MOLECULAR AND CELLULAR BIOLOGY, Nov. 1996, p. 6532–6541 Vol. 16, No. 11
0270-7306/96/$04.00+0

Binding of Human Immunodeficiency Virus Type 1 to CD4 Induces Association of Lck and Raf-1 and Activates Raf-1 by a Ras-Independent Pathway

WALDEMAR POPIK[1] AND PAULA M. PITHA[1,2]*

Oncology Center[1] and Department of Molecular Biology & Genetics,[2] The Johns Hopkins University School of Medicine, Baltimore, Maryland 21231

Received 6 June 1996/Accepted 17 July 1996

We have analyzed CD4-mediated signaling during the early stages of human immunodeficiency virus type 1 (HIV-1) infection. Binding of purified HIV-1 virions or recombinant HIV-1 glycoprotein gp120 to CD4 receptors resulted in association and tyrosine phosphorylation and activation of tyrosine kinase Lck and serine/threonine kinase Raf-1. The association between Lck and Raf-1 was mediated by stimulation of the CD4 receptors, since it was abolished by preincubation of the virus with soluble CD4 and was not detected in CD4-negative A201 T cells. However, the Lck–Raf-1 association was restored in A201 cells permanently transfected with human CD4 cDNA and stimulated with anti-CD4 antibodies. In addition, a catalytically active Lck was required for the association of Lck and Raf-1. Surprisingly, the CD4-mediated signaling, induced by the HIV-1 binding, did not result in stimulation of the Ras GTP-binding activity or its association with Raf-1, indicating that the signaling pathway generated by the HIV-1 binding is not identical to the classical Ras/Raf-1 pathway. Furthermore, overexpression of activated Raf-1 in Jurkat T cells stimulated the HIV long terminal repeat promoter activity and significantly enhanced HIV-1 replication. This suggests that the Lck–Raf-1 pathway, rapidly stimulated by the binding of HIV-1 or gp120 to CD4 receptors, may play an essential role in the transcriptional activation of the integrated HIV-1 provirus as well as in its pathogenicity.

The CD4 antigen is an integral membrane protein expressed predominantly on the surface of helper T lymphocytes. It serves as an adhesion molecule that stabilizes the major histocompatibility class II–T-cell receptor complex interaction (23, 33). In addition, by its association with the protein tyrosine kinase p56^{lck} (65, 81), CD4 provides costimulatory signals for T-cell receptor-mediated activation. CD4 has also been recently identified as a receptor for interleukin-16 (IL-16) (20), which suggests that it may also activate and transmit signals independently of the T-cell receptor. Thus, CD4, a primary receptor for the human immunodeficiency virus (HIV) (22, 46, 54), has the potential to affect cellular signaling upon binding of HIV-1.

The cytoplasmic domain of CD4 plays a critical role during early stages of HIV-1 infection in T cells, although it is not necessary for binding and subsequent internalization of the HIV-1 virions (7). A significant delay in HIV-1 replication has been demonstrated in cells expressing a truncated cytoplasmic domain of CD4 that resulted from the inability of the receptor to associate with p56^{lck} and to stimulate the nuclear expression of NF-κB (79). These results implicate CD4-associated tyrosine kinase Lck in the transduction of signals that may target T-cell-specific transcription factors and subsequently the expression of cellular genes. Although stimulation of the Lck activity by HIV-1/gp120 binding is well documented (38, 73, 88), other studies argue against HIV-1/gp120-induced signaling (39, 45). Therefore, the molecular mechanisms of the CD4-Lck-mediated signaling and its role in HIV-1 pathogenesis need further clarification.

The mechanism by which Lck generates intracellular signals upon HIV binding remains unclear. Lck has the potential to interact through its src homology-2 (SH2) and SH3 domains with various downstream target proteins including LckBP1 (78), phospholipase Cγ (82), mitogen-activated protein kinase (26), ZAP-70 (16), phosphatidylinositol 3- and 4-kinases (63), p95^{vav} (36), and Ras GAP (3). In addition, some proteins encoded by viruses infecting T cells, such as herpesvirus saimiri Tip protein (43) or HIV-1 accessory protein Nef (18), bind to Lck. However, little is known about cellular proteins which associate with and are activated by Lck in response to HIV-1 binding to CD4 receptors. One of them, the phosphatidylinositol 3-kinase, associates with Lck and becomes activated in response to HIV-1 envelope gp120 binding (63).

Activation of Raf-1 kinase has been implicated in the signaling pathways connecting the upstream receptor and nonreceptor tyrosine kinases to downstream serine/threonine kinases. At least three modes of Raf-1 activation have been characterized. First, Raf-1 can be activated by the receptor tyrosine kinases as a result of activation of Ras through adaptor protein-guanine nucleotide exchange factor complexes (14, 53, 74). Activated, GTP-bound Ras recruits Raf-1 to the plasma membrane (51, 75), where Raf-1 becomes fully activated, possibly by phosphorylation on tyrosine (27, 51, 55, 86). Second, certain isoforms of protein kinase C can activate Raf-1 by direct phosphorylation on serine (15, 47). Third, the nonreceptor tyrosine kinases (27), as well as receptor tyrosine kinases (40), can activate Raf-1 through mechanisms that do not involve Ras activation. Thus, the signals generated by the stimulation of the classical Ras/Raf pathway may not necessarily be identical to signals generated by Raf-1 in the absence of Ras activation (24, 85).

Activated Raf-1 triggers a kinase cascade by phosphorylating a dual-specificity threonine/tyrosine kinase Mek (mitogen-activated protein kinase kinase), which then phosphorylates mitogen-activated protein kinase/ERK (41). This pathway leads

* Corresponding author. Mailing address: The Johns Hopkins University Oncology Center, 418 N. Bond St., Baltimore, MD 21231-1001. Phone: (410) 955-8871. Fax: (410) 955-0840. Electronic mail address: parowe@welchlink.welch.jhu.edu.

to the induction of AP-1 activity by stimulating transcription of the c-*fos* gene and by phosphorylating the c-Jun protein (72). Raf-1 is also an important component of signal transduction pathways that lead to the activation of the NF-κB/Rel family of transcription factors (29).

We and others have shown that NF-κB plays a dominant role in the activation of both Tat-independent (2, 61, 62, 77) and Tat-dependent HIV-1 gene expression (2, 77). In addition, an NF-κB-binding motif in the HIV-1 long terminal repeat (LTR) has been identified as a Raf-1-responsive element (13, 31). These results suggest a direct link between activation of Raf-1 and transcriptional stimulation of the HIV-1 promoter. Activation of promoters of the cytokine genes expressed in HIV-1-infected T cells requires cooperation of several inducible transcription factors including NF-AT, AP-1, and NF-κB. While AP-1 and NF-κB are expressed by many cell types (3, 52), the NF-AT complex is specifically expressed in lymphocytes (19), and its assembly requires the presence of AP-1 (42, 58). Induction of NF-κB (7) and AP-1 (17) has been demonstrated in cells infected with HIV-1 and stimulated with HIV-1 envelope gp160, respectively. This may result in the aberrant cytokine expression observed in AIDS (21). Accordingly, the interaction of the HIV-1 gp120 with CD4 receptors was shown to induce IL-6 and IL-10, gamma interferon, tumor necrosis factor alpha, and upregulated expression of the Fas antigen (9, 59, 60). Therefore, the activation or alteration of the signaling pathway by binding of HIV-1/gp120 to CD4 receptors may have important consequences for the HIV-1-induced pathogenicity (6, 28, 30, 83).

In this report, we demonstrate that binding of HIV-1 or its envelope gp120 to CD4 receptors activates Lck and Raf-1 and induces their association through a Ras-independent pathway.

MATERIALS AND METHODS

Antibodies and reagents. Recombinant HIV-1 gp120 produced in a baculovirus expression system and rabbit anti-gp120 polyclonal antibodies were purchased from Intracel Corp. (Cambridge, Mass.). Mouse monoclonal anti-human CD4 (Q4120) and anti-human immunoglobulin G–peroxidase conjugate were from Sigma Immunochemicals. Anti-mouse and anti-rabbit immunoglobulin peroxidase antibodies and the enhanced chemiluminescence (ECL) kit were obtained from Amersham Life Science. Protein A/G Plus-Agarose and anti-Ras antibodies (Y13-238 and Y13-259) were obtained from Oncogene Science. Mouse monoclonal antiphosphotyrosine antibody PY20 was from Transduction Laboratories. The following antibodies were purchased from Santa Cruz Biotechnology: C-12, a rabbit polyclonal antibody raised against a peptide corresponding to the 12 carboxy-terminal amino acid residues of Raf-1; Lck (3A5), a mouse monoclonal antibody raised against a peptide corresponding to amino acid residues 1 to 222 of human Lck; and Lck (CT), rabbit polyclonal antibodies raised against a peptide corresponding to amino acids 476 to 505 within the carboxy-terminal domain of human Lck and antibodies against amino-terminal epitope of Sos (Sos1/2). A peptide substrate for Raf-1, Syntide-2 (PLARTLSV AGLPGKK), was also purchased from Santa Cruz Biotechnology.

Plasmids, transfections, and CAT activity assay. HIV LTR CAT plasmid contains the U3 and R region of the LTR from HIV-1 HXB2 inserted upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene. The HIV κBmt CAT plasmid contains point mutations (GGG→CTC) in both NF-κB sites of the HIV LTR. The HIV AP-1mt CAT contains the *Nde*I-*Xho*I-*Sal*I polylinker substituting for a deletion from −453 to −328. All these plasmids were obtained from J. C. Alwine (University of Pennsylvania) and were described previously (89). Plasmid Raf-BXB expressing the kinase domain of Raf-1 (CR3) was obtained from U. R. Rapp (National Cancer Institute, Frederick, Md.) (12). Plasmid pLNC:hrafER was obtained from M. McMahon (DNAX, Palo Alto, Calif.). Human CD4-expressing plasmids were obtained from J. Skowronski (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) (67). Jurkat cells were transfected by electroporation, cellular extracts were prepared 24 h after electroporation, and CAT activity was assayed as described previously (35).

Cell lines. Jurkat T cells, clone E6-1 (ATCC TIB-152), JCaM1.6 (ATCC CRL-2063), and A201 and A201.CD4 (obtained from K.-T. Jeang, National Institutes of Health) were maintained in RPMI 1640 (Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and gentamicin (50 μg/ml). The cells were cultured to a density of 0.5×10^6 to 1.0×10^6 cells per ml. Jurkat cell lines, JLNRaf, expressing activated Raf-1 were constructed by transduction of Jurkat cells with a murine replication-defective retroviral vector, pLNCX, containing a fusion gene of the Raf-1 (CR3) kinase domain and a human estrogen receptor (hER). pLNC:hrafER was described previously (68). Transduced cells were selected in the presence of G418 (1 mg/ml) and used as a pool in experiments. In addition, four clones expressing an enhanced level of Raf-1 were selected by a limiting-dilution method.

Viral stock and infection. An infectious viral stock of the NL4-3 clone of HIV-1 (1) was prepared by transfecting plasmid DNA into Jurkat cells by electroporation. Culture supernatants from infected cells, collected on day 7 after electroporation, were clarified by low-speed centrifugation and filtration through a 0.45-μm-pore-size filter. Virus was then pelleted by ultracentrifugation through a cushion of sucrose buffer (20% sucrose, 20 mM Tris [pH 7.4], 100 mM NaCl). The pelleted virus was resuspended in 500 μl of ice-cold RPMI–0.5% FBS and used immediately or kept frozen at −135°C until use. The virus titer was monitored by the reverse transcriptase activity assay (77) and the infectivity titer, based on the 50% tissue culture infectious dose, was assessed by a terminal-dilution microassay on C8166 cells. End point titers were determined by the observation of the virus-induced cytopathic effect. The 50% tissue culture infectious dose was calculated by the method of Reed and Muench (25). For infection, aliquots of 2×10^7 Jurkat cells in 1 ml of ice-cold RPMI–0.5% FBS were incubated for 1 h on ice with purified NL4-3 virus at a multiplicity of infection (MOI) of 0.5. After virus adsorption, the cells were incubated at 37°C for various times and then cooled on ice, washed with excess ice-cold phosphate-buffered saline, and collected by centrifugation.

Cross-linking of CD4 receptors by HIV-1 gp120. The cells were harvested, resuspended in ice-cold RPMI–0.5% FBS (2×10^7 cells per ml), and treated for 1 h at 4°C with recombinant HIV-1 gp120 (Intracel) at 20 μg/ml (63). The cells were washed once with excess ice-cold RPMI–0.5% FBS, treated with rabbit anti-gp120 polyclonal antibody (20 μg/ml) for 30 min at 4°C, and then incubated at 37°C for the indicated times.

Immunoprecipitation and Western blotting. Cell aliquots (2×10^7 cells) were solubilized in 1 ml of 1% Triton X-100 lysis buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4), 100 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, and 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) supplemented with the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), leupeptin (10 μg/ml), aprotinin (10 μg/ml), and soybean trypsin inhibitor (10 μg/ml) and the phosphatase inhibitors sodium orthovanadate (1 mM), sodium fluoride (25 mM), and β-glycerophosphate (20 mM). The lysates were clarified by centrifugation at 14,000 × *g*, and the supernatants were precleared for 1 h at 4°C with 50 μl of protein A/G Plus-Agarose (1:1) suspension. Protein concentrations of the precleared lysates were determined with the Pierce bicinchoninic acid protein assay kit. Cellular lysates (0.5 to 1 mg of protein) were incubated overnight at 4°C with 1 to 2 μg of appropriate antibodies and 50 μl of protein A/G Plus-Agarose. Immunoprecipitated complexes were washed five times with 1 ml of ice-cold 0.1% Triton X-100 lysis buffer, resuspended in 2× reducing sodium dodecyl sulfate (SDS) sample buffer, and boiled for 5 min. The proteins were resolved by SDS-PAGE (7.5 to 12.5% polyacrylamide), transferred to a nitrocellulose membrane, and probed with specific antibody. Bound antibodies were detected with the ECL system, using a secondary horseradish peroxidase-conjugated antibody. The Western blots (immunoblots) were reprobed with other antibodies as specified by the manufacturer (Amersham). For reprecipitation experiments, the immunoprecipitated proteins were released from protein A/G Plus-Agarose beads by boiling for 5 min in 50 μl of washing buffer supplemented with 2% SDS and 5% 2-mercaptoethanol, 1 ml of ice-cold lysis buffer was added to each tube, and, after centrifugation, supernatants were subjected to a new round of immunoprecipitation. Protein A/G Plus-Agarose-bound complexes were washed as described above and boiled in Laemmli reducing sample buffer, and proteins were resolved by SDS-PAGE and probed with specific antibodies.

Raf-1 immunocomplex kinase assay. Protein A/G Plus-Agarose–Raf-1 immune complexes, prepared as described above, were washed three times in lysis buffer, twice in washing buffer [10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.0), 0.1 M NaCl, 0.5% Nonidet P-40], and once in kinase buffer (10 mM PIPES [pH 7.0], 0.1 M NaCl, 10 mM $MgCl_2$, 2 mM $MnCl_2$). The complexes were resuspended in 25 μl of kinase buffer supplemented with 10 μCi of [γ-^{32}P]ATP, 10 μM ATP, and 200 μM Syntide-2. After 20 min at 30°C, the protein A/G Plus-Agarose was pelleted, and the supernatants were mixed with an equal volume of 10% trichloroacetic acid and 10 μl of bovine serum albumin (BSA; 10 mg/ml) as a carrier. The tubes were then incubated for 10 min on ice. Precipitated proteins were removed by centrifugation, and 25-μl portions of the supernatants were spotted on Whatman P81 phosphocellulose paper. The paper was washed five times with 75 mM phosphoric acid, dried, and counted. Background was corrected for by subtracting the counts per minute bound to phosphocellulose paper in assays performed in the absence of the enzyme from those bound in assays performed in the presence of enzyme.

Lck autokinase activity assay. Immunoprecipitates of Raf-1 were prepared as described above. After being washed three times with lysis buffer, twice with wash buffer, and once with kinase buffer, immune complexes were incubated for 20 min at 30°C in 25 μl of kinase buffer supplemented with 10 μCi of [γ-^{32}P]ATP. The labeled immunoprecipitates were washed three times with ice-cold kinase buffer, the immunoprecipitated proteins were released from protein A/G Plus-Agarose beads by boiling for 5 min in 50 μl of washing buffer supplemented with 2% SDS and 5% 2-mercaptoethanol, and then 1 ml of ice-cold lysis buffer was

added to each tube. After centrifugation, supernatants were subjected to a new round of immunoprecipitation with anti-Lck(3A5) antibody. Protein A/G Plus-Agarose-bound complexes were washed as described above and boiled in Laemmli reducing sample buffer, and labeled proteins were resolved by SDS-PAGE (10% polyacrylamide) and detected by autoradiography.

Ras GTP-binding activity assay. Jurkat cells were depleted of cellular phosphate by extensive washing with phosphate-free RPMI 1640 supplemented with 2% dialyzed fetal calf serum (complete medium). After a 15-min incubation in complete phosphate-free RPMI, the cells were metabolically labeled for 2 h at 37°C with 2 mCi of $^{32}P_i$ (Amersham PBS13) and then treated with 20 ng of phorbol ester (tetradecanoyl phorbol acetate [TPA]) per ml or incubated with HIV-1 or gp120 on ice for 1 h as described above. The cells were then transferred to 37°C and incubated for various times. They were harvested by centrifugation and lysed in 1 ml of ice-cold lysis buffer (50 mM Tris [pH 7.5], 5 mM $MgCl_2$, 1% Triton X-114, 100 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol, 1 mg of BSA per ml, 10 μg of trypsin inhibitor per ml, 10 μg of aprotinin per ml, and 10 μg of leupeptin per ml) as described previously (37). Clarified cell lysates were transferred to new tubes containing 100 μl of 5 M NaCl and then warmed to 37°C for 2 min to promote phase partition of the detergent. After brief centrifugation, the aqueous supernatants were discarded and the lower, detergent layers were redissolved in 1 ml of cold lysis buffer containing 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 1% Triton X-100, 0.5% deoxycholate, 0.05% SDS, 0.5 M NaCl, 1 mM EGTA, 1 mM dithiothreitol, 1 mg of BSA per ml, 10 mM benzamidine, 10 μg of trypsin inhibitor per ml, 10 μg of aprotinin per ml, and 10 μg of leupeptin per ml. The samples were then precleared for 10 min with 50 μl of a 1:1 slurry of Sepharose beads. The final supernatants were immunoprecipitated with anti-Ras Y13-259 antibody. The resulting precipitates were washed six times with 1 ml of ice-cold buffer (50 mM HEPES [pH 7.5], 0.5 M NaCl, 0.1% Triton X-100, 0.005% SDS, 5 mM $MgCl_2$). The Ras-bound nucleotides were eluted from pellets with 15 μl of 5 mM dithiothreitol–5 mM EDTA–0.2% SDS, 0.5 mM GTP–0.5 mM GDP at 68°C for 20 min. The eluted samples were loaded onto polyethyleneimine (PEI)-cellulose thin-layer chromatography plates, and nucleotides were separated by chromatography in a mixture of 1.2 M ammonium formate and 0.8 M HCl. Separated nucleotides, GTP and GDP, were visualized by autoradiography and quantitated by counting in a liquid scintillation counter (37).

RESULTS

Binding of HIV-1 virions to Jurkat T cells activates serine/threonine Raf-1 kinase activity. One of the earliest events in the CD4 receptor-mediated signal transduction is activation of the Lck tyrosine kinase activity noncovalently associated with CD4 receptors (34, 81). Since serine/threonine kinase Raf-1 was shown to transduce cellular signals from both receptor- and nonreceptor tyrosine kinases (36, 56, 57, 70), we examined whether Raf-1 participates in the signal transduction induced by binding of HIV-1 to the CD4 receptors. Therefore, we asked whether the binding of HIV-1 to CD4 receptors results in the activation of Raf-1 kinase. To measure the activation of Raf-1 kinase, we used in vitro kinase assays with the synthetic substrate Syntide-2 (amino acid sequence PLARTLSVAGLP GKK), which served as an exogenous substrate for Raf-1 (48). Since Syntide-2 can be phosphorylated only on serine and/or threonine, it serves as a substrate for serine/threonine kinases but not tyrosine kinases like Lck. Cellular lysates prepared from unstimulated Jurkat cells and the cells incubated with purified HIV-1 virions were immunoprecipitated with anti-Raf-1 (C-12) and then analyzed for the presence of serine/threonine kinase activity (Fig. 1). A ca. 2.5-fold increase in the levels of kinase activity was observed 5 min after HIV-1 binding. The levels of kinase activity progressively decreased after longer incubation with the virus; after 30 min, the kinase levels detected were the same as in unstimulated cells. We then examined whether the serine/threonine kinase activity was present in the CD4 receptor complex. Immunoprecipitation of the cell lysates with anti-CD4 antibodies (Q4120) showed more than a threefold increase in the level of serine/threonine kinase activity in CD4 immunoprecipitates 5 min after HIV-1 binding to the cells (Fig. 1). Thus, the kinetics of the serine/threonine kinase induction detected in Raf-1 and CD4 immunoprecipitates were similar. These results indicate that HIV-1 binding rapidly activates Raf-1 kinase which may be associated with the CD4 receptor complex. However, we cannot exclude the possibility that in addition to Raf-1, another serine/threonine kinase also associates with CD4 and becomes activated upon HIV-1 binding.

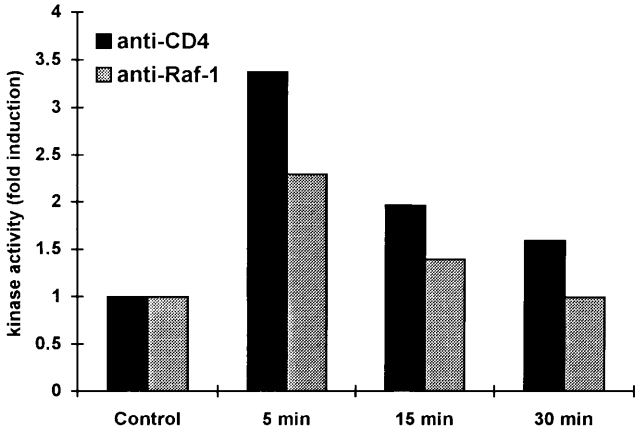

FIG. 1. Stimulation of the Raf-1 serine/threonine kinase activity by HIV-1 binding. Jurkat cells were incubated with purified HIV-1 (MOI, 0.5) for the indicated times at 37°C and then lysed in 1% Triton X-100 lysis buffer. The CD4 and CD4-associated proteins were immunoprecipitated from lysates of unstimulated (Control) or HIV-stimulated cells with monoclonal antibody Q4120 (anti-CD4). Raf-1 was precipitated with polyclonal antibody C-12 (anti-Raf-1). The immunoprecipitates were assayed for the serine/threonine kinase activity with a synthetic substrate Syntide-2 (amino acid sequence PLARTLSVAGLPGKK) as described in Materials and Methods.

Enhanced association between Raf-1 and Lck in response to CD4 binding. Since the cytoplasmic tail of CD4 interacts with an amino-terminal domain of the protein tyrosine kinase Lck (81) and since serine/threonine Raf-1 kinase activity was detected in CD4 immunoprecipitates, we examined whether the binding of HIV-1 or gp120 induces the association between Raf-1 and Lck. Lysates from unstimulated (control) Jurkat cells and cells stimulated with purified HIV-1 virions (Fig. 2A) or recombinant HIV-1 envelope gp120 (Fig. 2B) were immunoprecipitated with anti-Raf-1 (C-12) antibodies. The presence of Lck in these precipitates was determined by Western hybridization with monoclonal anti-Lck (3A5) antibodies. The results presented in Fig. 2A and B show an association between Raf-1 and Lck kinases in unstimulated cells; however, incubation with HIV-1 or gp120 for 5 min further increased (about threefold) the association between Lck and Raf-1. Higher levels of these associating kinases were observed in cells in which CD4 receptors were cross-linked with monoclonal anti-CD4 (Q4120) antibodies (Fig. 2C).

Several observations indicated that induction of the Raf-1–Lck association was mediated by the CD4 receptors. Preincubation of HIV-1 virions with soluble CD4 abrogated the stimulatory effect of HIV-1 (Fig. 3A). Furthermore, in A201 T cells expressing only very low levels of CD4 receptors (Fig. 3B), stimulation with anti-CD4 antibodies (Q4120) resulted only in very low levels of association between Lck and Raf-1. In contrast, in A201 cells transduced with the CD4 cDNA (A201/CD4) (8), stimulation with anti-CD4 antibodies resulted in a significant increase in the association between Raf-1 and Lck (Fig. 3B). Reprobing of the same membranes with anti-Raf-1 antibody shows constant levels of Raf-1 proteins in all samples.

To establish the specificity of the association between Raf-1 and Lck, we examined whether the anti-Lck (CT) antibody 2102 that recognized the carboxy-terminal domain of Lck (amino acids 476 to 505) could coprecipitate both Lck and

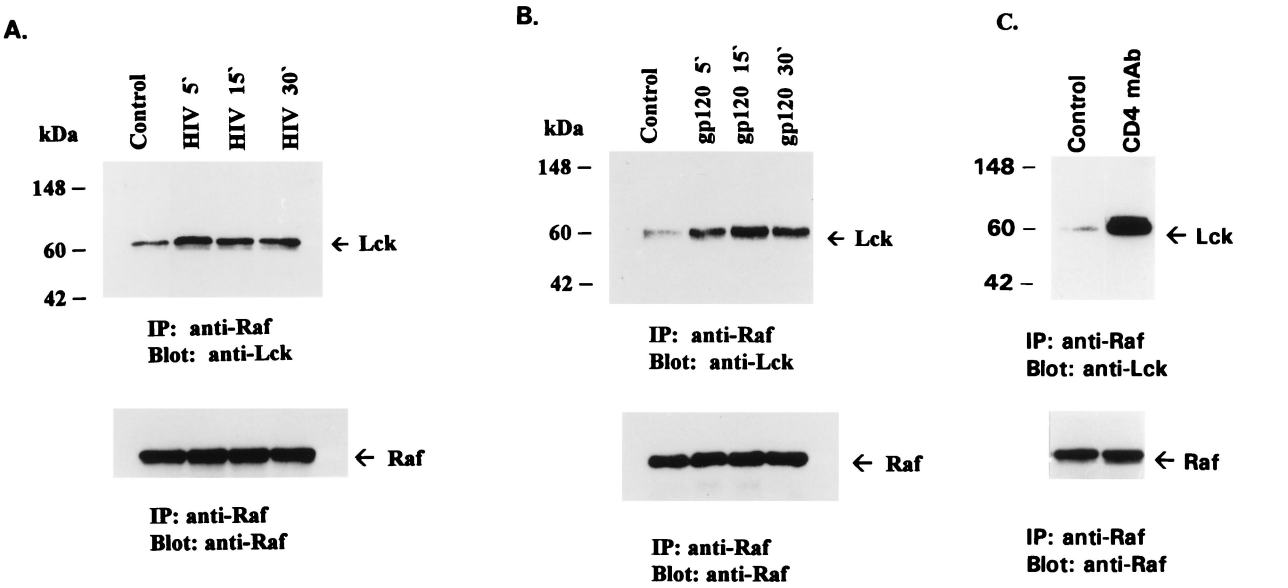

FIG. 2. Enhanced association between Raf-1 and Lck in response to binding of HIV-1, gp120, or anti-CD4 antibodies to CD4 receptor. Jurkat cells were incubated for the indicated times (for 5 min with anti-CD4) with HIV-1 (MOI, 0.5) or gp120 (30 μg/ml). They were then washed and lysed in 1% Triton X-100 lysis buffer, and cellular lysates were subjected to immunoprecipitation (IP) with anti-Raf-1 antibodies. Immunoprecipitated proteins were resolved by SDS-PAGE (10% polyacrylamide), transferred to a nitrocellulose membrane, and probed with anti-Lck antibody. The blots were stripped and reprobed with anti-Raf antibodies and detected by the ECL method with secondary antibodies coupled to horseradish peroxidase. The positions of molecular mass markers (in kilodaltons) and immunoprecipitated proteins are indicated. mAb, monoclonal antibody.

Raf-1. While Raf-1 was detected in anti-Lck (CT) immunoprecipitates in unstimulated cells (Fig. 4A), a further (about twofold) increase in the level of Raf-1 associated with Lck was observed in cells incubated for 5 min with HIV-1. Immunoprecipitation of Lck and Raf-1 by anti-Lck antibody was prevented in the presence of an excess of a peptide (Lck P) used as an immunogen to generate anti-Lck (CT) antibodies. Thus, we conclude that the association between Lck and Raf-1 is significantly increased upon HIV-1 binding to CD4 receptors, suggesting that Raf-1 may play a role in transmitting signals generated by the interaction of HIV-1 with CD4.

HIV-1 binding to Jurkat T cells results in a transient tyrosine phosphorylation of Lck and associated Raf-1. We examined whether the increased association between Raf-1 and Lck induced by binding of HIV-1 results in an increased tyrosine phosphorylation and activation of Raf-1. A nitrocellulose membrane containing the resolved proteins immunoprecipitated with anti-Lck (CT) antibodies and probed previously with anti-Raf-1 antibody (Fig. 4A) was stripped and reprobed with antiphosphotyrosine antibody PY20. As shown in Fig. 4B, in uninfected cells, the tyrosine phosphorylation of Raf-1 associated with Lck was detected at a very low level. In contrast, after HIV-1 binding, phosphorylation of Raf-1 on tyrosine was transiently increased and reached its maximal level 5 min after HIV-1 binding. This observation, together with the parallel increase in association between Lck and Raf-1, suggests that Raf-1 may be a direct target for activated Lck. Since only a fraction of the cellular Lck pool is associated with CD4 receptors (71), we examined whether the fraction of Lck that is associated with Raf-1 is also phosphorylated on tyrosine after HIV-1 binding. Cellular lysates were precipitated with anti-Raf-1 antibodies, and immunoprecipitated proteins were then subjected to a second round of precipitation with anti-Lck antibodies. Tyrosine phosphorylation of Lck was detected by Western analysis with antiphosphotyrosine PY20 antibodies (Fig. 4C). The results show that Lck, associated with Raf-1, was also transiently phosphorylated in response to HIV-1 binding, with the phosphorylation reaching a maximal level at 5 min after HIV-1 binding and decreasing thereafter. The autokinase activity assay performed on a fraction of anti-Raf-1-precipitated proteins (Fig. 4D) showed that Lck, associated with

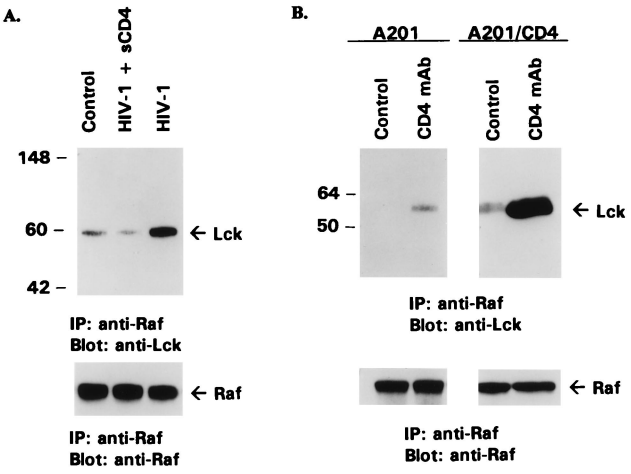

FIG. 3. The Lck-Raf-1 association is mediated by the CD4 receptors. (A) Pretreatment of HIV-1 virions with soluble CD4 (sCD4) abrogates Lck–Raf-1 association. Jurkat cells were stimulated for 5 min at 37°C with HIV-1 preincubated for 60 min at 37°C either with (HIV-1 + sCD4) or without (HIV-1) soluble CD4 (10 μg/ml) or left untreated (Control). (B) Expression of the CD4 cDNA in CD4-negative cells restores Lck–Raf-1 association. CD4-negative A201 cells and A201 cells permanently transfected with CD4 cDNA (A201/CD4) were incubated with anti-CD4 (Q4120) monoclonal antibodies (mAb) (10 μg/ml) for 5 min at 37°C or left untreated (Control). Cellular lysates were immunoprecipitated (IP) with anti-Raf-1 antibodies, and coprecipitated Lck was visualized as described in the legend to Fig. 2. Blots were reprobed with anti-Raf-1 antibodies. Molecular weight markers (in thousands) and immunoprecipitated proteins are indicated.

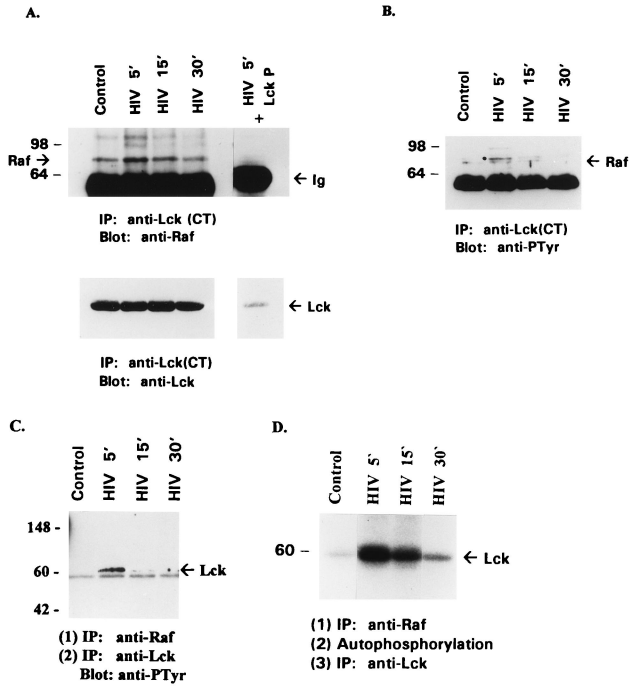

FIG. 4. Transient tyrosine phosphorylation of Lck and associated Raf-1 upon binding of HIV-1 to Jurkat T cells. Jurkat cells were incubated with HIV-1 (MOI, 0.5) for the indicated times and washed, and cellular lysates were prepared as described in Materials and Methods. (A) Raf-1 was precipitated with anti-Lck (CT) antibodies that recognize the carboxy-terminal (amino acids 476 to 505) region of Lck. Precipitated proteins were resolved by SDS-PAGE (10% polyacrylamide) and probed with anti-Raf-1 antibody (C-12). To prove the specificity of the Lck–Raf-1 association, the cellular lysates obtained from cells stimulated for 5 min with HIV-1 were also precipitated with anti-Lck (CT) antibodies in the presence of Lck peptide (Lck P, 500 μg/ml) used as an immunogen to generate these antibodies. As a control for protein loading, the blot was stripped and reprobed with anti-Lck (3A5) antibodies. (B) Raf-1 associated with Lck is transiently phosphorylated on tyrosine. A nitrocellulose membrane with resolved proteins immunoprecipitated with anti-Lck (CT) antibodies was probed with antiphosphotyrosine (anti-pTyr) antibody PY20. Tyrosine-phosphorylated Raf-1 is marked. (C) Lck associated with Raf-1 is transiently phosphorylated on tyrosine. Proteins immunoprecipitated with anti-Raf-1 antibodies were boiled, reprecipitated with anti-Lck antibodies, and probed with antiphosphotyrosine PY20. (D) Enhanced autophosphorylation activity of Lck associated with Raf-1. Proteins immunoprecipitated with anti-Raf-1 antibodies were subjected to autokinase activity assay, and ^{32}P-labeled immunoprecipitates were boiled and reprecipitated with anti-Lck (3A5) antibody as described in Materials and Methods. Immunoprecipitated ^{32}P-labeled proteins were resolved by SDS-PAGE (10% polyacrylamide) and detected by autoradiography. The positions of molecular weight markers (in thousands) and immunoprecipitated (IP) Lck and Raf-1 are indicated. Ig, immunoglobulin heavy chain.

Raf-1 in response to HIV-1 binding, was not only phosphorylated on tyrosine but also activated. Together, these results suggest that the association of Raf-1 with activated Lck results in tyrosine phosphorylation of Raf-1, indicating that Raf-1 can transmit signals generated by binding of HIV-1 to CD4 receptors.

A catalytically active Lck kinase domain is required for association with Raf-1. Elimination of the kinase domain of Lck does not completely abolish the coreceptor function of CD4-Lck complex (87). We therefore examined whether a catalytically active kinase domain of Lck is required for its association with Raf-1 by using a mutant of Jurkat cells, JCaM1.6, expressing a truncated and kinase-inactive form of Lck (76). We showed that HIV-1 binding to JCaM1.6 cells did not stimulate Lck kinase activity (autophosphorylation) (Fig. 5A). In addition, stimulation of these cells with anti-CD4 monoclonal antibody for 5 min did not result in increased association between Lck and Raf-1 (Fig. 5B). However, to rule out the possibility that this effect results from a low level of CD4 receptors expressed by the cells, JCaM1.6 cells were transfected with the wild-type human CD4 cDNA (67) and after 2 weeks in culture, cells expressing CD4 antigen were selected with Dynabeads M-450 (magnetizable polystyrene beads coated with monoclonal antibodies specific for the CD4). After selection, the cells were released from the beads with DETACHaBEAD as recommended by the manufacturer (Dynal), washed with complete medium, and cultured for another week before use in experiments. Selected cells were then stimulated with anti-CD4 monoclonal antibody for 5 min. As shown in Fig. 5C, Lck was not detected in anti-Raf-1 immunoprecipitates, suggesting that the catalytically active kinase domain of Lck was required for the association between Lck and Raf-1.

Association between Lck and Raf-1 does not involve Ras. It has been recently demonstrated that the active GTP-bound Ras directly recruits Raf-1 to the plasma membrane, where Raf-1 becomes fully activated, possibly by phosphorylation on tyrosine (55). To determine whether the observed association between Lck and Raf-1 is mediated by activated Ras, we analyzed the activation of Ras by measuring the guanine nucleotide levels bound to p21 Ras in $^{32}P_i$-labeled Jurkat cells which were either untreated (control) or stimulated with purified HIV-1 or gp120. Stimulation with TPA was used as a positive control. As shown in Fig. 6, the amount of GTP bound to Ras was significantly increased (2.7-fold) in Jurkat cells stimulated with TPA; however, stimulation with HIV-1 or gp120 did not result in a significant increase of GTP bound to Ras. To further analyze whether Ras is involved in the CD4-mediated signaling in Jurkat cells, cells were stimulated with anti-CD4 (Q4120) antibodies that induced the association between Raf-1 and Lck (Fig. 2C). Cellular lysates were immunoprecipitated with anti-Raf-1, anti-SOS, and anti-Ras (Y13-238) antibodies, and the presence of Raf-1 in immunoprecipitates was detected by Western blotting (Fig. 7A). It was previously shown that Y13-238 did not interfere with the interaction between Ras and Raf-1 (37). While we have shown that TPA stimulated the association between Ras and Raf-1 (Fig. 7B), we could not detect the presence of Raf-1 in either anti-Ras or anti-Sos immunoprecipitates. Similarly, Ras was not detected in anti-Raf or anti-Sos precipitates, although Raf-1, Ras, and Sos were efficiently immunoprecipitated. From these results, we conclude that Ras is not present in the complex with Raf-1 after CD4 receptor oligomerization in Jurkat T cells and does not participate in the activation of Raf-1.

Constitutively activated Raf-1 stimulates HIV-1 promoter activity and HIV-1 replication in Jurkat T cells. We next analyzed the significance of Raf-1 activation for HIV-1 replication. The Raf-1 responsive element has been located in the region of the HIV-1 LTR containing NF-κB-binding motifs, and a constitutively active Raf-1 has been shown to stimulate HIV-1 promoter activity (31). We extended this observation by showing that in Jurkat T cells, activated Raf-1 (Raf-BXB) synergizes with viral Tat transactivator in the stimulation of the HIV-1 promoter activity. Point mutations in the NF-κB-binding sites but not in the AP-1-binding element of the HIV-1 promoter completely abolished the observed synergism (Fig. 8). To further analyze the significance of the Raf-1-induced signaling pathway in viral replication, we developed Jurkat cell lines (JLNRaf) transduced with a replication-defective retroviral vector, LNCX, carrying a constitutively activated form of Raf-1 (68). As shown in Fig. 9A, the rate of HIV-1 replication, assayed by measuring the virion-associated reverse transcrip-

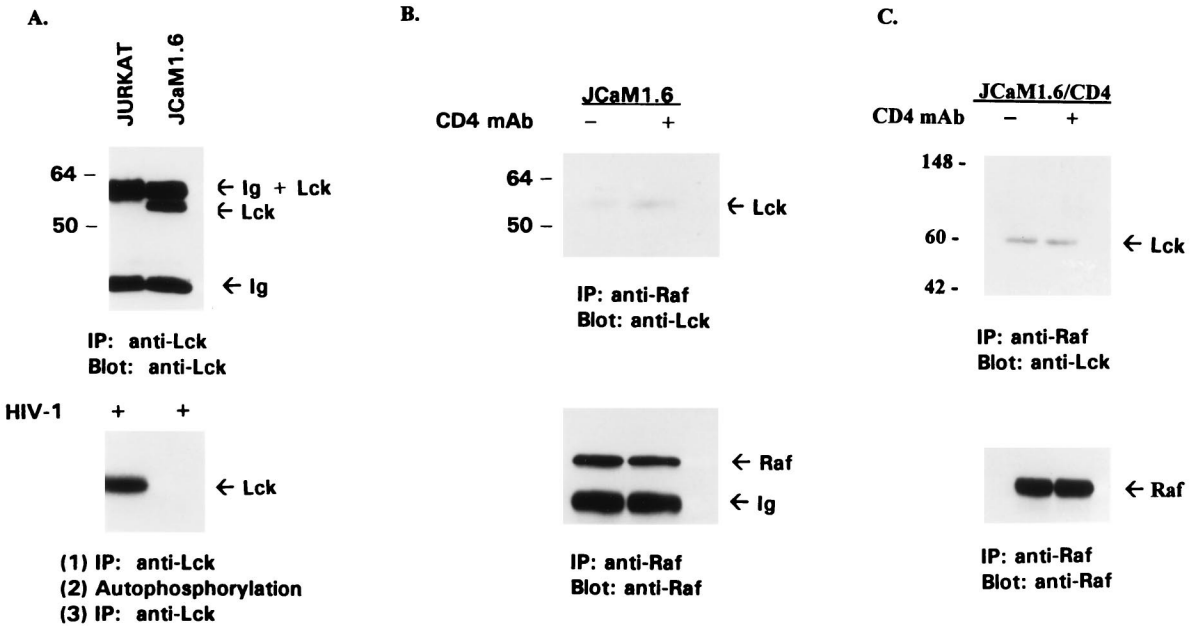

FIG. 5. A catalytic tyrosine kinase domain of Lck is required for association with Raf-1. (A) JCaM1.6 cells expressing truncated Lck have no kinase activity. Lysates prepared from unstimulated or HIV-1-stimulated (5 min) (MOI, 0.5) Jurkat and JCaM1.6 cells were immunoprecipitated with anti-Lck antibodies. Precipitates from unstimulated cells were resolved by SDS-PAGE (10% polyacrylamide), transferred to nitrocellulose, and blotted with anti-Lck antibodies (upper panel). Precipitates from stimulated cells were subjected to autokinase assay (lower panel) and reprecipitated with anti-Lck antibodies. (B) CD4 oligomerization does not induce Lck–Raf-1 association in Lck kinase-negative JCaM1.6 cells. Cell lysates prepared from cells stimulated with anti-CD4 monoclonal antibody (mAb) Q4120 for 5 min were immunoprecipitated with anti-Raf-1 antibodies. Proteins were resolved by SDS-PAGE (10% polyacrylamide) and blotted with anti-Lck antibodies. Stripped blots were rehybridized with anti-Raf-1 antibodies. (C) Expression of CD4 receptors in JCaM1.6 cells does not restore Lck–Raf-1 association after oligomerization with anti-CD4 antibodies. JCaM1.6 cells transfected with human CD4 cDNA (JCaM/CD4) were selected with Dynabeads M-450 (as described in the text) and stimulated with anti-CD4 antibodies. Cell lysates were immunoprecipitated with anti-Raf-1 antibodies, and Lck interacting with Raf-1 was detected by Western blot hybridization with anti-Lck antibodies. The positions of molecular weight markers (in thousands) and immunoprecipitated (IP) Lck and Raf-1 are indicated. Bands corresponding to immunoglobulin chains (Ig) are marked.

tase activity, was significantly increased in cells overexpressing Raf-1 (JLNRaf) compared with parental Jurkat cells. Similarly, the analysis of the level of viral proteins expressed in Jurkat cells and four selected JLNRaf cell lines showed a significant increase in the level of HIV-1 proteins 9 days postinfection in all JLNRaf clones tested when compared with parental Jurkat cells (Fig. 9B). These results indicate that activated Raf-1 upregulates HIV-1 replication in Jurkat T cells. The stimulation of HIV-1 replication in JLNRaf cells was not limited to the laboratory strain NL4-3 but was also observed with several primary isolates (49) (data not shown).

DISCUSSION

We have shown in this study that oligomerization of the CD4 receptors by purified HIV-1 virions or gp120/anti-gp120 complexes results in tyrosine phosphorylation and activation of both protein tyrosine kinase Lck and serine/threonine kinase Raf-1 and increases their association. These results suggest that Raf-1 may be a direct target for Lck and thus participate in signal transduction induced by HIV-1 binding to CD4 receptors. Raf-1 was previously shown to be a component of the signaling cascade induced by several cytokines and growth factors such as IL-2 (64, 80), IL-3, granulocyte-macrophage colony-stimulating factor (44), IL-9 (10), macrophage colony-stimulating factor (5), platelet-derived growth factor (57), epidermal growth factor (4), and insulin (48).

The CD4 receptor plays a critical role in the increased association between Lck and Raf-1. The association between Lck and Raf-1 was abolished in the presence of soluble CD4 and was not detected in CD4-negative A201 cells. Transfection of these cells with human CD4 cDNA restored the CD4-mediated association between Lck and Raf-1. The association between these two kinases was not specific for HIV-1 virions but could be also demonstrated in response to CD4 oligomerization by gp120/anti-gp120 complexes or by antibodies against CD4. However, the Lck–Raf-1 association induced as a response to binding of anti-CD4 antibodies was significantly higher than that induced by binding of HIV-1 or gp120/anti-gp120 complexes. This may be due to a higher affinity of binding of anti-CD4 antibodies and/or may be related to the fact that the epitope recognized by the anti-CD4 antibodies (amino acids 1 to 183) encompasses, in addition to the domain D1 recognized by HIV-1 and gp120, the domain D2 of the CD4 receptor.

Several observations indicate that the activation of Raf-1 upon binding of HIV-1 to CD4 receptors does not proceed through the classical Ras/Raf-1 signaling pathway. First, we were unable to show any significant increase in the level of Ras-bound GTP after binding of HIV-1 or gp120 whereas treatment with TPA, known to activate the Ras pathway, resulted in about threefold stimulation of the GTP-binding activity of Ras. Second, no direct association of Ras with Raf-1 or guanine nucleotide exchange factor, Sos, could be detected in cells stimulated with anti-CD4 antibodies or after HIV-1 binding (data not shown). These data indicate that Ras is not present in the Lck/Raf-1 complex and that tyrosine phosphorylation of Raf-1 and its activation are mediated by Lck. Recently, the presence of a cellular tyrosine kinase in HIV-1 virions was demonstrated (32), and the HIV-1-encoded matrix MA protein was shown to be a substrate for the virion-associated tyrosine kinase. However, it is unlikely that the observed

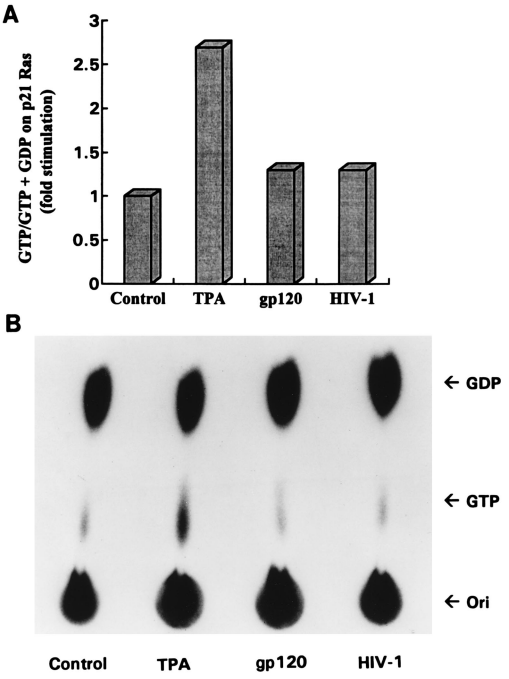

FIG. 6. Binding of HIV-1 and gp120 to CD4 receptors does not affect GTP-binding activity of Ras. Jurkat cells (control or stimulated with TPA, gp120, or HIV-1) were metabolically labeled with $^{32}P_i$. Cell lysates were immunoprecipitated with anti-Ras (Y13-259) antibodies, and nucleotides eluted from these immunoprecipitates were analyzed by PEI-cellulose thin-layer chromatography as described in Materials and Methods. Separated nucleotides, GTP and GDP, were visualized by autoradiography (B) and quantitated by counting in a liquid scintillation counter (A). Ori, origin.

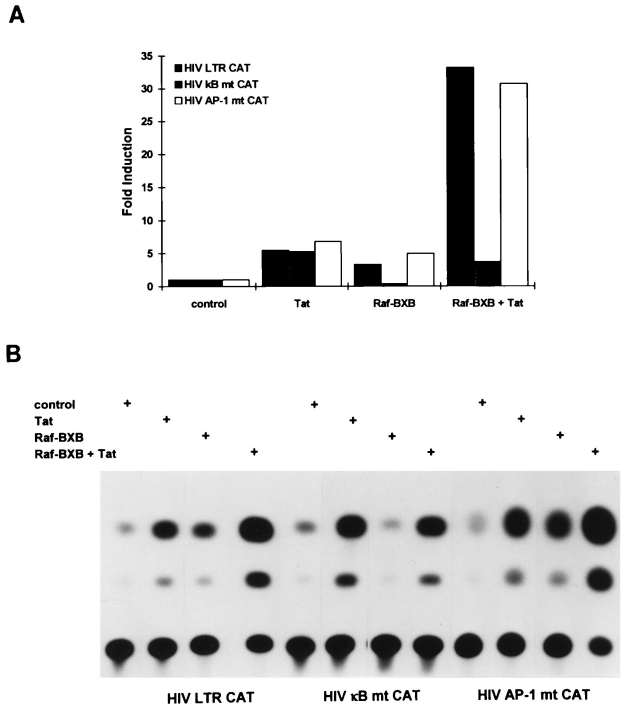

FIG. 8. Activated Raf-1 synergizes with Tat in the stimulation of the HIV-1 promoter activity. Jurkat cells were cotransfected with CAT reporter constructs (5 μg), HIV-1 LTR (HIV LTR CAT), or constructs in which HIV-1 LTR contained either mutated NF-κB sites (HIV kB mt CAT) or AP-1 sites (HIV AP-1 mt CAT), together with plasmid expressing Tat protein (CMV-Tat, 0.5 μg) and/or active Raf-1 (Raf-BXB, 10 μg). Cell extracts were harvested 24 h after transfection, and CAT activity (B) was determined as described in Materials and Methods. The ratio of the percent conversion induced in the presence of Tat and/or Raf-BXB over the percent conversion of unstimulated cells (fold induction) is also shown (A).

activation of the Lck/Raf-1 kinase pathway is triggered by this virion-associated kinase(s), since it is also induced by recombinant gp120/anti-gp120 complexes as well as monoclonal anti-CD4 antibodies. It is possible that the cellular Lck–Raf-1 signaling pathway induced by binding of HIV-1 to CD4 receptors is further modulated by the HIV-1 virion-associated kinases. In addition, it has been shown recently that the HIV-1 accessory protein Nef can associate with a cellular serine/threonine kinase (69) as well as src-related tyrosine kinases Hck, Lyn (50, 66), and Lck (18). Furthermore, Nef was shown to be incorporated into HIV-1 particles (84) and therefore could affect the CD4-mediated signal transduction. We are currently investigating this possibility by using HIV-1 in which the *nef* gene is nonfunctional. While this study was being completed, another membrane glycoprotein, fusin, was shown to be required for the entry of HIV-1 into the cells. This cofactor is a member of a superfamily of G-protein-coupled receptors. Although it is not known how the HIV-1 envelope interacts with fusin, it is likely that another cellular signaling may be induced through this G-protein-coupled receptor. However, the observation that the Lck–Raf-1 interaction depends on the presence of CD4 receptors and is also induced by anti-CD4 antibodies indicates that the interaction of HIV-1 or gp120 with fusin does not play a major role in the observed signaling.

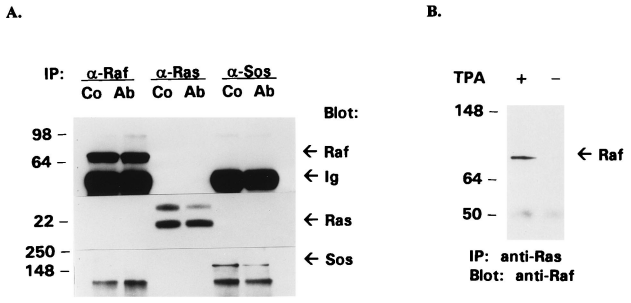

FIG. 7. Oligomerization of CD4 receptors does not induce association between Ras and Raf-1. (A) Cell extracts prepared from untreated Jurkat cells (Co) or cells stimulated with anti-CD4 antibodies (Ab) were immunoprecipitated (IP) with anti-Raf-1 (α-Raf) or anti-Ras (Y13-238; α-Ras) or anti-Sos1/2 (α-Sos) antibodies. Precipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with antibodies against Raf-1, Ras, and Sos. (B) Jurkat cells were treated for 5 min with TPA (20 ng/ml), cell extracts were immunoprecipitated with anti-Ras (Y13-238) antibodies, and proteins were resolved by SDS-PAGE. The presence of Raf-1 was detected by Western blotting with anti-Raf-1 antibodies. Molecular weight markers (in thousands) and immunoprecipitated proteins are indicated. Ig, immunoglobulin heavy chain.

The significance of the stimulation of the Lck/Raf-1 signaling pathway in the pathogenicity of HIV-1 remains to be determined. However, the observation that both the laboratory strain of HIV-1, NL4-3, and primary isolates (unpublished results) replicate much more effectively in Jurkat cells overexpressing activated Raf-1 than in parental cells suggests that the Lck–Raf-1 activation stimulated by binding of HIV-1 to CD4 receptors supports viral replication. There are at least three mechanisms by which activated Raf-1 can stimulate HIV-1 replication. First, the observations that the overexpression of activated Raf-1 in Jurkat T cells significantly enhanced the HIV-1 promoter activity and that activated Raf-1 synergizes

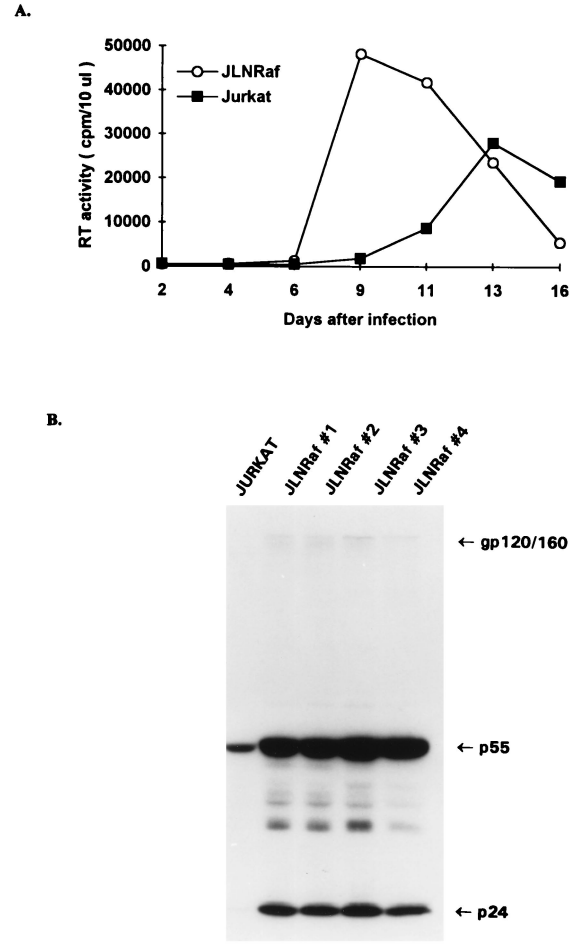

FIG. 9. Replication of the HIV-1 in Jurkat and JLNRaf cells overexpressing activated Raf-1. Jurkat cells and a pool of JLNRaf cells generated as described in Materials and Methods were infected with HIV-1 at a MOI of 0.01. (A) Medium from infected cells was collected every 2 or 3 days and analyzed for the presence of HIV-1 virions by the reverse transcriptase (RT) activity assay. (B) Jurkat cells and four selected JLNRaf clones were collected on day 9 after infection, and the presence of viral proteins in cellular lysates was detected by Western blotting with anti-HIV-1 serum as described previously (62). HIV-1 proteins were visualized by ECL as described in Materials and Methods. HIV-1-specific proteins are marked.

with the viral transactivator Tat in the stimulation of HIV-1 LTR activity suggest that the Lck/Raf-1 pathway may play an essential role in the transcriptional activation of the HIV-1 provirus. A recent observation (11) which shows that the binding of HIV-1 virions to latently infected and quiescent peripheral blood mononuclear cells stimulates HIV-1 production from these cells is in agreement with our observations. Second, activation of the Lck–Raf-1 pathway upon binding of HIV-1 to CD4 receptors may also provide the first stimulatory signal for activation of transcription of the integrated HIV-1 provirus before virus-encoded transactivator Tat is synthesized. Accordingly, we have found that activation of the Lck–Raf-1 pathway induced by binding of HIV-1 to CD4 receptors is functional and results in the nuclear expression of transcription factors NF-κB and AP-1 (unpublished data). We and others (2, 61, 62, 77) have previously shown that NF-κB-mediated transactivation of the HIV-1 LTR expression can occur in a Tat-independent manner. Third, the activation of NF-κB leads to upregulation of the expression of several cytokines and chemokines, all of which contain the NF-κB-binding motif in their promoter regions. Some of these cytokines (e.g., tumor necrosis factor alpha, IL-1, granulocyte-macrophage colony-stimulating factor, and IL-6) were shown to upregulate transcription of the HIV-1 provirus (28). Thus, the cytokines induced upon the activation of Lck–Raf-1 pathway may further contribute to the first round of transcription of HIV-1 provirus that occurs in the absence of Tat.

Infection with HIV-1 results in the activation of the immune system, exemplified by the expression of T-cell activation antigens and increased cytokine production. It has been shown that oligomerization of the CD4 receptors by gp120 not only induces the synthesis of IL-6 in T-cell clones (59) and IL-10 in human monocytes and macrophages (9) but also upregulates expression of the Fas antigen in lymphocytes by inducing gamma interferon and tumor necrosis factor alpha (60). The CD4-induced aberrant cytokine secretion and upregulation of Fas antigen expression may play a critical role in triggering T-cell apoptosis and may thus contribute to AIDS pathogenesis.

In summary, our results suggest that signaling events initiated by the oligomerization of CD4 receptors by HIV-1 or its envelope complexes result in the activation of the Lck–Raf-1 signaling pathway. This may lead to aberrant expression of different inflammatory cytokines, apoptosis of uninfected cells, and direct upregulation of HIV-1 expression. Finally, our preliminary results indicate that the signal transduction pathway described occurs not only in Jurkat T cells but also in primary CD4-positive T cells isolated from peripheral blood mononuclear cells (unpublished data).

ACKNOWLEDGMENTS

This study was supported by grant AI26123 from the National Institutes of Health (NIAID) (to P.M.P.) and by grant IRG 11-23 from the American Cancer Society and an Institutional Research Grant Project (IRGPC) (to W.P.).

We thank J. C. Alwine, M. McMahon, U. R. Rapp, and J. Skowronski for their generous gifts of plasmids and K.-T. Jeang for A201/CD4 cells. We also thank R. Siliciano and S. Desiderio for their helpful comments and review of the manuscript.

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Alcami, J., T. L. de Lera, L. Folgueira, M.-A. Pedraza, J.-M. Jacque, F. Bachelerie, A. R. Noriega, R. T. Hay, D. Harrich, R. B. Gaynor, J.-L. Virelizer, and F. Arenzana-Seisdedos.** 1995. Absolute dependence on κB responsive elements for initiation and Tat-mediated amplification of HIV transcription in blood CD4 T lymphocytes. EMBO J. **14:**1552–1560.
3. **Amrein, K. E., N. Flint, B. Panholzer, and P. Burn.** 1992. Ras GTPase-activating protein: a substrate and a potential binding protein of the protein-tyrosine kinase $p56^{lck}$. Proc. Natl. Acad. Sci. USA **89:**3343–3346.
4. **App, H., R. Hazan, A. Zilberstein, A. Ullrich, J. Schlessinger, and U. Rapp.** 1991. Epidermal growth factor (EGF) stimulates association and kinase activity of Raf-1 with the EGF receptor. Mol. Cell. Biol. **11:**913–919.
5. **Baccarini, M., D. M. Sabatini, H. App, U. R. Rapp, and E. R. Stanley.** 1990. Colony stimulating factor-1 (CSF-1) stimulates temperature dependent phosphorylation and activation of the RAF-1 proto-oncogene product. EMBO J. **9:**3649–3657.
6. **Banda, N. K., J. Bernier, D. K. Kurahara, R. Kurrle, N. Haigwood, R.-P. Sekaly, and T. H. Finke.** 1992. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J. Exp. Med. **176:**1099–1106.
7. **Benkirane, M., D. Blanc-Zouaoui, M. Hirn, and C. Devaux.** 1994. Involvement of human leukocyte antigen class I molecules in human immunodeficiency virus infection of CD4-positive cells. J. Virol. **68:**6332–6339.
8. **Benkirane, M., H. Schmid-Antomarchi, D. R. Littman, M. Hirn, B. Rossi, and C. Devaux.** 1995. The cytoplasmic tail of CD4 is required for inhibition of human immunodeficiency virus type 1 replication by antibodies that bind to the immunoglobulin CDR3-like region in domain 1 of CD4. J. Virol. **69:** 6904–6910.

9. **Borghi, P., L. Fantuzzi, B. Varano, S. Gessani, P. Puddu, L. Conti, M. R. Capobianchi, F. Ameglio, and F. Belardelli.** 1995. Induction of interleukin-10 by human immunodeficiency virus type 1 and its gp120 protein in human monocytes/macrophages. J. Virol. **69:**1284–1287.
10. **Brennscheidt, U., D. Riedel, W. Kolch, R. Bonifer, M. A. Brach, A. Ahlers, R. H. Mertelsmann, and F. Herrmann.** 1994. Raf-1 is a necessary component of the mitogenic response of the human megakaryoblastic leukemia cell line MO7 to human stem cell factor, granulocyte-macrophage colony-stimulating factor, interleukin 3, and interleukin 9. Cell Growth Differ. **5:**367–372.
11. **Briant, L., N. Coudronniere, V. Robert-Hebmann, M. Benkirane, and C. Devaux.** 1996. Binding of HIV-1 virions or gp120-anti-gp120 immune complexes to HIV-1-infected quiescent peripheral blood mononuclear cells reveals latent infection. J. Immunol. **156:**3994–4004.
12. **Bruder, J. T., G. Heidecker, and U. R. Rapp.** 1992. Serum-, TPA-, and Ras-induced expression from Ap-1/Ets-driven promoters requires Raf-1 kinase. Genes Dev. **6:**545–556.
13. **Bruder, J. T., G. Heidecker, T.-H. Tan, J. C. Weske, D. Derse, and U. R. Rapp.** 1993. Oncogene activation of HIV-LTR-driven expression via the NF-kappa B binding sites. Nucleic Acids Res. **21:**5229–5234.
14. **Buday, L., and J. Downward.** 1993. Epidermal growth factor regulates $p21^{ras}$ through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cell **73:**611–620.
15. **Carroll, M. P., and W. S. May.** 1994. Protein kinase C-mediated serine phosphorylation directly activates Raf-1 in murine hematopoietic cells. J. Biol. Chem. **269:**1249–1256.
16. **Chan, A. C., B. A. Irving, J. D. Fraser, and A. Weiss.** 1991. The zeta chain is associated with a tyrosine kinase and upon T-cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein. Proc. Natl. Acad. Sci. USA **88:**9166–9170.
17. **Chirmule, N., H. Goonewardena, S. Pahwa, R. Pasieka, V. S. Kalyanaraman, and S. Pahwa.** 1995. HIV-1 envelope glycoproteins induce activation of activated protein-1 in CD4+ T cells. J. Biol. Chem. **270:**19364–19369.
18. **Collette, Y., H. Dutartre, A. Benziane, F. Ramos-Morales, R. Benarous, M. Harris, and D. Olive.** 1996. Physical and functional interaction of Nef with Lck. HIV-1 Nef-induced T-cell signaling defects. J. Biol. Chem. **271:**6333–6341.
19. **Crabtree, G. R.** 1989. Contingent genetic regulatory events in T lymphocyte activation. Science **243:**355–361.
20. **Cruikshank, W. W., D. M. Center, N. Nisar, M. Wu, B. Natke, A. C. Theodore, and H. Kornfeld.** 1994. Molecular and functional analysis of a lymphocyte chemoattractant factor: association of biologic function with CD4 expression. Proc. Natl. Acad. Sci. USA **91:**5109–5113.
21. **Dalgleish, A. G.** 1995. The immune response to HIV: potential for immunotherapy? Immunol. Today **16:**356–358.
22. **Dalgleish, A. G., P. C. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature (London) **312:**763–767.
23. **Doyle, C., and J. L. Strominger.** 1987. Interaction between CD4 and class II MHC molecules mediates cell adhesion. Nature (London) **330:**256–259.
24. **Downward, J.** 1995. KSR: a novel player in the RAS pathway. Cell **83:**831–834.
25. **Dulbecco, R., and H. S. Ginsberg.** 1988. Virology: the nature of viruses, 2nd ed., p. 22–25. J. B. Lippincott Co., Philadelphia.
26. **Ettehadieh, E., J. S. Sanghera, S. L. Pelech, D. Hess-Bienz, J. Watts, N. Shastri, and R. Aebersold.** 1992. Tyrosyl phosphorylation and activation of MAP kinases by p56lck. Science **255:**853–855.
27. **Fabian, J. R., A. B. Vojtek, J. A. Cooper, and D. K. Morrison.** 1994. A single amino acid change in Raf-1 inhibits Ras binding and alters Raf-1 function. Proc. Natl. Acad. Sci. USA **91:**5982–5986.
28. **Fauci, A. S.** 1988. The human immunodeficiency virus: infectivity and mechanisms of pathogenesis. Science **239:**617–622.
29. **Finco, T. S., and A. S. Baldwin, Jr.** 1993. κB site-dependent induction of gene expression by diverse inducers of nuclear factor κB requires Raf-1. J. Biol. Chem. **268:**17676–17679.
30. **Finkel, T. H., G. Tudor-Williams, N. K. Banda, M. F. Cotton, T. Curiel, C. Monks, T. W. Baba, R. M. Ruprecht, and A. Kupfer.** 1995. Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. Nat. Med. **1:**129–134.
31. **Flory, E., A. Hoffmeyer, U. Smola, U. R. Rapp, and J. T. Bruder.** 1996. Raf-1 kinase targets GA-binding protein in transcriptional regulation of the human immunodeficiency virus type 1 promoter. J. Virol. **70:**2260–2268.
32. **Gallay, P., S. Swingler, C. Aiken, and D. Trono.** 1995. HIV-1 infection of nondividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator. Cell **80:**379–388.
33. **Gay, D., P. Maddon, R. Sekaly, M. A. Talle, M. Godfrey, E. Long, G. Goldstein, L. Chess, R. Axel, J. Kappler, et al.** 1987. Functional interaction between human T-cell protein CD4 and the major histocompatibility complex HLA-DR antigen. Nature (London) **328:**626–629.
34. **Goldman, F., W. A. Jensen, G. L. Johnson, L. Heasley, and J. C. Cambier.** 1994. gp120 ligation of CD4 induces p56lck activation and TCR desensitization independent of TCR tyrosine phosphorylation. J. Immunol. **153:**2905–2917.
35. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
36. **Gupta, S., A. Weiss, G. Kumar, S. Wang, and A. Nel.** 1994. The T-cell antigen receptor utilizes Lck, Raf-1, and MEK-1 for activating mitogen-activated protein kinase. Evidence for the existence of a second protein kinase C-dependent pathway in an Lck-negative Jurkat cell mutant. J. Biol. Chem. **269:**17349–17357.
37. **Hallberg, B., S. I. Rayter, and J. Downward.** 1994. Interaction of Ras and Raf in intact mammalian cells upon extracellular stimulation. J. Biol. Chem. **269:**3913–3916.
38. **Hivroz, C., F. Mazerolles, M. Soula, R. Fagard, S. Graton, S. Meloche, R. P. Sekaly, and A. Fischer.** 1993. Human immunodeficiency virus gp120 and derived peptides activate protein tyrosine kinase p56lck in human CD4 T lymphocytes. Eur. J. Immunol. **23:**600–607.
39. **Horak, I. D., M. Popovic, E. M. Horak, P. J. Lucas, R. E. Gress, C. H. June, and J. B. Bolen.** 1990. No T-cell tyrosine protein kinase signalling or calcium mobilization after CD4 association with HIV-1 or HIV-1 gp120. Nature (London) **348:**557–560.
40. **Hou, X. S., T. B. Chou, M. B. Melnick, and N. Perrimon.** 1995. The torso receptor tyrosine kinase can activate Raf in a Ras-independent pathway. Cell **81:**63–71.
41. **Howe, L. R., S. J. Leevers, N. Gomez, S. Nakielny, P. Cohen, and C. J. Marshall.** 1992. Activation of the MAP kinase pathway by the protein kinase raf. Cell **71:**335–342.
42. **Jain, J., P. G. McCaffrey, Z. Miner, T. K. Kerppola, J. N. Lambert, G. L. Verdine, T. Curran, and A. Rao.** 1993. The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature (London) **365:**352–355.
43. **Jung, J. U., S. M. Lang, U. Friedrich, T. Jun, T. M. Roberts, R. C. Desrosiers, and B. Biesinger.** 1995. Identification of Lck-binding elements in Tip of herpesvirus saimiri. J. Biol. Chem. **270:**20660–20667.
44. **Kanakura, Y., B. Druker, K. W. Wood, H. J. Mamon, K. Okuda, T. M. Roberts, and J. D. Griffin.** 1991. Granulocyte-macrophage colony-stimulating factor and interleukin-3 induce rapid phosphorylation and activation of the proto-oncogene Raf-1 in a human factor-dependent myeloid cell line. Blood **77:**243–248.
45. **Kaufmann, R., D. Laroche, K. Buchner, F. Hucho, C. Rudd, C. Lindschau, P. Ludwig, A. Hoer, E. Oberdisse, J. Kopp, et al.** 1992. The HIV-1 surface protein gp120 has no effect on transmembrane signal transduction in T cells. J. Acquired Immune Defic. Syndr. **5:**760–770.
46. **Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J. C. Gluckman, and L. Montagnier.** 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature (London) **312:**767–768.
47. **Kolch, W., G. Heidecker, G. Kochs, R. Hummel, H. Vahidi, H. Mischak, G. Finkenzeller, D. Marme, and U. R. Rapp.** 1993. PKCα activates Raf-1 by direct phosphorylation. Nature (London) **364:**249–251.
48. **Kovacina, K. S., K. Yonezawa, D. L. Brautigan, N. K. Tonks, U. R. Rapp, and R. A. Roth.** 1990. Insulin activates the kinase activity of the Raf-1 proto-oncogene by increasing its serine phosphorylation. J. Biol. Chem. **265:**12115–12118.
49. **Kunzi, M. S., H. Farzadegan, J. B. Margolick, D. Vlahov, and P. M. Pitha.** 1995. Identification of human immunodeficiency virus primary isolates resistant to interferon-α and correlation of prevalence to disease progression. J. Infect. Dis. **171:**822–828.
50. **Lee, C.-H., B. Leung, M. A. Lemmon, J. Zheng, D. Cowburn, J. Kuriyan, and K. Saksela.** 1995. A single amino acid in the SH3 domain of Hck determines its high affinity and specificity in binding to HIV-1 Nef protein. EMBO J. **14:**5006–5015.
51. **Leevers, S. J., H. F. Paterson, and C. J. Marshall.** 1994. Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature (London) **369:**411–414.
52. **Lenardo, M. J., and D. Baltimore.** 1989. NF-kappa B: a pleiotropic mediator of inducible and tissue-specific gene control. Cell **58:**227–229.
53. **Li, W., R. Nishimura, A. Kashishian, A. G. Batzer, W. J. H. Kim, J. A. Cooper, and J. Schlessinger.** 1994. A new function for a phosphotyrosine phosphatase: linking GRB2-Sos to a receptor tyrosine kinase. Mol. Cell. Biol. **14:**509–517.
54. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47:**333–348.
55. **Marais, R., Y. Light, H. F. Paterson, and C. J. Marshall.** 1995. Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. EMBO J. **14:**3136–3145.
56. **Maslinski, W., B. Remillard, M. Tsudo, and T. B. Strom.** 1992. Interleukin-2 (IL-2) induces tyrosine kinase-dependent translocation of active raf-1 from the IL-2 receptor into the cytosol. J. Biol. Chem. **267:**15281–15284.
57. **Morrison, D. K., D. R. Kaplan, J. A. Escobedo, U. R. Rapp, T. M. Roberts, and L. T. Williams.** 1989. Direct activation of the serine/threonine kinase

activity of Raf-1 through tyrosine phosphorylation by the PDGF beta-receptor. Cell **58:**649–657.
58. **Northrop, J. P., K. S. Ullman, and G. R. Crabtree.** 1993. Characterization of the nuclear and cytoplasmic components of the lymphoid-specific nuclear factor of activated T cells (NF-AT) complex. J. Biol. Chem. **268:**2917–2923.
59. **Oyaizu, N., N. Chirmule, Y. Ohnishi, V. S. Kalyanaraman, and S. Pahwa.** 1991. Human immunodeficiency virus type 1 envelope glycoproteins gp120 and gp160 induce interleukin-6 production in $CD4^+$ T-cell clones. J. Virol. **65:**6277–6282.
60. **Oyaizu, N., T. W. McCloskey, S. Than, R. Hu, V. S. Kalyanaraman, and S. Pahwa.** 1994. Cross-linking of CD4 molecules upregulates Fas antigen expression in lymphocytes by inducing interferon-gamma and tumor necrosis factor-alpha secretion. Blood **84:**2622–2631.
61. **Popik, W., and P. M. Pitha.** 1992. Transcriptional activation of the tat-defective human immunodeficiency virus type-1 provirus: effect of interferon. Virology **189:**435–447.
62. **Popik, W., and P. M. Pitha.** 1993. Role of tumor necrosis factor alpha in activation and replication of the *tat*-defective human immunodeficiency virus type 1. J. Virol. **67:**1094–1099.
63. **Prasad, K. V., R. Kapeller, O. Janssen, H. Repke, J. S. Duke-Cohan, L. C. Cantley, and C. E. Rudd.** 1993. Phosphatidylinositol (PI) 3-kinase and PI 4-kinase binding to the CD4-$p56^{lck}$ complex: the $p56^{lck}$ SH3 domain binds to PI 3-kinase but not PI 4-kinase. Mol. Cell. Biol. **13:**7708–7717.
64. **Riedel, D., U. Brennscheidt, M. Kiehntopf, M. Brach, and F. Herrmann.** 1993. The mitogenic response of T cells to interleukin-2 requires Raf-1. Eur. J. Immunol. **23:**3146–3150.
65. **Rudd, C. E., J. M. Trevillyan, J. D. Dasgupta, L. L. Wong, and S. F. Schlossman.** 1988. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. Proc. Natl. Acad. Sci. USA **85:**5190–5194.
66. **Saksela, K., G. Cheng, and D. Baltimore.** 1995. Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef^+ viruses but not for downregulation of CD4. EMBO J. **14:**484–491.
67. **Salghetti, S., R. Mariani, and J. Skowronski.** 1995. Human immunodeficiency virus type 1 Nef and $p56^{lck}$ protein-tyrosine kinase interact with a common element in CD4 cytoplasmic tail. Proc. Natl. Acad. Sci. USA **92:**349–353.
68. **Samuels, M. L., M. J. Weber, J. M. Bishop, and M. McMahon.** 1993. Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human Raf-1 protein kinase. Mol. Cell. Biol. **13:**6241–6252.
69. **Sawai, E. T., A. Baur, H. Struble, B. M. Peterlin, J. A. Levy, and C. Cheng-Mayer.** 1994. Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. Proc. Natl. Acad. Sci. USA **91:**1539–1543.
70. **Siegel, J. N., R. D. Klausner, U. R. Rapp, and L. E. Samelson.** 1990. T cell antigen receptor engagement stimulates c-raf phosphorylation and induces c-raf-associated kinase activity via a protein kinase C-dependent pathway. J. Biol. Chem. **265:**18472–18480.
71. **Sieh, M., J. B. Bolen, and A. Weiss.** 1993. CD45 specifically modulates binding of Lck to a phosphopeptide encompassing the negative regulatory tyrosine of Lck. EMBO J. **12:**315–321.
72. **Smeal, T., B. Binetruy, D. Mercola, A. Grover-Bardwick, G. Heidecker, U. R. Rapp, and M. Karin.** 1992. Oncoprotein-mediated signalling cascade stimulates c-Jun activity by phosphorylation of serines 63 and 73. Mol. Cell. Biol. **12:**3507–3513.
73. **Soula, M., R. Fagard, and S. Fischer.** 1992. Interaction of human immunodeficiency virus glycoprotein 160 with CD4 in Jurkat cells increases p56lck autophosphorylation and kinase activity. Int. Immunol. **4:**295–299.
74. **Stephens, R. M., D. M. Loeb, T. D. Copeland, T. Pawson, L. A. Greene, and D. R. Kaplan.** 1994. Trk receptors use redundant signal transduction pathways involving SHC and PLC-γ1 to mediate NGF responses. Neuron **12:**691–705.
75. **Stokoe, D., S. G. Macdonald, K. Cadwallader, M. Symons, and J. F. Hancock.** 1994. Activation of Raf as a result of recruitment to the plasma membrane. Science **264:**1463–1467.
76. **Straus, D. B., and A. Weiss.** 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. Cell **70:**585–593.
77. **Su, Y., W. Popik, and P. M. Pitha.** 1995. Inhibition of human immunodeficiency virus type 1 replication by a Tat-activated, transduced interferon gene: targeted expression to human immunodeficiency virus type 1-infected cells. J. Virol. **69:**110–121.
78. **Takemoto, Y., M. Furuta, X.-K. Li, W. J. Strong-Sparks, and Y. Hashimoto.** 1995. LckBP1, a proline-rich protein expressed in haematopoietic lineage cells, directly associates with the SH3 domain of protein tyrosine kinase p56Lck. EMBO J. **14:**3403–3414.
79. **Tremblay, M., S. Meloche, S. Gratton, M. A. Wainberg, and R. P. Sekaly.** 1994. Association of $p56^{lck}$ with the cytoplasmic domain of CD4 modulates HIV-1 expression. EMBO J. **13:**774–783.
80. **Turner, B., U. Rapp, H. App, M. Greene, K. Dobashi, and J. Reed.** 1991. Interleukin 2 induces tyrosine phosphorylation and activation of p72-74 Raf-1 kinase in a T-cell line. Proc. Natl. Acad. Sci. USA **88:**1227–1231.
81. **Veillette, A., M. A. Bookman, E. M. Horak, L. E. Samelson, and J. B. Bolen.** 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56lck. Nature (London) **338:**257–259.
82. **Weber, J. R., G. M. Bell, M. Y. Han, T. Pawson, and J. B. Imboden.** 1992. Association of the tyrosine kinase LCK with phospholipase C-gamma 1 after stimulation of the T cell antigen receptor. J. Exp. Med. **176:**373–379.
83. **Weinhold, K. J., H. K. Lyerly, S. D. Stanley, A. Austin, T. J. Mathews, and D. Bolognesi.** 1989. HIV-1 gp120-mediated immune suppression and lymphocyte destruction in the absence of viral infection. J. Immunol. **142:**3091–3097.
84. **Welker, R., H. Kottler, H. R. Kalbitzer, and H.-G. Krausslich.** 1996. Human immunodeficiency virus type 1 Nef protein is incorporated into virus particles and specifically cleaved by the viral proteinase. Virology **219:**228–236.
85. **White, M. A., C. Nicolette, A. Minden, A. Polverino, L. Van Aelst, M. Karin, and M. H. Wigler.** 1995. Multiple Ras functions can contribute to mammalian cell transformation. Cell **80:**533–541.
86. **Williams, N. G., T. M. Roberts, and P. Li.** 1992. Both $p21^{ras}$ and $pp60^{v\text{-}src}$ are required, but neither alone is sufficient, to activate the Raf-1 kinase. Proc. Natl. Acad. Sci. USA **89:**2922–2926.
87. **Xu, H., and D. R. Littman.** 1993. A kinase-independent function of lck in potentiating antigen-specific T cell activation. Cell **74:**633–643.
88. **Yoshida, H., Y. Koga, Y. Moroi, G. Kimura, and K. Nomoto.** 1992. The effect of p56lck, a lymphocyte specific protein tyrosine kinase, on the syncytium formation induced by human immunodeficiency virus envelope glycoprotein. Int. Immunol. **4:**233–242.
89. **Zeichner, S. L., J. Y. Kim, and J. C. Alwine.** 1991. Linker-scanning mutational analysis of the transcriptional activity of the human immunodeficiency virus type 1 long terminal repeat. J. Virol. **65:**2436–2444.