman, 1993) could be explained by a loss of additional internalization or sorting motifs in the distal region of the tail. Multiple sorting signals have been demonstrated in the cytoplasmic domains of several cellular molecules including the LDLr (Matter et al., 1993), cation-dependent mannose-6-phosphate receptor (Johnson et al., 1990), asialoglycoprotein receptor (Fuhrer et al., 1991), insulin receptor (Rajagopalan et al., 1991), CD3 γ and δ chains (Letourneur and Klausner, 1992), and the MHC class II invariant chain (Odorizzi et al., 1994). The SIV and HIV TM cytoplasmic domains do exhibit a number of other conserved Tyr residues as well as pairs of Leu/Ile residues, which have also been implicated as internalization and/or sorting signals for several cellular molecules (Shin et al., 1991; Letourneur and Klausner, 1992; Corvera et al., 1994; Odorizzi et al., 1994). In addition, although Tyr-723 mutations in the CD4/SIV-TM chimeric proteins reduced internalization, their rates of endocytosis were still greater than that of a CD4 construct lacking a cytoplasmic domain (Fig. 7). This finding suggests that even when Tyr-723 is mutated, there is still some residual internalization signal in a prematurely truncated TM. The assays we have developed to quantitate endocytosis in these viral molecules will be valuable tools to address these issues.

These findings indicate that on SIV-infected or *env*-expressing cells, viral glycoproteins on the plasma membrane may be targeted either to budding virions or to endocytic vesicles. It remains to be determined what viral and cellular factors are involved in regulating trafficking along these two distinct pathways. By analogy with the LDL, asialoglycoprotein and epidermal growth factor receptors (Pearse, 1988; Glickman et al., 1989; Pearse and Robinson, 1990; Beltzer and Spiess, 1991; Sorkin and Carpenter, 1993; Lamaze and Schmid, 1995), it is likely that internalization of the SIV TM glycoprotein is mediated by an interaction with clathrin adaptor molecules. These protein complexes are thought to bind to the cytoplasmic tails of some cellular proteins and to recruit these proteins into clathrin-coated pits (Schmid, 1993; Robinson, 1994; Ohno et al., 1995). Because the SIV TM has also been implicated in interacting with the viral matrix protein (MA) during virion formation (Gonzalez et al., 1993), it is possible that a TM-MA interaction may be required to displace envelope molecules from the adaptor proteins or other components of the endocytic pathway. Indeed, recent findings by Mammano et al. have indicated that some determinants in the HIV TM may interact with cellular factors and inhibit the association of envelope glycoproteins with virions (Mammano et al., 1995). Thus, it is likely that envelope glycoproteins are incorporated into virions through a complex series of events that involve interactions of their cytoplasmic domains with other viral proteins and with cellular components that affect their surface expression and determine their availability for uptake into virions (Hunter, 1994).

It is unclear if the region of the TM cytoplasmic domain that includes Tyr-723 could also be directly involved in the incorporation of envelope molecules into virions. Indeed, the finding for HIV and SIV that TM molecules lacking a cytoplasmic domain can still be incorporated into virions suggests that this region may not be critical for viral assembly (Gabuzda et al., 1992; Salzwedel et al., 1993; Weiss and White, 1993; Johnston et al., 1995). Moreover, envelope glycoproteins with mutations of this residue are also incorporated into virions at similar or even increased levels compared to wild-type proteins (LaBranche et al., 1994, 1995). However, findings for Moloney murine leukemia virus have shown that molecules on the plasma membrane may be incorporated into virions by two distinct mechanisms, one that is highly efficient and probably dependent on a specific interaction between the TM cytoplasmic domain and MA and another that is passive, less efficient, and dependent primarily on the level of envelope expression on the cell surface (Soumalainen and Garoff,

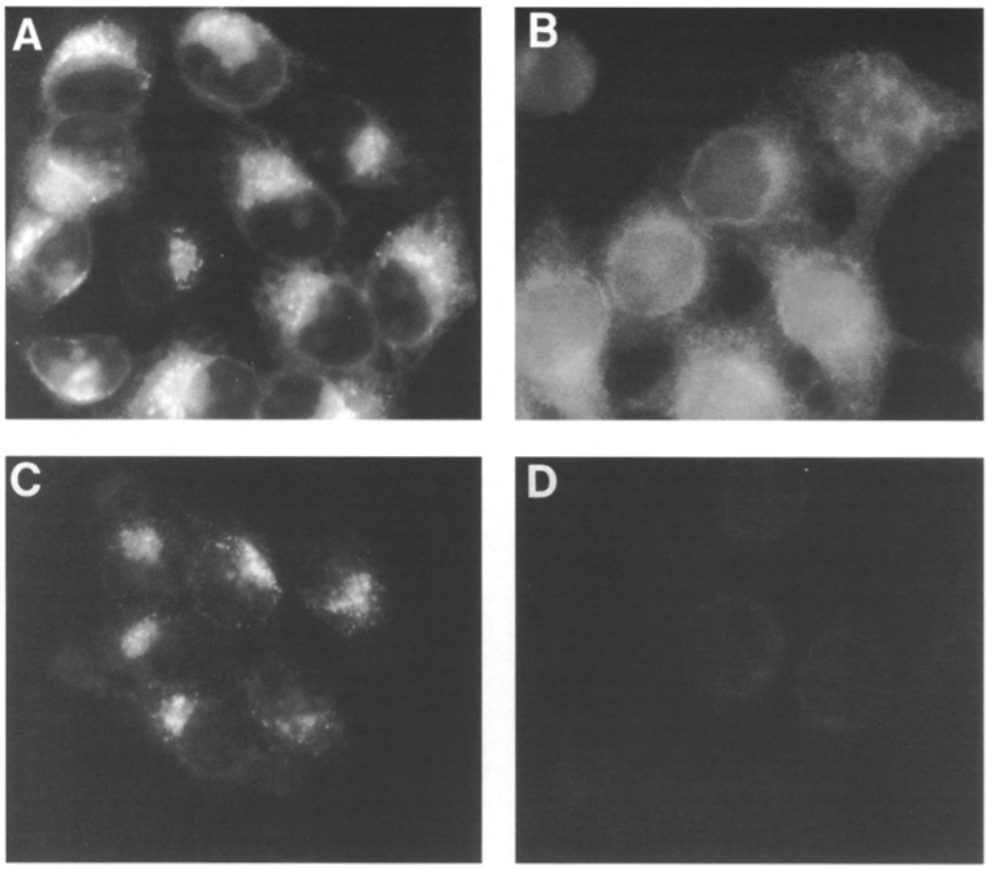

Figure 8. Distribution of CD4/SIV-TM and CD4/LDLr chimeras in HeLa cells. HeLa cells stably expressing the CD4/spacer-723Y (*A*), CD4/spacer-723C (*B*), and CD4/LDLr chimeras (*C*), are shown after permeabilization and labeling with an anti-CD4 mAb. Staining of HeLa cells not expressing a chimeric molecule are also shown as a control (*D*). For both CD4/spacer-723Y and the CD4/LDLr, staining is concentrated in a perinuclear and vesicular pattern with little expression seen on the cell surface, while for the CD4/spacer-723C chimera, staining is diffuse and detectable on the cell surface.

1994). Given the paucity of surface envelope glycoproteins on cells infected by SIVs containing the Tyr signal (Fig. 5), it is likely that these molecules are incorporated into virions with high efficiency. Conversely, envelope molecules containing mutated or absent cytoplasmic tails could be incorporated into virions simply as a reflection of their increased level of expression on the cell surface. Interestingly, it has recently been shown for alphaviruses that a highly conserved Tyr in the cytoplasmic domain of the SFV E2 protein, located eight amino acids from the predicted membrane spanning domain, is essential for the active incorporation of envelope glycoproteins into virions (Zhao et al., 1994). It is possible that the endocytosis motif involving Tyr-723 could also play a role in directing envelope glycoproteins to virions.

Finally, the biological effects of a mutation that disrupts the SIV internalization signal were evaluated on infectious viruses. By comparing two SIVs that differed in the presence or absence of a Tyr at position 723, it was shown that loss of the internalization signal resulted in accelerated infection kinetics and a striking increase in syncytia formation during viral replication. These effects are likely a direct result of the increased cell surface expression of envelope glycoproteins, which have been clearly shown to mediate cell fusion (Lifson et al., 1986; Bosch et al., 1989; Steffy et al., 1992) and likely facilitate the spread of virus

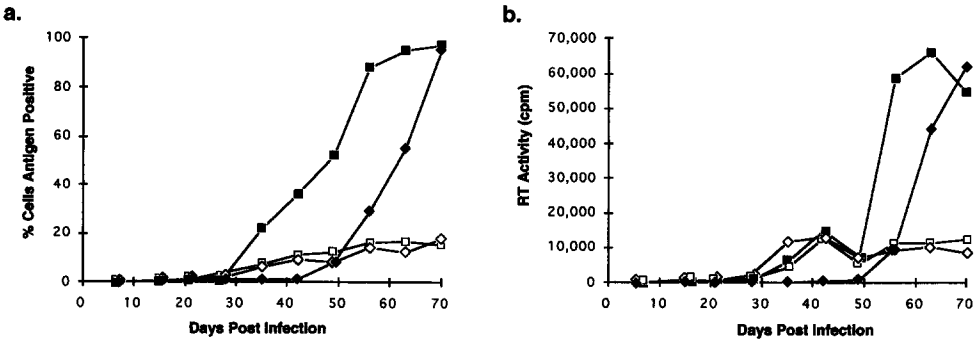

Figure 9. Effects of a Tyr-723 mutation on SIV infection kinetics. Viral supernatants of BK/NC-723Y (*open squares, open diamonds*) and BK/NC-723C (*closed squares, closed diamonds*) were normalized to a RT level of 10,000 cpm (*open squares, closed squares*) or 1,000 cpm (*open diamonds, closed diamonds*), and used to inoculate Sup-T1 cells. Cultures were monitored for infection by: (*a*) immunofluorescence using antibodies against $p27^{gag}$; and (*b*) RT activity in culture supernatants at the indicated time points. As shown, at high and low inoculations, viruses containing the Tyr to Cys mutation at position 723 exhibit more rapid infection kinetics and more extensive spread of infection in the culture.

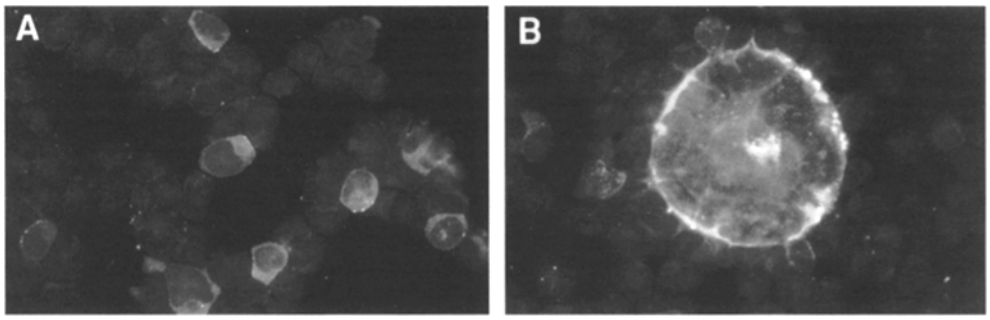

Figure 10. Effects of a 723 Tyr to Cys mutation on syncytia formation. Sup-T1 cells from the experiment shown in Fig. 9 were stained by IFA for viral antigen expression ($p27^{gag}$). (*A*) Cells infected by BK/NC-723Y on day 49 after inoculation; (*B*) Cells infected by BK/NC-723C on day 35 after inoculation. A large syncytium is evident for BK/NC-723C, while only single antigen-positive cells are present during BK/NC-723Y infection.

infection from cell to cell (Dimitrov et al., 1993). Interestingly, although BK/NC-723C infection in the culture was more rapid and eventually involved nearly all cells, the onset of infection remained delayed, suggesting that the infectivity of the viral particles per se was not enhanced. Infection of Sup-T1 cells by NC-MAC has been shown to be noncytopathic and slow, thus enabling the effects of this mutation to be readily apparent. Whether or not similar mutations would alter infectivity of viruses that exhibit rapid and cytopathic infection kinetics remains to be determined. Nonethess, it is apparent that in some circumstances, mutations that disrupt this internalization signal can produce biological alterations in the virus. Although the significance of these findings for in vivo infection remains unclear, an HIV-1 containing a Tyr to Cys mutation at the analogous position in the TM cytoplasmic domain has recently been reported from a patient with advanced HIV infection and a high viral load (Gao et al., 1996). The relationship of this mutation to the in vivo properties of this virus remains to be determined.

These findings indicate that a determinant in the SIV TM cytoplasmic tail has evolved to mimick a cellular signal that is widely used to remove proteins from the cell surface. Although just one component of the complex biology of the virus, it is clear from the experiments described here that this signal can contribute to the infectivity and cytopathic properties of SIV in culture, and may also be important for the pathogenesis of these viruses in vivo. By reducing the level of envelope glycoprotein on the surface of infected cells this signal could modulate the susceptibility of infected cells to humoral immune responses and contribute to the ability of the virus to establish a persistent infection in vivo. Thus, only proteins actively being incorporated into progeny virions would be present on the cell surface, while the remainder would be removed via the endocytic pathway. Endocytosis of viral glycoproteins could also play a role in altering cellular anti–viral immune responses by enabling these proteins to be processed for presentation by HLA class II molecules (Rowell et al., 1995). Finally, it is possible that an endocytosis signal could play a role during viral entry by helping to clear envelope glycoproteins that are deposited in the plasma membrane following fusion of the virion with the cell. Of note, SIVs that contain mutations of Tyr-723 are replication competent in macaque peripheral blood lymphocytes (Fultz, P., personal communication). It will therefore be possible to evaluate the effects of this mutation on the natural history of viral infection in vivo as well as on the immune responses in infected animals.

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