Binding of HIV-1 to Its Receptor Induces Tyrosine Phosphorylation of Several CD4-Associated Proteins, Including the Phosphatidylinositol 3-Kinase

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Cell surface CD4 molecules are known to be important in several physiological responses of T lymphocytes. The use of human immunodeficiency virus (HIV) particles or purified gp120 molecules as CD4 cross-linking agents has been shown to result in a cascade of intracellular biochemical events. In addition, we and others have provided evidence suggesting that virus-mediated CD4 multimerization can lead to modulation of HIV-1 long terminal repeat-dependent activity and virus production. We were thus interested in measuring the effect of HIV-1 particles on intracellular tyrosine-phosphorylation levels, mostly of CD4-associated proteins. Using the T cell line CEM-T4, we observed that HIV-1 induces an increase in tyrosine phosphorylation of four major proteins physically complexed to the CD4 molecule. Immunoblot analysis permitted the identification of two of these proteins, p56

INTRODUCTION

The CD4 molecule is the primary cellular receptor for the human immunodeficiency virus (HIV) and thus plays an essential role in infection of CD4-expressing cells, such as lymphocytes and macrophages (Dalgleish et al., 1984; Klatzmann et al., 1984). CD4 is a member of the immunoglobulin family, consisting of four extracellular domains, a membrane-spanning region, and a cytoplasmic tail (Maddon et al., 1985). The HIV-1 binding sites have been identified by mapping studies as the second complementary determining region located in the first extracellular domain of CD4 (Arthos et al., 1989). The normal physiological role of CD4 during the antigenic response is to stabilize interaction between T cells and antigen-presenting cells (Doyle and Strominger, 1987) and to transduce an independent signal upon binding to nonpolymorphic regions of the class II major histocompatibility complex molecules (Julius et al., 1991). The integral membrane CD4 glycoprotein is physically associated with the lymphocyte-specific protein tyrosine kinase p56

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(1990; Veillette et al., 1989a,b). The mechanism by which p56

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... downstream molecules. Putative substrates include CD5 (Burgess et al., 1992), glycoposphatidylinositol-anchored proteins (Resh, 1994; Shenoy-Scaria et al., 1993; Stefannová et al., 1991), MAP-2 kinase (Ettehadieh et al., 1992), phospholipase Cγ (Weber et al., 1992), p120 (Reedquist et al., 1994), phosphatidylinositol 3-kinase and 4-kinase (PI 3-kinase and PI 4-kinase) (Prasad et al., 1993; Vogel and Fujita, 1993), p95vav (Gulbins et al., 1993; Gupta et al., 1994), Raf-1-related p110 polypeptide (Prasad and Rudd, 1992), rasGap (Amrein et al., 1992), Syk (Couture et al., 1994), ZAP-70 (Chan et al., 1991; Duplay et al., 1994; Iwashima et al., 1994), and the ζ subunit of the TCR/CD3 complex (Veillette et al., 1989a). There is some debate regarding the ability of the virus external envelope glycoprotein gp120 and/or the virus itself to transduce CD4-mediated biochemical signals in T cells. Some studies have shown activation of AP-1, MAP-kinase, and PI 4-kinase, as well as an increase in intracellular calcium, hydrolysis of phosphatidylinositol, and protein-tyrosine phosphorylation (Chimirurle et al., 1995; Cruikshank et al., 1990; Hivroz et al., 1993; Kornfeld et al., 1988; Schmid-Antomarchi et al., 1996), while others have failed to observe some of these events (Horak et al., 1990; Orioff et al., 1991). Differences in technical approaches might account for such contradictory observations.

On the basis of the findings discussed above and on the reported rapid stimulation of CD4-associated p56

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and increase in intracellular protein-tyrosine phosphorylation mediated by anti-CD4 antibodies (Horak et al., 1990; Luo and Sefton, 1990; Veillette et al., 1989a,b,c, 1988), it is likely that the viral-binding event might create a CD4-dependent cascade of signal transduction events. In this report, we demonstrate that, in the human CD4-expressing T lymphoid cell line CEM-T4, binding of HIV-1 results in rapid and strong tyrosine phosphorylation of at least four major CD4-associated cellular substrates. Two of the four prominent species were identified as the tyrosyl kinase p56lck and the PI 3-kinase p85α regulatory subunit.

MATERIALS AND METHODS

Cells and culture condition

CEM-T4 is a naturally isolated subclone of the parental CEM cell line and expresses high levels of surface CD4 (Foley et al., 1965); H9 is a subclone of the human cutaneous T cell line HUT 78 (Mann et al., 1989); Jurkat E6-1 is a CD3/Ti-bearing leukemic T cell line, which expresses low levels of CD4 (Weiss et al., 1984); J45.01 is a mutant Jurkat E6-1 clone selected for low expression of the transmembrane tyrosine phosphatase CD45 (Koretzky et al., 1991); Molt-4 clone 8 is a CD4+ T cell line which is highly susceptible to HIV-1 infection (Kikutaka et al., 1986); and the T lymphocyte Sup-T1 cells express high levels of surface CD4 (Smith et al., 1984). These cell lines (CEM-T4, H9, Molt-4 clone 8, and Sup-T1) were provided by the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH (Rockville, MD). Jurkat E6-1 and J45.01 were supplied by Dr. A. Weiss (Howard Hughes Medical Center, San Francisco, CA). Cell lines were maintained in complete culture medium made of RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (100 μg/ml).

Antibodies

The anti-CD4 monoclonal antibody SIM.4 was obtained through the AIDS Research and Reference Reagent Program. The mouse anti-human p56lck (clone TPICA1) has been kindly provided by Dr. Rafick-P. Sékaly (Institut de Recherches Cliniques de Montréal, Montréal, Canada) and is directed against the COOH-terminal domain of p56lck and has been described previously (Ansotegui et al., 1991). These monoclonal antibodies were isolated from hybridoma culture supernatants and purified by protein G-Sepharose affinity chromatography according to manufacturer’s instructions (Gibco BRL, Gaithersburg, MD). Polyclonal anti-CD4 antibodies were raised in rabbits following immunization with recombinant soluble CD4 (scCD4; kindly provided by R. Sweet, SmithKline Beecham). Monoclonal anti-phosphotyrosine (clone 4G10) and polyclonal anti-PI 3-kinase p85α antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Peroxidase-conjugated goat anti-mouse and peroxidase-conjugated sheep anti-rabbit antibodies were purchased from Amersham (Arlington Heights, IL).

Immunoblot assay

Cells were resuspended in lysis buffer consisting of 20 mM Tris- HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 10% glycerol, 0.025 mM p-nitrophenyl guanidinobenzoate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, and 10 mM sodium fluoride. The homogenate was vortexed and incubated for 45 min on ice with intermittent mixing before centrifugation to remove cellular debris. Protein content was determined by the commercial BCA protein assay reagent (Pierce, Rockford, IL). Cleared cellular lysates were electrophoresed on 10% SDS–PAGE and the gel was transferred to a nitrocellulose sheet. Immunoblot detection was performed with a 1:5000 dilution of monoclonal anti-phosphotyrosine (clone 4G10), a 1:1000 dilution of monoclonal anti-p56lck (clone TPICA1), or a 1:3000 dilution of rabbit anti-PI 3-kinase p85α antibodies. Detection was carried out using either peroxidase-conjugated goat anti-mouse at a 1:20,000 dilution or peroxidase-conjugated sheep anti-rabbit antibodies at 1:5000 dilution. Immunoreactive bands were visualized using the ECL detection system according to the manufacturer’s instructions (Amersham).

Immunoprecipitation analyses

Cleared cell lysates were first incubated with rabbit anti-CD4 antibodies overnight at 4°C with intermittent mixing. Thereafter, protein G-agarose beads (Santa Cruz Biotechnology, Inc.) were added and incubation at 4°C with intermittent mixing was pursued for 3 hr. Immune complexes were washed three times in lysis buffer without NP-40 before analysis by SDS–PAGE.

Antibody-mediated CD4 cell surface receptor stimulation

CEM-T4 cells (1 x 10^6) were incubated with saturating concentrations of SIM.4 (10 μg/10^6 cells) for specific periods of time at 37°C (dimerization of CD4) or for 30 min at 4°C (multimerization of CD4). Cells were next treated with goat anti-mouse antibodies (20 μg/10^6 cells) at 37°C for the indicated times (multimerization of CD4). After stimulation, cells were immediately lysed and precleared cell lysates were used for immunoblot.

Exposure of CEM-T4 cells to HIV-1

The Molt-4 clone 8 cell line was infected with HIV-1 (Adachi et al., 1986) and fresh cell-free supernatant from such virally infected cells was used for our experiments. This combination of producer cell line and strain of HIV-
1 was used as it produces high amounts of viruses ranging from 0.5 to 1.5 μg/ml p24 (unpublished observations from our laboratory). Moreover, it is possible to maintain an acute infection with high levels of viral production simply by a weekly addition of uninfected cells. Binding of HIV-1 to surface CD4 was carried out as follows: CEM-T4 cells (7 × 10^6) were first incubated for 30 min on ice with HIV-1NL4-3 (50 ng of p24) contained in fresh cell-free supernatant of acutely infected Molt-4 clone 8 cells. This initial incubation period on ice is to allow maximal viral binding (Horak et al., 1990) and to minimize activation of signaling intermediates. Thereafter, cells were incubated at 37°C for 0, 1, 5, 10, and 15 min, washed twice with cold phosphate-buffered saline (pH 7.4), and immediately solubilized in lysis buffer for 45 min on ice. The pre-cleared cell lysates were then used for immunoprecipitation. In some experiments, soluble CD4 (20 μg/ml) was added to Molt-4 clone 8 cells prior the addition of HIV-1.

RESULTS

Determination of p56^{lck} protein expression and basal tyrosine phosphorylation levels in various CD4+ T cell lines

In an attempt to shed light on the controversy surrounding protein-tyrosine phosphorylation events induced by the occupancy of CD4 by HIV-1, we used a combination of immunoprecipitation and immunoblotting techniques. To specifically identify putative HIV-1-mediated CD4-derived signals, we first screened several HIV-1-susceptible CD4-expressing T lymphoid cell lines as we reasoned that the most appropriate cell line for our needs should contain high amounts of p56^{lck} physically linked to CD4 and low basal levels of tyrosine-phosphorylated p56^{lck} found associated with CD4. Thus, expression and basal tyrosine phosphorylation levels of p56^{lck} were measured in the human CD4+ tumor T cell lines CEM-T4, Jurkat E6-1, J45.01, H9, and Sup-T1. This was achieved by immunoprecipitating CD4 with the use of predetermined saturating concentrations of polyclonal anti-CD4 antibodies. As depicted in Fig. 1A, the relative abundance of p56^{lck} physically linked to CD4 is highly variable among lymphoid cell lines. Our measurements demonstrate that the levels of p56^{lck} protein expression are relatively low in Jurkat E6-1, J45.01, and H9, while p56^{lck} protein expression levels are quite substantial in CEM-T4 and Sup-T1 cells. These results were expected based on a previous report showing a marked variability in p56^{lck} protein expression levels in different lymphoid cell lines (Olszowy et al., 1995). Next, we monitored the basal tyrosine phosphorylation levels of CD4-associated p56^{lck} with the use of a monoclonal anti-phosphotyrosine antibody. Again, marked differences were seen among these different CD4-expressing T lymphoid cell lines (Fig. 1B). Values for phosphorylation of p56^{lck} on tyrosine ranged from undetectable levels in CEM-T4, Jurkat E6-1, and H9 to high levels in Sup-T1 cells. The highest basal tyrosine phosphorylation level of CD4-associated p56^{lck} was seen in J45.01, a cell line containing 8% of the level of the CD45 tyrosine phosphatase normally found in the parental Jurkat E6.1 cell line (Koretsky et al., 1991). This result was expected considering that p56^{lck} is a known substrate of p56^{lck} expressed in the parental Jurkat E6.1 cell line (Koretsky et al., 1991). This result was expected considering that p56^{lck} is a known substrate of p56^{lck} expressed in the parental Jurkat E6.1 cell line (Koretsky et al., 1991).

Induction of tyrosine phosphorylation of p56^{lck} by dimerization and multimerization of CD4

Previous studies have shown that antibody-mediated cross-linking of the CD4 receptor induces a rapid increase of tyrosine phosphorylation of p56^{lck} (Horak et al., 1990; Luo and Sefton, 1990; Veillette et al., 1989b,c, 1988). To determine whether occupancy of cell surface CD4 by antibodies can also affect phosphorylation of p56^{lck} in CEM-T4, these cells were treated either with SIM.4 alone (dimerization) or with cross-linked SIM.4 (multimerization), an anti-CD4 antibody known to be specific for the HIV-1 gp120-binding epitope on CD4 (McCallus et al., 1992). In this set of experiments, cells were incubated with an experimentally predetermined saturating concentration of SIM.4 for 30 min on ice and washed, half of the samples being further treated at 37°C with an excess of goat anti-mouse antibodies. Total cellular proteins were then prepared, and tyrosine-phosphorylation events were determined by using a combination of rabbit anti-CD4 antibodies with protein G-agarose beads. Immunoprecipitated proteins were separated by SDS-PAGE followed by transfer to nitrocellulose. The membrane was immunoblotted sequentially with anti-p56^{lck} (clone TP1CA1) (A) and anti-phosphotyrosine antibodies (clone 4G10) (B). Detection was achieved with the ECL Western blotting detection system (Amersham, Oakville, Ontario). The positions of molecular mass standards (in kilodaltons) are given on the left.
merization of the same molecule, is associated with a greater increase in the intensity of protein-tyrosine phosphorylation in CEM-T4 cells (Fig. 2).

Initially, we searched for HIV-1-mediated induction of tyrosine phosphorylation in total cellular proteins and found no significant changes in the pattern of protein-tyrosine phosphorylation (data not shown). We then decided to monitor tyrosine-phosphorylation levels of CD4-associated cellular proteins exclusively. This was achieved by incubating an immune complex made of rabbit anti-CD4 antibodies and protein G-agarose beads together with CEM-T4 cell lysates previously put in contact with HIV-1. This technical approach allowed us to detect a rapid increase (1 min) in protein-tyrosine phosphorylation levels of several CD4-associated cellular proteins that was sustained for 5 to 10 min (Fig. 3, top). An enhancement of phosphorylation on tyrosine residues of CD4-linked cellular proteins was not due to marked changes in the amounts of precipitated p56<sup>Lck</sup>, as demonstrated by immunoblotting of the same membrane with a monoclonal anti-p56<sup>Lck</sup> antibody (Fig. 3, bottom). Negative controls consisting of CEM-T4 cells incubated at 4°C and were analyzed by Western blotting. Dimerization of CD4 induced a slow and weak increase in total tyrosine phosphorylation (Fig. 2, left). However, multimerization of CD4 led to a rapid (within 1 min) and strong increase in protein-tyrosine phosphorylation of several substrates (Fig. 2, right). The reprobing of the membrane with an anti-p56<sup>Lck</sup> monoclonal antibody (clone TPICA1) revealed that p56<sup>Lck</sup> is one of the major tyrosine-phosphorylated proteins following antibody-mediated cross-linking of CD4 on CEM-T4 cells (data not shown). These data indicate that multimerization of CD4 on CEM-T4 cells induces greater alterations in the tyrosine phosphorylation of p56<sup>Lck</sup> and other cellular proteins than dimerization of cell surface CD4.

Protein-tyrosine phosphorylation of several cellular substrates following HIV-1-induced CD4 ligation

To explore the ability of HIV-1 to induce intracellular tyrosine phosphorylation events, we incubated CEM-T4 cells with HIV-1<sub>NL4-3</sub> for various periods of time at 37°C. We deliberately used unfrozen fresh cell-free supernatant from acutely infected cells as a source of HIV-1 due to the reported weak association between the external envelope gp120 and the transmembrane gp41 proteins, which frequently results in shedding of surface gp120 spikes from the virion (Gelderblom et al., 1985; McKeating et al., 1991). Indeed, it is logical to believe that a virion partly denuded of gp120 will lead to a diminished multivalency state of cell surface CD4 and can thus be considered a less potent inducer of signal transduction. This postulate is supported by our observations that antibody-mediated multimerization of CD4, compared to dimerization of the same molecule, is associated with a greater increase in the intensity of protein-tyrosine phosphorylation in CEM-T4 cells (Fig. 2).

FIG. 2. Anti-CD4-mediated protein-tyrosine phosphorylation in CEM-T4 cells. CEM-T4 cells were stimulated with anti-CD4 antibodies (clone SIM.4) followed by secondary cross-linking with goat anti-mouse IgG for various periods of time at 37°C. Signaling was terminated by detergent lysis at designated times and total cellular proteins were resolved on 10% SDS-PAGE gel. Detection was achieved using a primary monoclonal anti-phosphotyrosine antibody (clone 4G10) followed by peroxidase-conjugated goat anti-mouse antibodies. Numbers to the left indicate the molecular mass in kilodaltons.

FIG. 3. Kinetics of HIV-1-mediated tyrosine phosphorylation of CD4-associated proteins. CEM-T4 cells were incubated for various periods of time at 37°C with HIV-1<sub>NL4-3</sub>. After detergent lysis, immunoprecipitation was carried out with rabbit anti-CD4 antibodies. Immunoprecipitated proteins were separated by reducing SDS–PAGE electrophoresis followed by transfer to nitrocellulose. Proteins were immunoblotted sequentially with anti-phosphotyrosine (clone 4G10) (top) and anti-p56<sup>Lck</sup> antibodies (clone TPICA1) (bottom). Numbers to the left indicate the molecular mass in kilodaltons. One representative experiment of three is shown.
of integrated optical density at Time 0 (Fig. 4B). Tyrosine phosphorylation of p56\(^{\text{ck}}\) (pp56) was rapid and transient, reaching maximum phosphorylation levels within 1 min (3.5-fold increase) and returning to basal level at 15 min following incubation with HIV-1 (1.14-fold increase). A stronger increase of tyrosine phosphorylation was detected for pp60 with a 9.6-, 15.4-, and 8.6-fold enhancement at 1-, 5-, and 10-min time, respectively. Significant changes in tyrosine phosphorylation were also observed for pp76 with a 10.6-, 10.4-, and 6.8-fold increase at 1, 5, and 10 min, respectively, following incubation at 37\(^\circ\) with HIV-1\(_{NL4-3}\). The return to basal levels of tyrosine-phosphorylation was also detected at the 15-min time. Finally, the most dramatic changes in tyrosine-phosphorylation levels were seen for pp85 with a maximum value reached at 5 min postincubation with HIV-1\(_{NL4-3}\) (20.2-fold increase) and a 1.7-fold increase still detected at the 15-min time. In conclusion, antiphosphotyrosine immunoblotting of immunoprecipitated CD4-associated proteins revealed that HIV-1\(_{NL4-3}\) induces protein-tyrosine phosphorylation of several CD4-associated cellular proteins in CEM-T4 cells.

Identification of pp85 as the \(\alpha\) regulatory subunit of the PI 3-kinase

The PI 3-kinase enzymatic complex has been shown to be associated with p56\(^{\text{ck}}\) (Prasad et al., 1993; Vogel and Fujita, 1993). We thus attempted to determine whether pp85 could represent the p85\(^{\alpha}\) regulatory subunit of the PI 3-kinase by immunoblotting with an antibody that recognizes the candidate protein. After stripping and reprobing the membrane (Fig. 3) with polyclonal anti-PI 3-kinase p85\(^{\alpha}\) antibodies, a CD4-associated protein with a molecular mass of 85 kDa was detected (Fig. 5). Thus, these results strongly suggest that pp85 is indeed the p85\(^{\alpha}\) regulatory subunit of the PI 3-kinase. It is important to specify that comparable levels of p85\(^{\alpha}\) were found to be physically linked to CD4 following incubation of CEM-T4 cells with HIV-1\(_{NL4-3}\) (Fig. 5), similar to

![Graph A](image1.png)

**Fig. 4.** Quantitation of HIV-1-induced tyrosine phosphorylation of CD4-associated proteins. The autoradiograms from CEM-T4 cells incubated for various periods of time at 37\(^\circ\) with HIV-1\(_{NL4-3}\) were scanned and analyzed using a phosphorimager devised by the ImageQuant software package (Molecular Dynamics, Sunnyvale, CA). (A) Ratio of integrated optical density was determined as follows. Optical density for selected tyrosine-phosphorylated proteins was divided by the optical density for p56\(^{\text{ck}}\) immunoblot at each time point. (B) Results are shown relative to ratio of integrated optical density at Time 0.

1 has resulted in an inhibition of HIV-1-induced tyrosine phosphorylation (data not shown), indicating that the observed phenomenon is indeed resulting from the virus-binding step.

The relative band intensities (integrated optical density) for four major CD4-associated phosphotyrosyl proteins, which are modulated over time by the presence of HIV-1, are shown in Fig. 4A. The measurement for each selected tyrosine-phosphorylated protein has been divided by the value for p56\(^{\text{ck}}\) at each specific time point. Strong intensity changes in tyrosine phosphorylation were seen for pp85, pp76, pp60, and pp56. Fold enhancement of HIV-1-mediated induction of tyrosine phosphorylation was also determined by dividing the ratio of integrated optical density at 1, 5, 10, and 15 min by the ratio of integrated optical density at Time 0 (Fig. 4B). Tyrosine phosphorylation of p56\(^{\text{ck}}\) (pp56) was rapid and transient, reaching maximum phosphorylation levels within 1 min (3.5-fold increase) and returning to basal level at 15 min following incubation with HIV-1 (1.14-fold increase). A stronger increase of tyrosine phosphorylation was detected for pp60 with a 9.6-, 15.4-, and 8.6-fold enhancement at 1-, 5-, and 10-min time, respectively. Significant changes in tyrosine phosphorylation were also observed for pp76 with a 10.6-, 10.4-, and 6.8-fold increase at 1, 5, and 10 min, respectively, following incubation at 37\(^\circ\) with HIV-1\(_{NL4-3}\). The return to basal levels of tyrosine-phosphorylation was also detected at the 15-min time. Finally, the most dramatic changes in tyrosine-phosphorylation levels were seen for pp85 with a maximum value reached at 5 min postincubation with HIV-1\(_{NL4-3}\) (20.2-fold increase) and a 1.7-fold increase still detected at the 15-min time. In conclusion, antiphosphotyrosine immunoblotting of immunoprecipitated CD4-associated proteins revealed that HIV-1\(_{NL4-3}\) induces protein-tyrosine phosphorylation of several CD4-associated cellular proteins in CEM-T4 cells.

**Fig. 5.** Detection of CD4-associated pp85 in CEM-T4 cells incubated with HIV-1. Immunoprecipitated CD4-associated proteins from CEM-T4 cells incubated for various periods of time at 37\(^\circ\) with HIV-1\(_{NL4-3}\) were subsequently immunoblotted with rabbit anti-p85\(^{\alpha}\) antibodies. Numbers to the left indicate the molecular mass in kilodaltons, while the positions of p85\(^{\alpha}\) and immunoglobulin (Ig) heavy chains are indicated.
our results obtained with p56\textsuperscript{ck} (Fig. 3, bottom), and suggest no variation in the protein levels of p85\textsubscript{\alpha} following HIV-1-dependent CD4 cross-linking.

**DISCUSSION**

The major objective of this study was to investigate changes in tyrosine-phosphorylation levels of CD4-associated proteins following cross-linking of CD4 by whole HIV-1 particles. Our analytical study is justified by the debate surrounding the putative signal transduction events mediated following the interaction between the virus and its cell surface receptor, the CD4 glycoprotein. For example, we and others have shown that different sources of multimerizing agents, including HIV-1 particles, could down-regulate virion production (Benkirane et al., 1995a,b; Haffar et al., 1992; Moore et al., 1992; Tremblay et al., 1994). More recently, we have demonstrated that HIV-1 itself was also capable of down-modulating transcription of a reporter gene placed under the control of its own regulatory elements (long terminal repeat) (Bérubé et al., 1996). In contrast to these results, Benkirane et al. have reported that HIV-1-mediated CD4 multimerization rather induces virus replication (Benkirane et al., 1994). Another contrasting set of results regarding gp120- or virus-mediated CD4 cross-linking has also been documented and includes increase in intracellular calcium level; phosphatidylinositol hydrolysis; activation of AP-1; MAP-kinase, and phosphatidylinositol 4-kinase; as well as protein-tyrosine phosphorylation (Chirimurle et al., 1995; Cruikshank et al., 1990; HIVroz et al., 1993; Kornfeld et al., 1988; Schmid-Antomarchi et al., 1996), some of which have not been corroborated in other studies (Horak et al., 1990; Orloff et al., 1991).

Our analysis of CD4-dependent tyrosine phosphorylation was performed with CEM-T4, a cell line demonstrating a fairly high level of CD4-associated p56\textsuperscript{ck} but a low basal level of tyrosine-phosphorylated p56\textsuperscript{ck}. After addition of anti-CD4 antibodies to the CEM-T4 cell line, an increase in the level of tyrosine phosphorylation of total cell extracts was observed after 15 min and was markedly enhanced in terms of time and intensity following addition of anti-IgG antibodies. However, no such increase in tyrosine phosphorylation of total CEM-T4 extracts could be observed when HIV-1 was used as the multimerization agent in agreement with the results obtained by Horak et al. (1990). We next used anti-CD4-immunoprecipitated proteins from CEM-T4 incubated with HIV-1 based on the possibility that more subtle changes in tyrosine phosphorylation might then be perceived. An enhancement in tyrosine phosphorylation was now seen for four different phosphoproteins in a time lapse of less than 5 min. Immunoblot analysis of the CD4-immunoprecipitated complex in this study has permitted the identification of the tyrosine-phosphorylated protein p56\textsuperscript{ck}: a tyrosine kinase known to be activated after CD4 cross-linking by anti-CD4 antibodies and gp120 molecules (Goldman et al., 1994; HIVroz et al., 1993; Juszczak et al., 1991; Veillette et al., 1989a,c). Furthermore, this increase in tyrosine phosphorylation was not due to change in the levels of CD4-linked p56\textsuperscript{ck}. Measurement of p56\textsuperscript{ck} kinase activity was not determined in our study and we cannot be certain of a concomitant enhancement in tyrosine kinase activity. However, previous studies have reported that the binding of viral envelope proteins or anti-CD4 antibodies to CD4 triggers an increase in autophosphorylation of p56\textsuperscript{ck}, which is paralleled by an enhancement of its activity (Hivroz et al., 1993; Juszczak et al., 1991; Luo and Sefton, 1990; Soula et al., 1992; Veillette et al., 1991). The rapid increase in tyrosine phosphorylation of the other CD4-associated molecules after virus-induced CD4 multimerization argues for a possible role of p56\textsuperscript{ck} kinase activity in these events although modulation of other kinase(s) activity cannot be excluded.

Another CD4-associated molecule, which showed the most important increase in its phosphotyrosine content after the addition of HIV-1 particles, has been identified as most likely being the p85\textsubscript{\alpha} regulatory subunit of the PI 3-kinase enzyme complex. This is based on our results indicating that CD4 is found associated with the PI 3-kinase p85\textsubscript{\alpha} regulatory subunit. Our findings are supported by previous studies which have reported a physical association between p56\textsuperscript{ck} and PI 3-kinase in a CD4-linked form (Prasad et al., 1993; Vogel and Fujita, 1993). Similarly to p56\textsuperscript{ck}, no quantitative changes in the level of CD4-associated PI 3-kinase p85\textsubscript{\alpha} were detected following HIV-1-induced CD4 multimerization. To the best of our knowledge, this is the first report showing an increase in tyrosine phosphorylation of the PI 3-kinase p85\textsubscript{\alpha} subunit following CD4 multimerization mediated by whole HIV-1 particles. It should be noted that HIV-1-mediated cross-linking of CD4 on CEM cells has been previously reported to lead to activation of PI 4-kinase but not of PI 3-kinase (Schmid-Antomarchi et al., 1996). In this study, the use of a different cell line and serum starvation prior to incubation with HIV-1 might help explain such discrepant results.

On the basis of our findings and on the described p56\textsuperscript{ck}-PI 3-kinase interaction, we favor the hypothesis that the virus-mediated CD4 multimerization induces an increase in tyrosine phosphorylation of the PI 3-kinase p85\textsubscript{\alpha} regulatory subunit mediated by the activation of p56\textsuperscript{ck} kinase activity. Previous studies have implicated the PI 3-kinase in several signal transduction pathways (Fantl et al., 1992; Okada et al., 1994; Ruderman et al., 1990) and have suggested that an increase in tyrosine phosphorylation of the PI 3-kinase complex induces an activation of its enzymatic activity (Kaplan et al., 1987; Soltoff et al., 1992), which thus renders it a potential important element in CD4-mediated signal transduction pathways. The use of Wortmannin, a selective PI 3-kinase inhibitor (Nakanishi et al., 1992), and of a dominant negative construct of PI 3-kinase (Hara et al., 1994) will help
us to decipher the potential role of this enzyme in various CD4-dependent signal transduction pathways. Moreover, it will also permit assessment the involvement of PI 3-kinase in the virus-mediated repression of HIV-1 LTR-driven activity (Bérubé et al., 1996).

In the present study, two other CD4-associated cellular proteins have been shown to be phosphorylated on tyrosine residues by HIV-1, one of 60 kDa and another of 76 kDa. Recently, the binding of HIV-1 particles to CD4-bearing Jurkat cells was shown to lead to tyrosine phosphorylation and activation of Raf-1 kinase (Popik and Pitha, 1996), a 72- to 76-kDa cytoplasmic serine–threonine protein kinase. It is thus tempting to speculate that the 76-kDa tyrosine-phosphorylated substrate is Raf-1. The pp60 protein may represent the immunoreactive form of p56\(^{ck}\) that has been shown to migrate to approximately 60 kDa due to serine-phosphorylation events (Veillette et al., 1988). The inability to detect a specific band migrating at 60 kDa with the monoclonal anti-p56\(^{ck}\) antibody (Fig. 3, bottom) might be related to the fact that the amount of phosphorylated protein was sufficient to allow its detection by the anti-phosphotyrosine blot but was below the level of detection using an anti-p56\(^{ck}\) blot. Although the direct identification of these cellular substrates remains to be established, it seems likely that they also participate in CD4-mediated signaling and could represent putative substrates of p56\(^{ck}\) following the HIV-1-mediated CD4 multimerization. Identification of these two proteins is currently in progress.

CD4 cell surface molecules have been known to be important in T cell response to antigen presentation leading either to anergy or greater level of T lymphocyte activation (Bank and Chess, 1985; Eichmann et al., 1987; Emmrich et al., 1987; Julius et al., 1991; Tite et al., 1986). The biochemical cascade involved in these physiological changes are mostly dependent on p56\(^{ck}\) activity (Veillette et al., 1989a,b) and, on the basis of the presented data, could also implicate other CD4-associated proteins, including the PI 3-kinase. As mentioned above, cross-linking of CD4 molecules by gp120 molecules or HIV-1 particles can induce different cellular changes and can also regulate HIV-1 LTR-driven activity and virus replication (Benkirane et al., 1994; Bérubé et al., 1996; Haffar et al., 1992; Tremblay et al., 1994). The contradictions arising from some studies might be reminiscent of the various cellular components that are complexed to CD4, which included in the present study at least four different phosphoproteins. Variation in expression levels and/or the presence of different isoforms of these proteins depending on the cell lines studied might thus explain the possible controversial set of results obtained after CD4 cross-linking. Furthermore, the choice of the oligomerization agent might also be another important parameter leading to opposite results. The fact that, in total cell extracts, HIV-1 particles could not lead to noticeable changes in tyrosine phosphorylation while marked changes in levels of protein-tyrosine phosphorylation were induced by cross-linked anti-CD4 antibodies supports this notion. A further evidence is provided by the observation that gp120 molecules and different anti-CD4 antibodies can lead to distinct patterns of tyrosine phosphorylation of the SHC adaptor protein isoforms (Baldari et al., 1995).

It will thus be interesting to investigate whether the use of gp120 molecules or antibodies directed to various epitopes of the CD4 molecule could lead to different patterns of phosphorylation of CD4-associated proteins. These results might then help in our understanding of the pleiotropic and sometimes conflicting effects that are induced by the cross-linking of cell surface CD4.

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