Enhancement of HIV-1-Induced Syncytium Formation in T Cells by the Tyrosyl Kinase p56\textsuperscript{\textit{ck}}

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The CD4 glycoprotein is the primary cellular receptor for human immunodeficiency virus type 1 (HIV-1) and has also been reported to be physically associated with p56\textsuperscript{ck}, a tyrosyl protein kinase. p56\textsuperscript{ck} is a member of the src family of nonreceptor protein-tyrosine kinases and is expressed predominantly in T lymphocytes. Our objective was to study the effect of p56\textsuperscript{ck} on the biology of HIV-1. For this purpose, we have stably transfected two human p56\textsuperscript{ck}-negative T cell lines (C8166-45 and MT-2) with plasmids encoding for this cellular protein. Following coculture with HIV-1-infected cells or infection with cell-free virus, p56\textsuperscript{ck}-expressing cell lines showed a greater propensity for virus-mediated syncytium formation than parental p56\textsuperscript{ck}-negative cells. The enhancement of HIV-1-induced syncytium formation was not associated with the kinase activity of p56\textsuperscript{ck}, as demonstrated by experiments using a kinase-deficient mutant. However, the physical interaction between CD4 and p56\textsuperscript{ck} was shown to be necessary to obtain the enhancement of syncytium formation since a mutated version of p56\textsuperscript{ck}, which is deficient in its capacity to associate with CD4, did not lead to an increase in virus-mediated cell-to-cell fusion events. Finally, we determined that cells transfected with wild-type and kinase-negative mutant p56\textsuperscript{ck} showed a reduced rate of CD4 endocytosis compared to parental p56\textsuperscript{ck}-negative cells. Together, these results suggest that p56\textsuperscript{ck} can be seen as an accessory molecule facilitating HIV-1-mediated syncytium formation in T cells by a mechanism involving the stabilization of the CD4 molecule at the cell surface.

INTRODUCTION

CD4 is a differentiation antigen expressed primarily on T-helper cells and on some cells of the monocyte/macrophage lineage (Littman, 1987; Reinherz et al., 1983; Rudd et al., 1989). This cell surface structure interacts with class II major histocompatibility complex (MHC)-bearing cells during the normal process of antigen presentation. The CD4 molecule is a transmembrane glycoprotein of 55 kDa containing four immunoglobulin-like domains and is a member of the immunoglobulin family (Maddon et al., 1985). The cytoplasmic tail of CD4 is noncovalently associated with the src-family protein-tyrosine kinase p56\textsuperscript{ck} (Rudd, 1990). Although the expression of cell surface CD4 defines T cell subsets, the precise role of this molecule in T cell function is still unresolved. It has been reported that the association between CD4 and p56\textsuperscript{ck} is required for the optimal antigenic response of certain T cells (Abraham et al., 1991; Gaichenhau et al., 1991). The CD4–p56\textsuperscript{ck} complex can also physically associate with the TCR/CD3 complex (Burgess et al., 1991), leading to an enhancement of T cell proliferation (Anderson et al., 1987; Emmrich et al., 1986). Furthermore, anti-CD4 antibodies have been shown to inhibit antigen-stimulated T cell activation (Parnes, 1989). Taken together, these studies suggest that CD4 modulates the activity of the T cell receptor by enhancing low-affinity interactions between the TCR and the antigen-presenting cells following its binding to polymorphic determinants located on class II MHC molecules (Doyle and Strominger, 1987; Gay et al., 1987; Sleckman et al., 1987). The cross-linking of CD4 surface molecules with antibodies has been demonstrated to lead to a rapid and strong autophosphorylation of p56\textsuperscript{ck} and to an increase of its kinase activity on exogenous substrates (Luo and Selt, 1990). These studies suggest that p56\textsuperscript{ck} plays an integral part in the cascade leading to CD4-mediated signal transduction. In addition to its involvement in signal transduction, p56\textsuperscript{ck} has been shown to inhibit endocytosis of CD4 by preventing its endocytic uptake in lymphoid cells (Pelchen-Matthews et al., 1991, 1992).

CD4 is also recognized as the major cellular receptor for human immunodeficiency virus type 1 (HIV-1) (Dalgleish et al., 1984; Dugnall et al., 1984). Viral tropism is mediated by the high-affinity interaction between the major viral envelope glycoprotein gp120 and CD4 (Lasky et al., 1987; McDougal et al., 1986). The effect of the protein-tyrosine kinase p56\textsuperscript{ck} on the biology of HIV in T cells warrants investigation as this molecule shows both signaling and stabilizing properties with respect to CD4.
In an effort to determine whether p56\textsuperscript{ck} can influence some specific events in the replicative cycle of HIV-1, wild-type and several mutated forms of human p56\textsuperscript{ck} were expressed in different p56\textsuperscript{ck}-negative T cell lines (MT-2, C8166-45, and JCaM1.TAg). We have observed that p56\textsuperscript{ck}-positive transfected cells displayed an increased susceptibility to HIV-1-mediated syncytium formation compared to their p56\textsuperscript{ck}-negative parental cells, implying a modulatory role for p56\textsuperscript{ck} in HIV-1-induced syncytium formation in T cells.

MATERIALS AND METHODS

Cells, molecular constructs, and transfection

MT-2 and C8166-45, two human p56\textsuperscript{ck}-negative CD4+ leukemic T cell lines, were grown at 37° in complete culture medium consisting of RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (100 μg/ml). Similar growth conditions were used for two Jurkat-derived T cell lines: J1.1, which harbors latently integrated HIV-1 proviral DNA (Perez et al., 1991) and JCaM1.TAg, a derivative of JCaM1 which stably expresses the SV40 large T antigen (Straus et al., 1996). JCaM1 is a Jurkat leukemic T cell line derivative that is deficient in p56\textsuperscript{ck} expression (Goldsmith and Weiss, 1987; Straus and Weiss, 1992).

J1.1 cells were supplied by the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH (Rockville, MD), while JCaM1.Tag cells were provided by Dr. A. Weiss (Howard Hughes Medical Center, San Francisco, CA). The packaging cell line DAMP (Peterson and Seed, 1988) was grown in DMEM medium supplemented with 10% FBS and 100 μg/ml of the selective agent G418. The full-length human p56\textsuperscript{ck} cDNA, originating from pHK-28 (kindly provided by Dr R. M. Perlmutter, Seattle, WA), was subcloned into the retroviral vector MNC (Sleckman et al., 1987). This vector contains the neomycin (G418) resistance gene and a cytomegalovirus promoter for eu-karyotic gene expression. Human p56\textsuperscript{ck} cDNA was also introduced in the reverse orientation and was used as a control. A kinase-deficient p56\textsuperscript{ck}, made by substituting the lysine residue at position 273 with alanine (K273A) using a PCR overlap extension procedure, was also subcloned into the vector MNC. This construct has been kindly provided by Dr. R.-P. Sekaly (Institut de Recherches Cliniques de Montréal, Montréal, Canada). The MNC-p56\textsuperscript{ck} plasmids were then transfected into DAMP cells by calcium-phosphate precipitation and transfected cells were selected in 1 mg/ml of G418. Neomycin-resistant DAMP lines were used to infect MT-2 and C8166-45 cells. Stable G418-resistant cellular clones of MT-2 and C8166-45 cells were generated and used for our studies. pBluescript-based wild-type Ick and mutant IckC1 cDNAs (a generous gift from Dr. A. Shaw, Washington University, St. Louis, MO) were excised with EcoRI and then subcloned into the EcoRI site of the pBSRαEN expression vector (obtained from Dr. M. W. Olszowy, Washington University, St. Louis, MO). pSrα/Ickwt(s) and pSrα/IckC1 contain wild-type and mutated p56\textsuperscript{ck} in the sense orientation, while pSrα/Ickwt(as) encodes the antisense oriented wild-type p56\textsuperscript{ck} cDNA. Mutant IckC1 is characterized by the substitution of the cysteine residue at position 420 for a serine, rendering it ineffective in its association with CD4 (Shaw et al., 1990). These plasmids (25 μg) or an equal amount of carrier herring sperm DNA was then cotransfected along with the CD4 expressing vector pETE8/CD4wt (10 μg) (a generous gift from Dr. L. Poulin, Centre Hospitalier Universitaire de Québec, Pavillon CHUL, Québec, Canada) (Moir et al., 1996) in JCaM1.TAg using a previously described DEAE-dextran protocol (Bérubé et al., 1996).

Viruses

Stocks of infectious HIV-1\textsubscript{inB} and HIV-1\textsubscript{inRF} were prepared from acutely infected H9 cells. Titration of infectivity was performed by terminal dilution microassay using the highly susceptible MT-4 cell line (Harada et al., 1985). End-point titration was carried out in flat-bottomed microtiter wells using four parallel series of 10-fold dilutions. After 5 to 7 days of incubation, cell-free supernatants were harvested and tested for the major viral core p24 protein by a commercially available enzymatic assay. The TCID\textsubscript{50} was calculated by the method of Reed and Muench (1938). The IIIB and IIIRF strains of HIV-1 were kindly provided by Dr. R. C. Gallo through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH, Rockville, MD) as cell-free supernatant from infected H9 cells.

Syncytium formation

Parental (p56\textsuperscript{ck}-negative) and stably transfected (p56\textsuperscript{ck}-positive) MT-2 and C8166-45 cells were infected with HIV-1\textsubscript{inB} and HIV-1\textsubscript{inRF} at a multiplicity of infection of 0.1 (virus/target cell) or were coincubated for 24 hr with Jurkat cells chronically infected with HIV-1\textsubscript{inB} at a 1:5 ratio (infected:uninfected) in 100 μl of RPMI medium in 96-well plates. In some assays, at 24 hr posttransfection with DEAE – dextran, JCaM1.Tag cells (10\textsuperscript{4} cells per 100 μl per well in a 96-well plate) were incubated for 48 hr with an equal number and volume of J1.1 cells. The number of syncytia per field (40X) was determined with an inverted microscope. Syncytia were defined as giant cells having a diameter at least four times that of uninfected single cells (Lifson et al., 1986). These multinucleated giant cells are bound by a single membrane and are not disrupted by pipetting.

MTT assay

Cells (5 × 10\textsuperscript{4}/100 μl) were incubated at 37° for 48 hr. Thereafter, 10 μl of MTT solution (5 mg/ml) was added...
to each well before incubation for 4 hr at 37°C. Acid-isopropanol (150 μl, 0.04 N HCl in isopropanol) was added to each well. The plates were read on a spectrophotometer (540 nm).

Reverse transcriptase and p24 enzymatic assays

The reverse transcriptase activity was monitored as described previously (Bernier and Tremblay, 1995). Quantitative determination of the main viral core p24 protein was achieved with the use of a commercial enzyme-linked immunosorbent assay (Organon Teknika, Durham, NC).

Western blot analysis

Cells were resuspended in lysis buffer [20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Nonidet-P40, 10% glycerol, 0.025 mM p-nitrophenyl guanidino-benzoate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, and 10 mM sodium fluoride] and the homogenate was vortexed and incubated for 45 min on ice before centrifugation to remove cellular debris. Cellular lysates (50 μg) were electrophoresed on a 10% SDS–polyacrylamide gel. Thereafter, the gel was transferred to a nitrocellulose membrane (0.22 μm) and incubated with the appropriate combination of antibodies. Immunoreactive bands were visualized using the ECL Detection System (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. Antibodies used for our experiments consisted of monoclonal antibodies specific for human p56lk (clone TPICA1) (Anstegui et al., 1991) and phosphotyrosine (clone 4G10; UBI, Lake Placid, NY) proteins.

Flow cytometry analysis

Levels of CD4 surface molecules were detected by direct immunofluorescence using a cytofluorimeter. Briefly, 1 x 10⁶ cells were first incubated with an experimentally determined saturating concentration of a fluoresceinated Leu-3A antibody (clone S53; Becton Dickenson Immunocytometry, San Jose, CA) for 30 min on ice. After two washes with PBS, samples were fixed with 1% paraformaldehyde and analyzed by a cytofluorimeter. Controls consisted of commercial isotype matched murine mAb (Sigma).

Endocytosis assay

Anti-CD4 OKT4 antibody was radio-iodinated using IODO-GEN (Pierce) and Na¹²⁵I. Cells were resuspended at 4°C in binding medium (BM) (RPMI supplemented with 0.2% BSA and 10 mM HEPES, pH 7.4) and incubated with ¹²⁵I-labeled OKT4 for 2 hr at 4°C. Cells were washed several times to remove unbound ¹²⁵I-OKT4 and were divided into several aliquots, one of which was maintained on ice while the others were incubated at 37°C for various periods of time. Thereafter, cells were divided into two portions to measure the proportion of total and internalized ¹²⁵I-OKT4. The proportion of total cell-associated ¹²⁵I-OKT4 was obtained by resuspending the sample in cold BM at pH 7.4. To monitor the level of internalized ¹²⁵I-OKT4, cells were resuspended in BM adjusted to pH 2.0 to remove the cell surface ¹²⁵I-OKT4. Samples were incubated on ice for 3 min in BM (pH 7.4 or 2.0), washed, and collected by centrifugation through a cushion of 5% BSA in PBS. The amount of ¹²⁵I-OKT4 remaining in the cell pellet was measured by gamma counting. The percentage of acid-resistant ¹²⁵I-OKT4, in cells kept for 60 min at 37°C, was determined by subtracting acid-resistant counts at t = 0.

RESULTS

Establishment of p56lk-expressing T cell lines

To monitor if the presence of p56lk can affect the biology of HIV-1, MT-2 and C8166-45, two p56lk-nonexpressing CD4-positive T cell lines, were transfected with a human p56lk-expressing construct. The protein-tyrosine kinase p56lk was introduced into MT-2 and C8166-45 cells by infection with an amphotropic retrovirus vector that contained the human p56lk cDNA driven by the MuLV LTR and the neo gene driven by the CMV promoter. Several stable transfectants of MT-2 and C8166-45 cells were generated. The expression of p56lk, evaluated by Western blot analysis, was found to be variable among the clones obtained (Fig. 1). We next measured surface expression of CD4 glycoprotein by flow cytometry and detected comparable levels of CD4 on parental p56lk-negative and stably transfected p56lk-positive MT-2 and C8166-45 cells (Table 1).

Syncytium formation and virus replication in p56lk-negative and -positive cells

As parental MT-2 and C8166-45 cell lines are known to be highly prone to syncytium formation, it was of interest
Table 1

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<th>Cell line</th>
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<td>99.3</td>
<td>93.6</td>
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</tbody>
</table>

<sup>a</sup> Percentage of CD4<sup>+</sup> cells as measured by flow cytometry using the fluoresceinated Leu-3A anti-CD4 antibody.

<sup>b</sup> Mean fluorescence value.

To evaluate whether the presence of p56<sup>lck</sup> in such cells can modulate their susceptibility to virus-mediated syncytium formation, HIV-1-associated syncytium formation was first monitored by coculturing p56<sup>lck</sup>-negative or -positive MT-2 and C8166-45 cells with a cell line chronically infected with HIV-1 (Jurkat-HIV-1<sub>IIIB</sub>). Results from these experiments showed that the presence of p56<sup>lck</sup> enhanced the susceptibility of the target cells to HIV-1-induced syncytium formation (Fig. 2). Indeed, a two- to fourfold increase in the number of syncytia was observed when Jurkat-HIV-1<sub>IIIB</sub> cells were coincubated with p56<sup>lck</sup>-expressing MT-2 cells compared to coincubation with the parental p56<sup>lck</sup>-negative cell line (Fig. 3). The higher susceptibility to syncytium formation conferred by p56<sup>lck</sup> was also seen in C8166-45 cells, where a two- to fivefold increase in the number of syncytia was detected for cells stably expressing p56<sup>lck</sup>. No change in susceptibility to HIV-1-induced syncytium formation was seen in cells transfected with the control plasmid consisting of p56<sup>lck</sup> cDNA in the reverse orientation (data not shown). An increase in the number of syncytia was still observed when p56<sup>lck</sup>-positive MT-2 and C8166-45 cells were acutely infected with HIV-1<sub>IIIB</sub> (Fig. 4A). Infection with another strain of HIV-1 (IIIRF) resulted in a similar p56<sup>lck</sup>-mediated enhancement of syncytium formation (Fig. 4B), suggesting that the observed effect is not viral strain-specific.

To determine whether the presence of p56<sup>lck</sup> in T-lymphoid CD4-positive cells can also modulate the extent of virus replication, reverse transcriptase activity was assessed following virus infection. Infection of parental and stably transfected MT-2 and C8166-45 cells with HIV-1<sub>IIIB</sub> at a multiplicity of infection of 0.1 (virus/target cell) revealed that, soon after initial infection (24 hr), the extent of virus replication was greater in p56<sup>lck</sup>-positive cells compared to p56<sup>lck</sup>-negative parental cells (Figs. 5A and 5B), which is in correlation with syncytium formation. However, as the infection progressed, virus replication was greater in parental p56<sup>lck</sup>-negative MT-2 than in cells stably expressing p56<sup>lck</sup>. These results can be attributed to the degree of cell death caused by the formation of syncytia.

Mechanism(s) responsible for virus-mediated enhancement of syncytium formation in T cells expressing p56<sup>lck</sup>

To identify the putative mechanism responsible for the higher susceptibility to HIV-1-induced syncytium forma-

Fig. 2. Phase-contrast photomicrographs of syncytia formed when Jurkat cells chronically infected with HIV-1<sub>IIIB</sub> (A) were mixed with parental p56<sup>lck</sup>-negative (B) or transfected p56<sup>lck</sup>-positive C8166-45 (clone 11) (C) cells. The photographs were taken 24 hr after mixing (original magnification, 40×). Multinucleated giant cells are indicated by arrows.
responsible for the p56\textsubscript{ck}-associated enhancement of virus-mediated syncytium formation. The number of syncytia was still lower in parental p56\textsubscript{ck}-negative C8166-45 cells, which is consistent with our previous findings.

We next attempted to determine whether a physical CD4/p56\textsubscript{ck} association was necessary for the observed increase in HIV-1-mediated syncytium formation. We thus constructed pSR\textsubscript{α}-based expression vectors encoding either wild-type human p56\textsubscript{ck} (pSR\textsubscript{α}/lck\textsubscript{wt(s)}) or a modified p56\textsubscript{ck} into which cysteine at position 420 has been mutated for a serine (pSR\textsubscript{α}/lckC1). The latter p56\textsubscript{ck} construct has been shown to be unable to interact with cell surface CD4 (Shaw et al., 1990). As a negative control, we used the antisense wild-type p56\textsubscript{ck} construct (pSR\textsubscript{α}/lck\textsubscript{as}). The constructed plasmids contain the SV40 origin of replication. Therefore, our experiments were carried out in JCaM1.T\textsubscript{Ag} cells, a derivative of JCaM1HIV-IIIB-infected Jurkat cells expressing the SV40 large T antigen (Straus et al., 1996). This technical approach was used to obtain higher levels of p56\textsubscript{ck} expression. Since the JCaM1.T\textsubscript{Ag} cell line is expressing undetectable levels of surface CD4, the CD4 expressing vector pTEJ8/CD4\textsubscript{wt} (Moir et al., 1996) was cotransfected along with the p56\textsubscript{ck} encoding plasmids.

To further examine the underlying mechanism by which the tyrosyl kinase p56\textsubscript{ck} could influence HIV-1-induced syncytium formation in T cells, we used a p56\textsubscript{ck} vector encoding for a protein that is defective in phosphotransferase activity but still able to bind to CD4. In this construct, the lysine residue at position 273 is mutated for an alanine (K273A). This residue is located near the ATP binding site of p56\textsubscript{ck} and is thus essential for the kinase activity of the molecule (Xu and Littman, 1993). A stably transfected C8166-45 cellular clone expressing the kinase-negative p56\textsubscript{ck} protein was derived to test if the signaling property of p56\textsubscript{ck} was responsible for the enhancement of HIV-1-induced syncytium formation. Antibody-mediated multivalent occupancy of cell surface CD4 resulted in a strong and rapid autophosphorylation on tyrosine residues of wild-type p56\textsubscript{ck} (Fig. 6A, lane 1 vs lane 2), indicating that p56\textsubscript{ck} is both physically associated with CD4 and functional when introduced into C8166-45 cells. However, as expected, no increase in autophosphorylation of p56\textsubscript{ck} was detectable in cells carrying the kinase-inactive mutant (Fig. 6A, lane 3 vs lane 4). The degree of HIV-1-mediated syncytium formation was shown to be similar in C8166-45 stably transfected with either wild-type or kinase-inactive p56\textsubscript{ck} (Fig. 6B), suggesting that the signaling property of p56\textsubscript{ck} is not

![Graph](image-url)  
**Fig. 3.** Susceptibility of parental p56\textsubscript{ck}-negative and stably transfected p56\textsubscript{ck}-positive MT-2 and C8166-45 cells to HIV-1-induced syncytium formation. MT-2 and C8166-45 cells were mixed with chronically HIV-1\textsubscript{Iib}-infected Jurkat cells at a ratio of 1:5 (infected:uninfected). The number of syncytia per field (40X) was determined with an inverted microscope 18–20 hr after mixing. This experiment was repeated on three occasions and gave similar results. Results shown represent the mean ± SD of three different wells.

![Graph](image-url)  
**Fig. 4.** Susceptibility of p56\textsubscript{ck}-negative and -positive cells to HIV-1-induced syncytium formation. Parental and stably transfected cells were infected with HIV-1\textsubscript{Iib} (A) or HIV-1\textsubscript{Iib} (B) at a multiplicity of infection of 0.1. The number of syncytia per field (40X) was determined with an inverted microscope 3 days after initial infection. Results shown represent the mean ± SD of three different wells.
POTENTIATES HIV-1-MEDIATED SYNCTIUM FORMATION demonstrated that the association with p56lck prevented CD4 entry into coated pits (Pelchen-Matthews et al., 1991, 1992). Measurements of the rate of endocytosis of surface CD4 molecule in p56lck-negative and -positive cell lines were performed with the use of a radiolabeled anti-CD4 antibody. The results presented in Table 2 indicate that the percentage of internalized CD4 at steady state (60 min at 37°C) was weakly but reproducibly greater in p56lck-negative C8166-45 cells (19.2%) than in cells expressing either wild-type (clone 7, 6.2%; clone 8, 4.4%; clone 9, 7.4%; and clone 11, 10.1%) or kinase-deficient p56lck (K273A, 8.4%). These results suggest that the increase in virus-mediated syncytium formation, which is due to the interaction between p56lck and surface CD4, might be dependent on p56lck-mediated lowering of the CD4 internalization process that normally occurs in T cells.

After transient transfection of these constructs in the p56lck-nonexpressing JCaM1.TAg cell line, a coculture experiment was performed between these cell lines and the latently HIV-1-infected J1.1 cell line. As depicted in Fig. 7, a similar number of syncytia were seen in JCaM1.TAg cells transfected with CD4 cDNA alone, with CD4 and p56lck cDNAs (antisense orientation), or with plasmids encoding for CD4 and mutated p56lck, which can no longer interact with CD4. However, the expression of surface CD4 along with wild-type p56lck (sense orientation) still led to an increase in HIV-1-mediated syncytium formation. It should be noted that the number of syncytia was higher in JCaM1.TAg cells carrying CD4 than in untransfected cells. Our data are thus indicative of a requirement for CD4/p56lck association for the enhancement of HIV-1-mediated syncytium formation.

We next monitored the stabilizing property of p56lck with respect to CD4 since previous observations had
upregulates the expression of a component facilitating syncytium formation. It is also possible that this signal generates conformational changes of a cell surface molecule, such as an adhesion molecule switching to its high-avidity form. A putative candidate is LFA-1, as this molecule has been reported to participate in HIV-1-mediated syncytium formation (James and Orentas, 1989). Furthermore, LFA-1 has been shown to switch to a state of high affinity for ICAM-1 following signaling through the antigen receptor (Dustin and Springer, 1989). The involvement of the signaling property of p56\(^{lck}\) in HIV-1-induced syncytium formation was monitored by introducing a kinase-deficient form of p56\(^{lck}\) within C8166-45 cells. The demonstration that cells expressing inactive p56\(^{lck}\) lose their enhanced susceptibility to syncytium formation would be an indication that the kinase activity of p56\(^{lck}\) is responsible for the observed phenomenon. On the contrary, an equal susceptibility to HIV-1-induced syncytium formation would suggest that the kinase activity of p56\(^{lck}\) is not required. Our experiments revealed that the susceptibility to syncytium formation was independent of the signaling property of p56\(^{lck}\) because kinase-deficient C8166-45 cells were as susceptible to syncytium formation as cells transfected with wild-type p56\(^{lck}\). In this respect our results are inconsistent with a previous study that has demonstrated that the tyrosine kinase inhibitor herbimycin A decreases HIV-1-associated syncytium formation (Cohen et al., 1992). However, it is of interest to note that this protein tyrosine kinase inhibitor has a broad range of activity and is not specific for only p56\(^{lck}\), which may account for such contradictory results.

Our results indicate that an interaction between CD4 and p56\(^{lck}\) is necessary to achieve an increase in HIV-1-mediated syncytium formation. Indeed, the inability of a mutated p56\(^{lck}\) construct, defective in its association with CD4, to cause an enhancement in the number of syncytia is suggestive of the need of such an association. Furthermore, the presence of p56\(^{lck}\) in transfected T cells was

### TABLE 2

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<th>Cell line</th>
<th>Rate of CD4 endocytosis</th>
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<td>C8166-45 lck+ K273A</td>
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</table>

\(^{a}\) Rates of endocytosis were monitored over the first 15 min at 37°C.

\(^{b}\) Levels of acid-resistant 125I-OKT4 were determined in cells incubated for 60 min at 37°C (see Materials and Methods for details).
found to be associated with a diminished rate of CD4 endocytosis. Our results are consistent with a previous study that demonstrated that the presence of p56\(^{ck}\) in lymphoid cells decreases CD4 endocytosis by preventing the entry of CD4 into coated pits. Indeed, the percentage of CD4 found intracellularly at equilibrium in our p56\(^{ck}\)-expressing (4.4 to 10.1%) and p56\(^{ck}\)-negative (19.2%) C8166-45 cellular clones lies in a similar range to the percentages observed for a CD4-negative T lymphoid cell line expressing either a wild-type (5 to 10%) or a truncated form of CD4 unable to interact with p56\(^{ck}\) (21%) (Pelchen-Matthews et al., 1991). On the basis of our results, we propose that the diminished rate of CD4 endocytosis observed in p56\(^{ck}\)-expressing cells, due to the physical association between CD4 and p56\(^{ck}\), will most likely result in a more prolonged interaction between CD4 and gp120. Based on this assumption, we believe that this mechanism could ultimately favor HIV-1-induced syncytium formation by allowing CD4 to remain on the cell surface long enough to engage in the fusion process. Taken together, our results suggest that p56\(^{ck}\) cannot be considered an essential element for cell-to-cell fusion but rather is an accessory cellular component facilitating virus-induced syncytium formation in T cells.

The present data are in agreement with another study which has demonstrated that p56\(^{ck}\)-positive U937 monocytoid cells are more susceptible to HIV-1-induced syncytium formation than p56\(^{ck}\)-negative parental cells (Yoshida et al., 1992). However, in contrast to our findings, they postulated that p56\(^{ck}\) affects syncytium formation in U937 cells through its kinase activity. Their hypothesis was based on experiments with genistein, a protein tyrosine kinase inhibitor that is known to possess a wide spectrum of activity.

The progression of disease in HIV-infected individuals is characterized by a steady depletion of CD4+ T cells (Fahey et al., 1984; Gottlieb et al., 1981; Masur et al., 1981). One of the putative mechanisms that has been postulated to explain this CD4-specific T cell depletion is that the virus exerts cytopathic effects on infected cells through its ability to induce cell fusion (Levy, 1993; Pantaleo et al., 1993b; Weiss, 1993). The clinical relevance of HIV-1-induced syncytium formation has yet to be documented in vivo. However, the detection of high concentrations of virus particles trapped within lymphoid organs of HIV-infected patients, even during the clinically latent stage of disease (Embreton et al., 1993; Pantaleo et al., 1993a), suggests that multinucleated giant cells may be found in these tissues. The inability to detect their presence in the peripheral blood is most likely attributed to their rapid elimination by scavenger cells of the immune system. While binding of envelope proteins to CD4 is required for the initiation of the fusion process, other membrane components, not yet clearly identified, are necessary for the merging of membranes. This is demonstrated by the fact that many animal cells, expressing human CD4, do not fuse with virus envelope-expressing cells (Aoki et al., 1991; Ashorn et al., 1990). Results from our studies permit more knowledge to be gained regarding cellular structures that can modulate virus-induced cell fusion.

It is important to derive more sensitive cell lines for the detection of syncytium-inducing isolates of HIV-1 as the appearance of a syncytium-inducing phenotype has been reported to be associated with a more rapid course of disease progression (Koot et al., 1993; Schellekens et al., 1992; Tersmette et al., 1989). The fact that one of the most commonly used biological assays to classify primary isolates of HIV as syncytium inducing or nonsyncytium inducing relies on the use of MT-2 cells (Boucher et al., 1992; Karlsson et al., 1994; Koot et al., 1992) renders our studies even more interesting. Indeed, the generation of cell lines that are more sensitive to HIV-1-induced syncytium formation may allow the detection of syncytium-inducing primary isolates that were initially classified as nonsyncytium isolates using the parental p56\(^{ck}\)-negative MT-2 cell line. Interestingly, a recent study has shown that primary isolates of HIV-1 produced typical syncytia in CD4+ T cell lines established from mononuclear cells of healthy donors despite being initially characterized as having a nonsyncytium-inducing phenotype using the p56\(^{ck}\)-negative parental MT-2 cell line (Todd et al., 1995). These data further reinforce our results, suggesting that p56\(^{ck}\) may be seen as an accessory cellular molecule that facilitates the process of virus-mediated cell-to-cell fusion.

In conclusion, the results of our paper contribute to our understanding of the nature of the accessory molecules required for virus-mediated cell-to-cell fusion, a mechanism that has been proposed to be responsible for the observed diminution of CD4-bearing T cells in individuals infected with HIV.

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