

Inhibition of T-Cell Receptor Signal Transduction and Viral Expression by the Linker for Activation of T Cells-Interacting p12^I Protein of Human T-Cell Leukemia/Lymphoma Virus Type 1[∇]

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The p12^I protein of human T-cell leukemia/lymphoma virus type 1 (HTLV-1) is a small oncoprotein that increases calcium release following protein kinase C activation by phorbol myristate acetate, and importantly, this effect is linker for activation of T cells (LAT) independent. Here, we demonstrate that p12^I inhibits the phosphorylation of LAT, Vav, and phospholipase C- γ 1 and decreases NFAT (nuclear factor of activated T cells) activation upon engagement of the T-cell receptor (TCR) with anti-CD3 antibody. Furthermore, we demonstrate that p12^I localizes to membrane lipid rafts and, upon engagement of the TCR, relocates to the interface between T cells and antigen-presenting cells, defined as the immunological synapse. A p12^I knockout molecular clone of HTLV-1 expresses more virus upon antigen stimulation than the isogenic wild type, suggesting that, by decreasing T-cell responsiveness, p12^I curtails viral expression. Thus, p12^I has contrasting effects on TCR signaling: it down-regulates TCR in a LAT-dependent manner on one hand, and on the other, it increases calcium release in a LAT-independent manner. The negative regulation of T-cell activation by p12^I may have evolved to minimize immune recognition of infected CD4⁺ T cells, to impair the function of infected cytotoxic CD8⁺ T cells, and to favor viral persistence in the infected host.

Human T-cell leukemia/lymphoma virus type 1 (HTLV-1) causes a rare, aggressive hematopoietic malignancy of mature T cells, designated adult T-cell leukemia/lymphoma, as well as a progressive myelopathy defined as HTLV-1-associated myelopathy/tropical spastic paraparesis (18, 20, 25, 42, 55). HTLV-1 infects human CD4⁺ and CD8⁺ (22, 33, 35, 39, 43) T cells of memory phenotype and is believed to confer a proliferative and survival advantage on infected memory T-cell clones, with consequent accumulation of somatic mutations and neoplastic transformation (16). The provirus is thought to be mostly latent, mainly in resting T cells (21). However, once T cells are stimulated by antigen, the proviral DNA can be expressed, endangering the survival of the infected cells because of immune recognition.

The HTLV-1 genome, in addition to structural (Gag and Env) and enzymatic (Pol) proteins, encodes positive regulators of viral expression from open reading frames (ORFs) III and IV, such as Tax and Rex, as well as two negative regulators, p30^{II} (ORF II) (40) and HBZ, which is encoded from the minus strand RNA

(19). ORF I encodes a 12-kDa protein (p12^I) that in cells is localized in the endoplasmic reticulum (ER)/Golgi (13, 28, 32). The p12^I protein has been shown to have multiple functions. p12^I interacts with the interleukin-2 receptor (IL-2R) β and γ_c chains, increases STAT-5 activation, and decreases the threshold of the IL-2 requirement for T-cell proliferation (38, 41) in primary human lymphocytes. In the ER/Golgi, p12^I binds both the IL-2 receptor chains and the major histocompatibility complex (MHC) class I heavy chain and interferes with their trafficking to the cell surface (28, 38). p12^I also binds to calreticulin and calnexin (13) and increases calcium release and nuclear factor of activated T cells (NFAT) activation upon phorbol myristate acetate (PMA) stimulation of T cells. This effect bypasses antigen receptor ligation, as it also occurs in linker for activation of T cells (LAT)-deficient T cells (1, 12, 29).

T-cell receptor (TCR) signaling occurs through a cascade of events (51, 52) that involve activation of the protein tyrosine kinases Lck and Fyn, which phosphorylate the tyrosines in the cytosolic domains of TCR ζ and the CD3 subunits. These phosphorylated domains become the docking sites for ZAP70, which is phosphorylated and activated when bound. Activated ZAP70 phosphorylates LAT, which then binds Grb2, phospholipase C- γ 1 (PLC- γ 1), and the p85 subunit of phosphatidylinositol 3-kinase and, indirectly, Vav, Cb1, and SLP76. The recruitment of these critical molecules to the membrane ultimately results in multiple events, including calcium release, dephosphorylation and nuclear translocation of NFAT, enhancement of transcription, cytokine production, and T-cell proliferation.

These events lead over time to protein and lipid rearrangements that occur during the formation of the immunological

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synapse between the T cell and the antigen-presenting cells (APCs) (7, 37). These membrane rearrangements include effects on the lipid rafts, a heterogeneous mixture of components on the surfaces of cells that includes the glycosphingolipid- and cholesterol-rich microdomains and proteins that are enriched in these regions (46). The function of immune synapse formation and the various molecular sorting events that accompany it are controversial but may include effects on signaling and down-regulation of various molecules (5, 48).

TCR stimulation of HTLV-1-infected T cells and IL-2 production could be deleterious to viral persistence, as the viral transactivator, Tax, increases viral expression by exploiting CREB/ATF that is activated by IL-2 (50). Thus, viral expression is likely maintained at a very low level in vivo to minimize the elimination of the infected T cells by the adaptive immune response. In support of this notion is the fact that the virus has evolved at least two negative regulators of viral expression: HBZ, which decreases Tax-mediated transcriptional activation (19), and p30^{II}, which decreases the cytoplasmic level of the Tax/Rex mRNA (40).

p12¹ has been shown to have an effect on T-cell activation. In the presence of strong antigens, such as phytohemagglutinin or PMA, p12¹ increases STAT-5 activation, NFAT transcriptional activity, and calcium release (12, 29, 41), and the last effect is LAT independent. Surprisingly, we found here that p12¹ down-regulates NFAT activity in a LAT-dependent manner following engagement of the TCR with either the anti-CD3 antibody or MHC-bound antigen. Furthermore, we demonstrate that p12¹ accesses and clusters within the lipid rafts (8, 46) and is recruited to the immunological synapse upon engagement of the TCR by MHC-bound antigen. p12¹ decreases the phosphorylation of PLC- γ 1 and Vav molecules involved in proximal TCR signaling and interacts with LAT. Furthermore, genetic knockout (KO) of p12¹ expression in an HTLV-1 molecular clone resulted in an augmentation of viral production only following engagement of the TCR. Thus, p12¹ indirectly regulates viral expression. Taken together, these results suggest that HTLV-1 has evolved p12¹ as a function to favor viral persistence by dampening the responsiveness to TCR stimulation of T cells carrying the provirus.

MATERIALS AND METHODS

Plasmids, immunoprecipitation, and Western blotting. The pME p12¹ expression plasmid used here was modified by deleting amino acids S and L within the HA1 tag carboxy terminus of pME p12¹ as described by Koralnik et al. (31), as these two amino acids revealed an endosomal sorting motif that affects protein expression. The plasmid pCEFL-LAT-Myc was described previously (6, 56, 57). pNFAT-TA-luciferase and pTK-Renilla were obtained from Clontech (Palo Alto, CA) and Promega (Madison, WI), respectively. The anti-HA1 (12CA5) and anti-HA1-horseradish peroxidase (3F10-hrp) antibodies were obtained from Roche (Indianapolis, IN); the anti-Lck (3A5), anti-c-Myc (9E10), anti-PLC- γ 1 (E-12), anti-Vav (C-14), and anti-Lyn (H-6) antibodies from Santa Cruz (Santa Cruz, CA); and the anti-CD71 antibody from Zymed (San Francisco, CA). Anti- β -tubulin (TUB 2.1) was from Sigma (St. Louis, MO); anti-LAT (2E9) and anti-PY (4G10) were from Upstate (Lake Placid, NY).

Total cell lysates were extracted with 1% NP-40 RIPA buffer with 50 mM Tris-HCl (7.5) and 150 mM NaCl in the presence of 20 μ g/ml leupeptin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 1 mM Na₃VO₄, and 20 μ g/ml aprotinin, and insoluble materials were removed by centrifugation. Immunoprecipitations were performed, after lysates were precleared with 10 μ l of protein G-agarose (Roche), by adding the appropriate antibodies and protein G-agarose and washing them three times in RIPA buffer. In some experiments, 60 mM N-octylglucoside was added in RIPA buffer (Sigma). The sample suspension was

boiled in the presence of 10% β -mercaptoethanol and loaded on a gel for electrophoresis.

Transfection and gradient flotation assays. Jurkat T, J.CaM2.5, and Raji cells were kept in RPMI 1640, and 293T cells were kept in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 5 mM L-glutamine. Jurkat T and J.CaM2.5 cells were transfected with DMRIE-C (Invitrogen, Carlsbad, CA) and 293T cells with Effectene (QIAGEN, Valencia, CA).

For the flotation assay, 2×10^7 cells were lysed at 0°C in 1 ml of 0.2% Triton X-100 in MNE buffer (25 mM MES [morpholineethanesulfonic acid], pH 6.5, 150 mM NaCl, 5 mM EDTA) containing 20 μ g/ml of aprotinin, 1 mM AEBSF, and 1 mM sodium orthovanadate; subjected to 10 strokes of a tight-fitting Dounce homogenizer; and mixed with 1 ml of 80% sucrose in MNE buffer. The lysate was transferred to a centrifuge tube and overlaid with 2 ml of 30% sucrose and 1 ml of 5% sucrose. After centrifugation for 20 h at 200,000 \times g, 0.4-ml fractions were collected, starting from the top of the gradient buffer. The pellet fraction was suspended in 0.4 ml of 1% Triton in MNE buffer and mixed with sodium dodecyl sulfate sample buffer. Five microliters of individual fractions was subjected to polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane, which was reacted with specific antibody.

T-cell stimulation and detection of the immunological synapse. Jurkat T cells were spun down and treated with 1 μ g/ml or the indicated concentrations of anti-CD3 (UCH-T1; EMD Biosciences, La Jolla, CA), anti-CD28 (BD, Franklin Lakes, NJ), or both in the serum-free medium. In some experiments, cells were treated with PMA at 20 ng/ml. After 1 h of incubation at 37°C, an equal amount of medium with 20% fetal calf serum was overlaid on the cells, and samples were incubated at 37°C to allow luciferase expression. For analysis of phosphorylated proteins following TCR stimulation, cells were lysed in RIPA buffer.

In the cocultivation experiments, Jurkat T and Raji cells were prepared separately at a concentration of 1×10^6 cells/ml in the fresh medium. Raji cells were prepulsed with or without 10 μ g/ml of superantigen staphylococcal enterotoxin E (SEE) (Toxin Technology, Sarasota, FL) for 1 h at 37°C. The cells were combined at a 1:1 ratio and fixed by adding 4% paraformaldehyde at each time point. In parallel, cells were incubated for the indicated time and lysates were obtained for the luciferase assay. In some experiments, cells were cocultivated in the presence of TransWell with a pore size of 0.4 μ m (Corning, Corning, NY).

Some of the cocultivation experiments were performed in the presence of Zerit (Bristol-Myers Squibb Virology, Princeton, NJ) (24). We treated long terminal repeat (LTR)-driven luciferase-expressing Raji cells with or without 35 μ M of Zerit the day before cocultivation with Jurkat T cells. The cocultures were maintained in the presence of Zerit for 72 h.

For the confocal microscopy, 1×10^5 cells were fixed and spun on a glass slide coated with poly-L-lysine. After being washed with phosphate-buffered saline, the cells were permeabilized with 0.1% saponin for 60 min at 37°C. The cells were then stained with fluorescein isothiocyanate (FITC)-conjugated cholera toxin A (Co-Tx) (FITC-Co-Tx) or anti-HA1 and anti-CD3, followed by FITC-labeled or Cy3-labeled anti-mouse antibodies. Cells were visualized with a Zeiss laser scanning confocal microscope (LSM 510; Carl Zeiss MicroImaging, Thornwood, NY) with a 100 \times /1.3 natural aperture Plan Apochromat oil objective and 3 \times zoom and visualized as maximum projections of Z stacks of focal-plane sections through entire cell volumes.

Luciferase assay. The Dual-Luciferase Reporter Assay System was obtained from Promega (Madison, WI), and all the measurements were performed according to the manufacturer's protocol. The same samples were used for the Bio-Rad (Hercules, CA) Protein Assay to determine the amount of protein used.

Quantification of HTLV p19 Gag. Direct or diluted cell culture supernatants were used in an HTLV p19 Gag enzyme-linked immunosorbent assay using a kit obtained from the ZeptoMetrix Corporation (Buffalo, NY). All the measurements were performed according to the manufacturer's protocol.

RESULTS

The HTLV-1 p12¹ protein down-regulates TCR signaling. Others have demonstrated that p12¹ increases calcium release and NFAT activity in T cells stimulated with PMA, which directly activates a protein kinase C (PKC) isoform downstream of TCR (12, 29). The PMA-mediated activation of NFAT by p12¹ is LAT independent and occurs in the LAT-deficient Jurkat T cells (J.CaM2.5), in which phosphorylation of PLC- γ 1 and Vav is also lost (12).

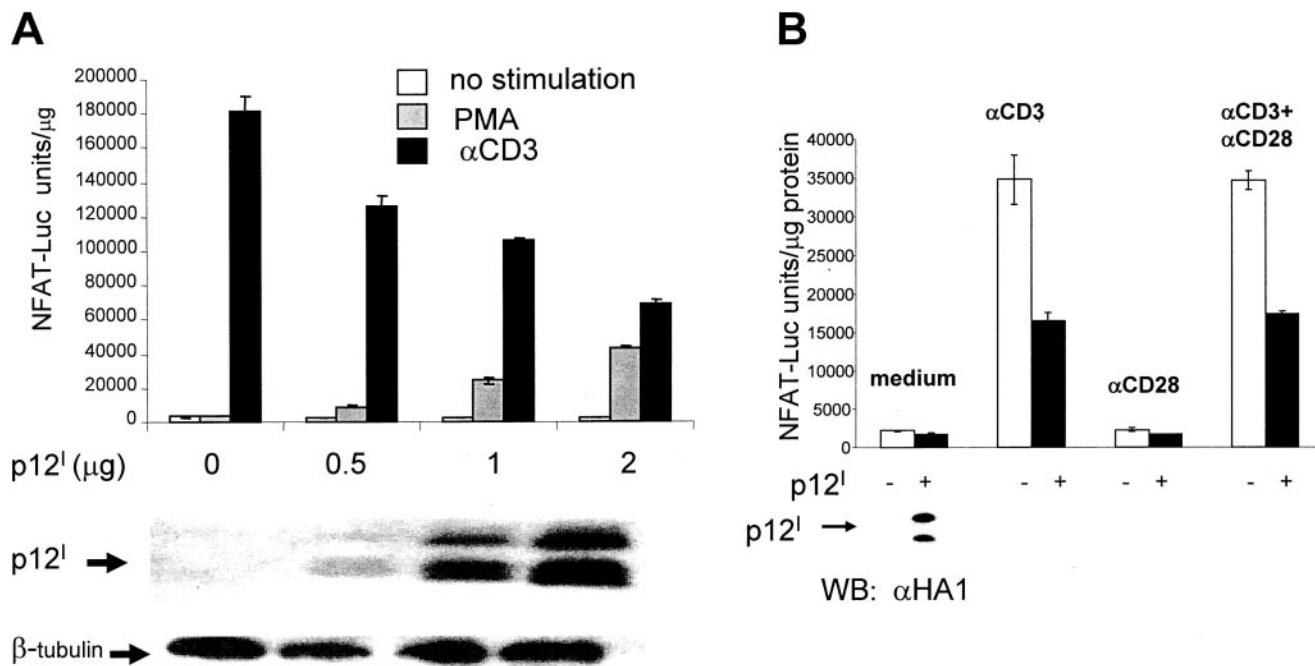


FIG. 1. Contrasting effects of the HTLV-1 p12^I protein on TCR and PKC signaling in Jurkat T cells. (A) (Top) Jurkat T cells were cotransfected with p12^I at different doses (μg/10⁶ cells) and with NFAT reporter plasmid and stimulated with nothing, with anti-CD3 (αCD3), or with PMA. NFAT activity was measured at 18 h by measuring the levels of luciferase in cell lysates. The level of luciferase activity was normalized to the protein content of the cell lysates. The average and the standard deviation of triplicate samples are shown. This is a representative experiment out of four independent experiments. (Bottom) The expression of the p12^I^{HA1}-tagged protein was verified by Western blotting on total cell lysate with antibodies to HA1. Antibody to β-tubulin was also used in Western blotting to verify equivalent protein loading. (B) Jurkat T cells cotransfected with the NFAT reporter plasmid and either p12^I (2 μg/10⁶ cells) or control vector were left unstimulated or were stimulated with either anti-CD3 or anti-CD28 alone or both for 18 h. NFAT activity was normalized to the protein content as in panel A, and a duplicate experiment is presented with the standard error. p12^I expression was assessed by Western blot (WB) analysis in transfected cells (bottom).

In the present study, we demonstrate that p12^I displays opposite effects upon engagement of the TCR through ligation with anti-CD3 antibody and decreases T-cell activation (Fig. 1A). Transfection of Jurkat T cells with p12^I resulted in NFAT activation when PMA was used as a stimulus, as observed previously (12, 29); however, CD3 ligation resulted in a decrease in NFAT activation, and both effects were dependent on the dose of p12^I (Fig. 1A). Costimulation with anti-CD28 antibody did not restore NFAT activity (Fig. 1B), and the down-regulation of TCR signaling by p12^I was LAT dependent, as coexpression of LAT (LAT-Myc) was required in LAT-deficient J.CaM2.5 cells to observe p12^I down-regulation of NFAT (data not shown).

The p12^I protein decreases phosphorylation of LAT and proximal TCR signaling molecules. To begin to understand the molecular mechanism of NFAT down-regulation induced by p12^I, we investigated whether the phosphorylation of cellular proteins involved in the TCR signaling cascade was affected (51, 52). The lysates of Jurkat T cells expressing p12^I or not were stimulated with the indicated doses of anti-CD3 antibody, and cell lysates were reacted with the anti-phosphotyrosine antibody 4G10. We observed a decreased level of phosphorylation of cellular proteins that ranged in mass between 36 and 145 kDa in cells expressing p12^I (Fig. 2A), which occurred within 1 min of anti-CD3 stimulation (Fig. 2B). Next, we analyzed by immunoprecipitation and Western blotting the expression and phosphorylation of proximal TCR signaling molecules, such as PLC-γ1, Vav, Lck, and LAT. Western blotting

with the specific antibodies was used to control for equivalent levels of immunoprecipitates.

LAT phosphorylation was decreased in the presence of p12^I, as demonstrated by the 4G10 anti-phosphotyrosine antibody, and this effect was not due to differential loading of proteins, as the same membrane reacted with anti-LAT antibody demonstrated an equivalent level of LAT in the immunoprecipitates (Fig. 2C, left). Similarly, decreased phosphorylation of PLC-γ1 and Vav, but not Lck, was observed in the presence of p12^I (Fig. 2C, right, and data not shown for Lck). Again, Western blotting on the same membranes with the specific antibodies to PLC-γ1 and Vav demonstrated equivalent levels of PLC-γ1 and Vav in the immunoprecipitate (Fig. 2C, right). Expression of the tagged p12^I was verified by Western blotting in the Jurkat T-cell lysates using the anti-HA1 antibody (Fig. 2C, right). Thus, p12^I expression resulted in decreased phosphorylation of proximal TCR signaling molecules, and these findings provide a mechanistic explanation for the decrease in NFAT activity observed in the presence of p12^I (Fig. 1A).

The HTLV-1 p12^I protein localizes to lipid rafts. When p12^I is expressed ectopically in transfected cells, it localizes mostly to the ER/Golgi (28, 34). Because TCR signaling involves recruitment of molecules within the lipid rafts (57), we hypothesized that p12^I may also translocate to the lipid rafts. To test this hypothesis, we used both immunofluorescence on entire cells and a biochemical approach following cell fractionation.

Jurkat T cells transfected with p12^I_{HA} were stained with

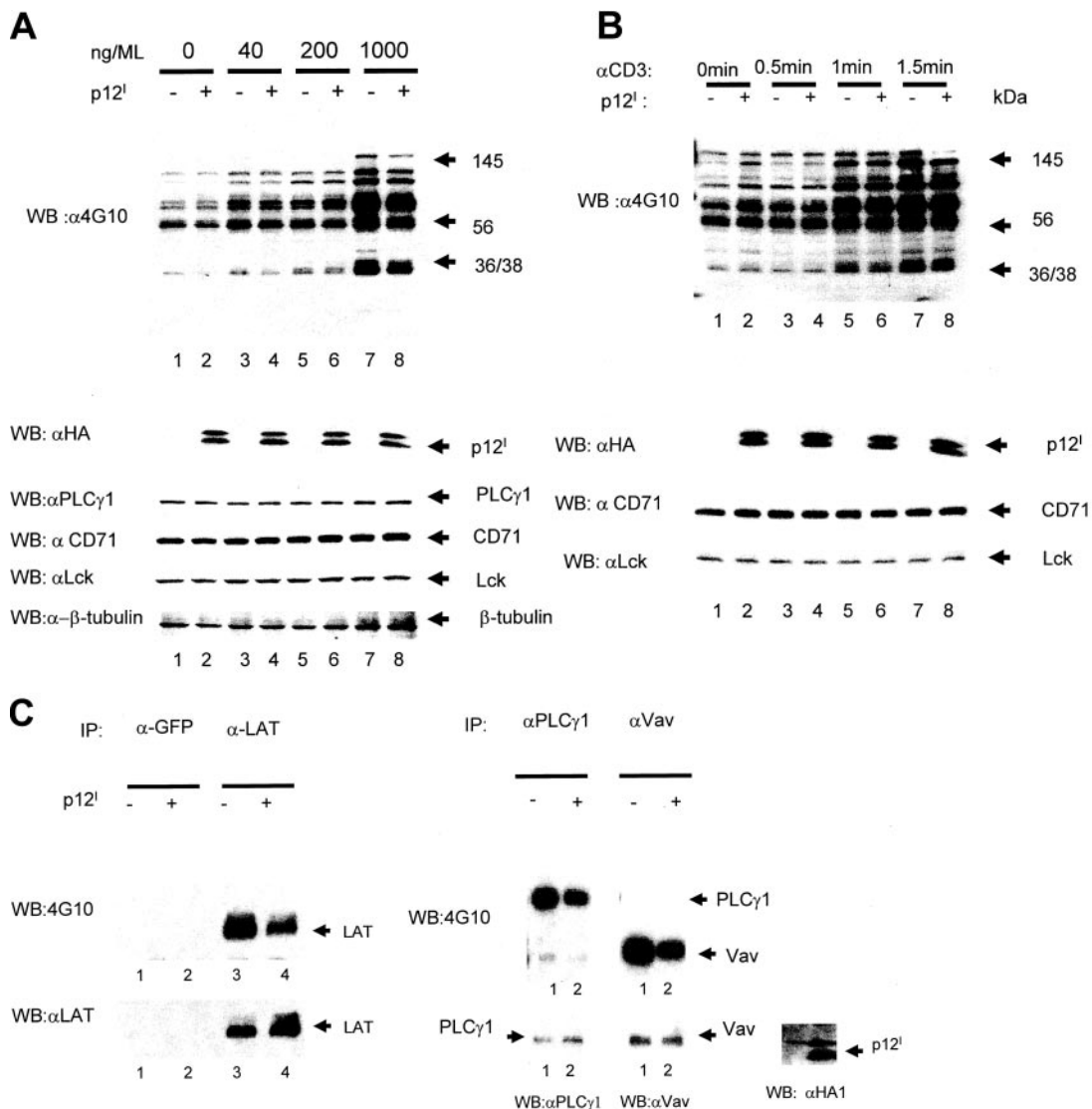


FIG. 2. The HTLV-1 p12¹ protein decreases the phosphorylation of PLC-γ1, Vav, and LAT upon TCR engagement. (A) (Top) Jurkat T cells transfected with p12¹ or sham DNA plasmids were treated with anti-CD3 antibody at increasing concentrations. Total cell lysates were obtained 2 min after pulse and analyzed by Western blotting (WB) using the anti-phosphotyrosine antibody (4G10). (Bottom) The membrane was also treated with anti-hemagglutinin (αHA) to verify p12¹ expression, as well as anti-PLC-γ1, anti-transferrin receptor, anti-Lck, and anti-β-tubulin to control for equivalent protein loading. (B) Jurkat T cells transfected with p12¹ or sham DNA plasmids were treated with 1 μg/ml anti-CD3, and cell lysates were harvested at the indicated time points. Equal amounts of lysates were analyzed by Western blotting with 4G10. The same membrane was probed with anti-HA to verify p12¹ expression and with anti-transferrin receptor (CD71) and anti-Lck to control for equivalent amounts of proteins. (C) The lysates of Jurkat T cells expressing p12¹ stimulated with anti-CD3 antibody (1 μg/ml) for 2 min were harvested, and aliquots were immunoprecipitated with antibodies against LAT, PLC-γ1, and Vav. The immunoprecipitates were first reacted in Western blotting with the 4G10 antibody to assess the phosphorylation status of each of the proteins. The same membranes were stripped and reacted with their specific antibodies to control for equivalent levels of protein in the immunoprecipitates. p12¹ expression was confirmed by Western blotting using the anti-HA1 antibody. The anti-green fluorescent protein (GFP) antibody was used as an irrelevant antibody control.

FITC-Co-Tx, which binds to the lipid rafts (2) and the anti-HA1 antibody. A subset of cells that were costained with both FITC-Co-Tx and anti-HA1 and image merging suggested colocalization of p12¹ and Co-Tx in the lipid rafts (Fig. 3A).

To confirm biochemically that p12¹ is in the lipid microdomains, we performed a flotation assay on T-cell lysates. The p12¹ protein was expressed in Jurkat T cells, and the total cell lysate was reacted in Western blotting with anti-HA1, anti-Lck (a protein located in the lipid rafts), and anti-CD71 (trans-

ferrin receptor localized in the cytoplasm and cellular membranes) (Fig. 3B). Cell lysates were prepared in 40% sucrose and overlaid with 30% and 5% sucrose cushions. The flotation assay yields the lipid rafts as a Triton-insoluble and buoyant fraction in the 5% and 30% sucrose interface (fractions 2 to 5), whereas solubilized proteins remain in 40% sucrose at fractions 8 to 12. Following centrifugation, the gradient fractions were analyzed by Western blotting with anti-HA1 and the anti-Lck and anti-CD71 antibodies as controls. Lipid raft frac-

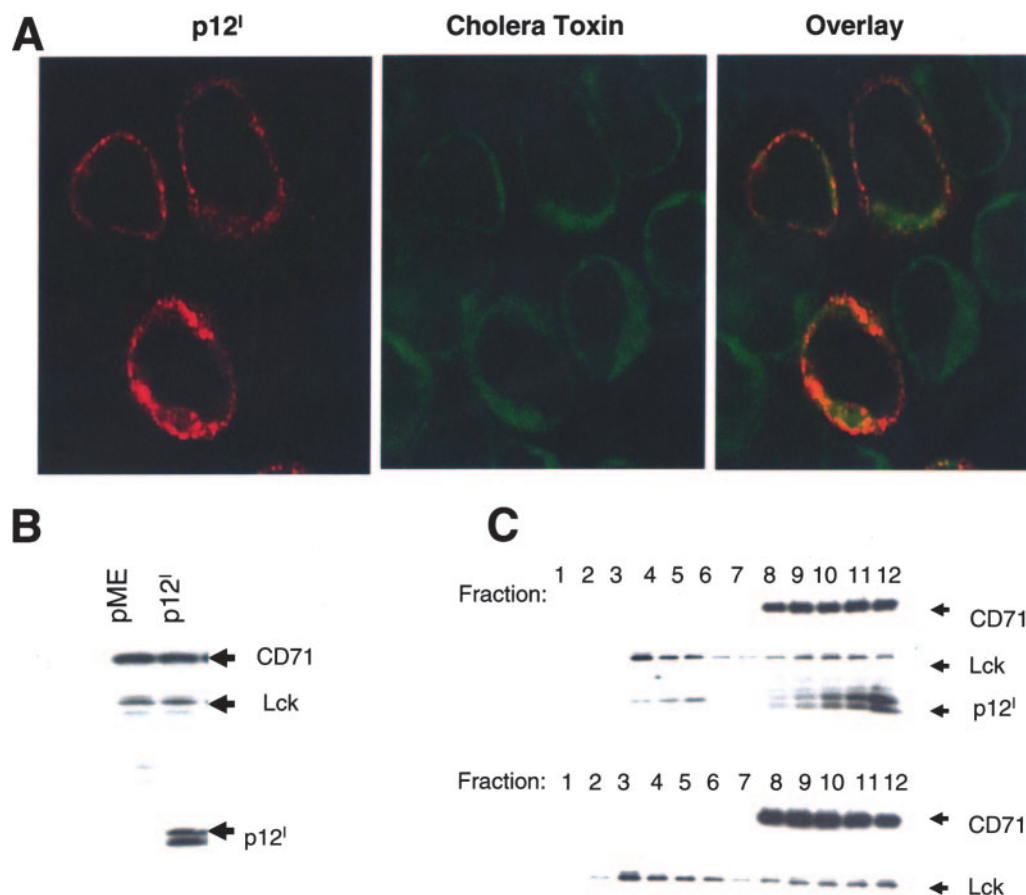


FIG. 3. p12¹ localizes to the lipid rafts. (A) p12¹ colocalization with Co-Tx was visualized by confocal microscopy using immunostaining with anti-HA1, followed by Cy3-labeled secondary antibody and FITC-Co-Tx, which binds to the rafts. (B) Total cell lysates of Jurkat T cells expressing p12¹ and control were analyzed by Western blotting to demonstrate equivalent amounts of CD71 and Lck. p12¹ expression was also verified. (C) Equivalent amounts of cell lysates from Jurkat T cells (B) were subjected to flotation assay to separate raft and nonraft fractions by biochemical means. The distribution of p12¹, as well as Lck and CD71 as raft and nonraft markers, was visualized by Western blot analysis in the collected fractions.

tions (4 to 6) contained Lck, a raft-associated protein in Jurkat T cells, whereas the solubilized fractions (8 to 12) contained CD71 (Fig. 3C, top). Interestingly, p12¹ was found not only in the soluble fractions 8 to 12, containing the cytoplasmic membrane, as expected, but also in fractions 4 to 6, which are enriched for Lck. No p12¹ was observed in cells transfected with the pME vector as a control (Fig. 3C, bottom). Lipid raft localization of p12¹ was also confirmed in 293T cells, where p12¹ colocalized in fraction 4, together with Lyn, a protein found in the lipid rafts in this cell type (data not shown). These data demonstrate that p12¹ localizes not only to the ER/Golgi, but also to the lipid rafts of different cell types.

p12¹ translocates to the immunological synapse upon MHC class II TCR engagement and down-regulates TCR signaling. Engagement of the TCR by peptide MHC on APCs leads to formation of immune synapses, complex structures that include the TCR, protein tyrosine kinases, and LAT. Because p12¹ is found in the lipid rafts and affects TCR signaling, we wished to investigate whether p12¹ was recruited to the immunological synapse upon TCR engagement. We used Jurkat T cells and Raji cells as an *in vitro* model of CD4⁺ T-cell and APC interaction that involves the TCR and MHC class II engagement. Polarization of the TCR could be visualized with the anti-CD3 antibody

within 15 min of cocultivation of Jurkat T cells with SEE-prepulsed Raji B cells (Fig. 4A). Transfection of p12¹ in Jurkat T cells demonstrated that, following cocultivation of the T cells with SEE-prepulsed Raji B cells, the p12¹ protein was present in the immunological synapse and colocalized with CD3 (Fig. 4B, b, c, e, f, h, and i). The formation of the immunological synapse was not observed in the absence of SEE (Fig. 4C, right).

To investigate whether in this *in vitro* model of TCR stimulation as well, p12¹ affects TCR signaling and NFAT activity, Jurkat T cells were cotransfected with the NFAT-luciferase reporter gene with or without p12¹ before cocultivation with Raji cells. p12¹ expression was verified by Western blotting (Fig. 4D, top). When Raji cells were prepulsed with SEE and cocultivated with Jurkat T cells, there was an increase of luciferase activity that decreased in the presence of p12¹ (Fig. 4D). Thus, p12¹ down-regulates TCR signaling, not only following engagement of the TCR by anti-CD3 ligation, but also under more physiological conditions with MHC class II.

p12¹ binds to exogenous LAT. The finding that p12¹ affects TCR signaling, translocates to the lipid rafts, and participates in immunological-synapse formation led us to formulate the hypothesis that p12¹ may physically interact with proximal TCR signaling molecules. We focused particularly on LAT,

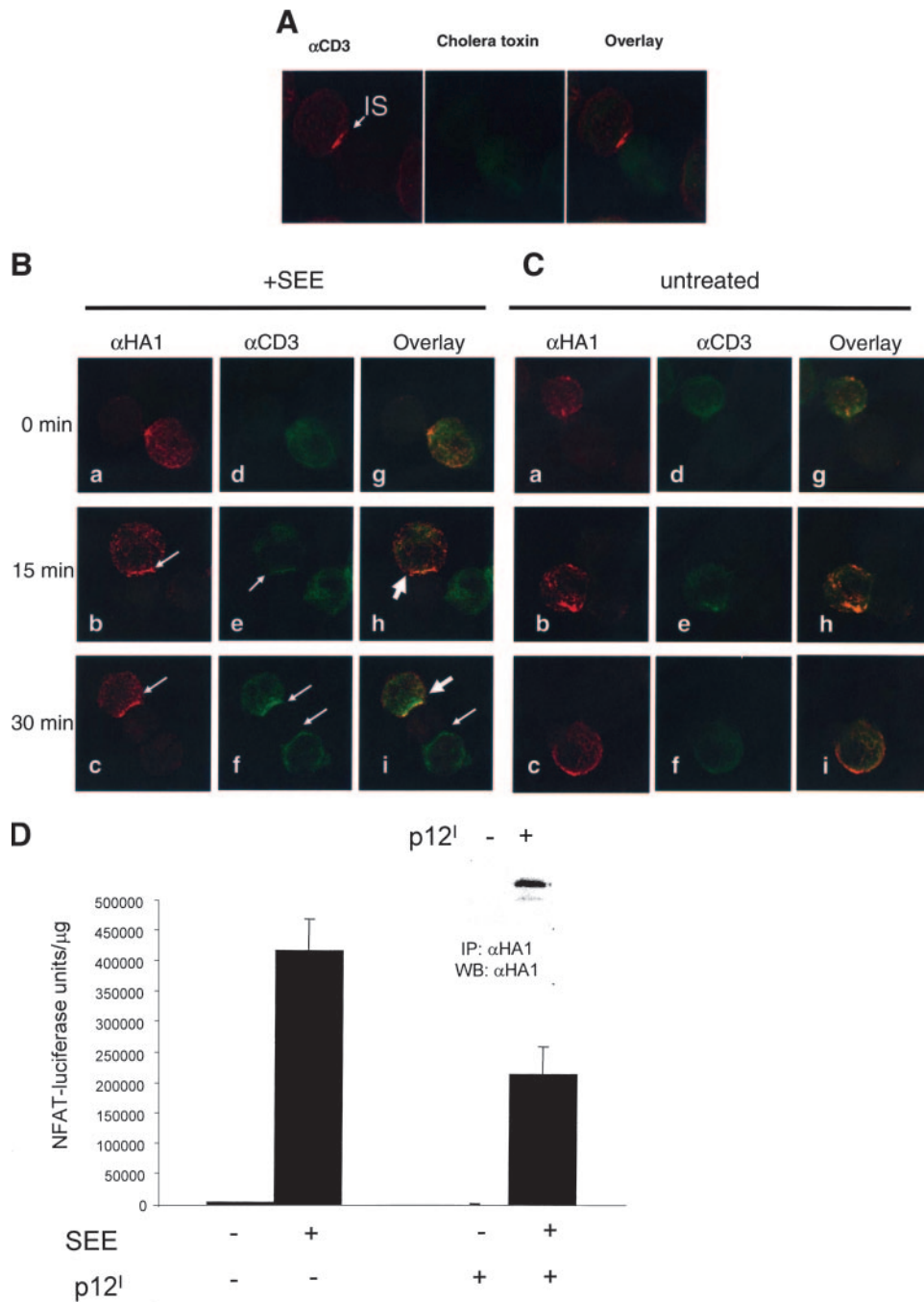


FIG. 4. Recruitment of the HTLV-1 p12¹ protein to the immunological synapse and down-regulation of TCR signaling in T cells. (A) Staining of Jurkat T cells cocultivated with SEE-prepulsed Raji cells for 15 min with the anti-CD3 antibody and FITC-Co-Tx. CD3 molecules aggregated at the immunological synapse (IS) following TCR engagement. (B and C) The distribution of p12¹ and CD3 was studied by immunofluorescence and microimaging analyses. Jurkat T cells expressing p12¹ were mixed with prepulsed Raji B cells in the presence (B) or absence (C) of 10 μ g/ml SEE and fixed at different time points. The arrows indicate the immunological synapse stained by anti-HA1 (α HA1) antibody (images b and c) or anti-CD3 antibody (images e and f). (D) The effect of p12¹ expression on NFAT-driven luciferase activity in Jurkat T cells was studied under the same experimental conditions as for panels B and C. Lysates were prepared after 20 h of cocultivation. Luciferase units were normalized for the lysate protein content. The average and the standard deviation of six duplicate samples are shown. Expression of p12¹ in the lysate was verified by immunoprecipitation (IP) and Western blotting (WB) (inset).

because LAT, like p12¹, resides in the lipid rafts. To investigate this possibility, we expressed a LAT-Myc-tagged protein together with p12¹ in Jurkat T cells. As demonstrated in Fig. 5A, the anti-Myc antibody coprecipitated p12¹ and the anti-HA1

(mouse monoclonal antibody 12CA5) coprecipitated LAT-Myc, whereas the irrelevant anti-AU1 control antibody precipitated neither p12¹ nor LAT-Myc (Fig. 5A). Binding of p12¹ to LAT-Myc was also confirmed in 293T cells, where antibody to

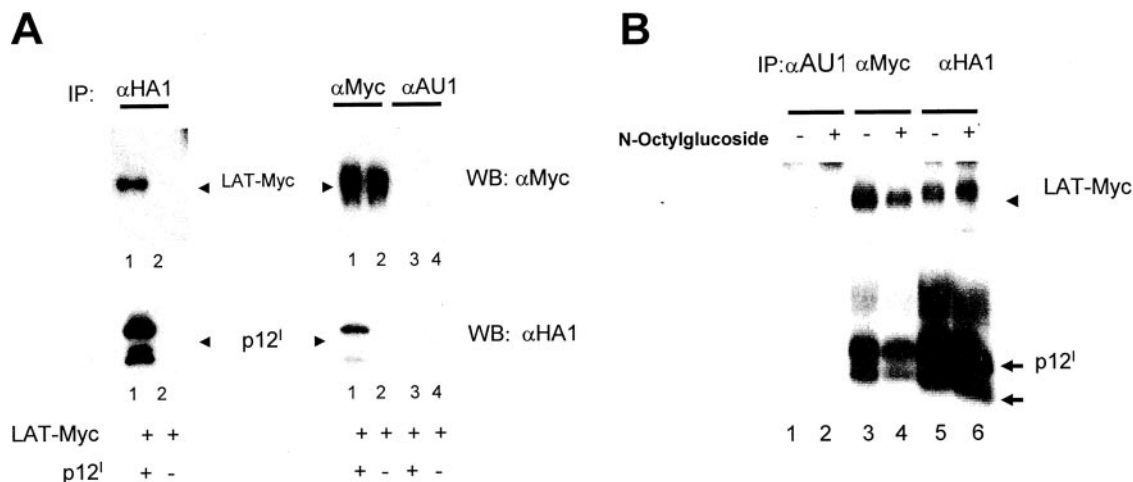


FIG. 5. p12^I interacts with exogenous LAT. (A) Jurkat T cells transfected with p12^I and LAT-Myc as indicated were analyzed by immunoprecipitation (IP), followed by Western blotting (WB) to assess the presence of association. (B) Lysates of 293T cells coexpressing p12^I and LAT-Myc were used for immunoprecipitation in the presence or absence of *N*-octylglucoside at 60 mM. Western blotting of the immunoprecipitates is shown.

p12^I coprecipitated LAT-Myc, anti-Myc antibody coprecipitated p12^I, and again, the anti-AU1 antibody coprecipitated neither (data not shown). Because p12^I is very hydrophobic and is found in the lipid microdomains, there is a possibility that its coprecipitation with LAT is due to the localization of both proteins to the glycosphingolipid-enriched membrane microdomains rather than to a real physical interaction within the cell. To attempt to address this question, Jurkat T cells were cotransfected with both p12^I and the tagged LAT-Myc construct and the cell lysate was solubilized with *N*-octylglucoside, a compound able to solubilize the glycosphingolipid-enriched components of the membrane (6). Treatment with *N*-octylglucoside did not affect the coprecipitation of p12^I and LAT, providing further support to their interaction in cells (Fig. 5B). We next investigated whether p12^I was able to coprecipitate endogenous LAT from Jurkat T-cell lysates using the anti-LAT antibody. p12^I did not coprecipitate endogenous LAT. While it is possible that the interactive site of p12^I with endogenous LAT may interfere with the binding of the anti-LAT antibody, there is also the alternative possibility that this interaction may result from overexpression of highly hydrophobic proteins. Thus, whether the interaction occurs in live cells and whether it is of any biological significance remain uncertain.

p12^I reduces HTLV-1 expression following TCR engagement by anti-CD3 or MHC class II. In vivo, HTLV-1 infects CD4⁺ T cells and, less frequently, CD8⁺ T cells (17). Because TCR ligation increases Tax-mediated transcription from the HTLV-1 LTR (36), we hypothesized that the ability of p12^I to down-regulate TCR signaling may have evolved to limit viral replication. To test this, we transfected Jurkat T cells with the proviral clone pACH (30) without or with increasing amounts of p12^I and stimulated them with the anti-CD3 antibody. We observed an increase in the activity of Tax-driven luciferase expression in transfected cells with pACH following TCR ligation, as expected (36) (Fig. 6A), and p12^I decreased Tax-driven luciferase levels in a dose-dependent manner (Fig. 6A). Decreased Tax activity was associated with decreased virus production measured as the p19 Gag level in cell supernatants (Fig. 6B). Likewise, while in unstimulated T

cells intracellular p24 Gag was barely detectable, in Jurkat T cells stimulated with anti-CD3 antibody, p24 Gag was readily detectable and was decreased by p12^I coexpression (Fig. 6C).

Next, we wished to assess whether the engagement of the TCR by MHC class II in the Jurkat T/Raji B-cell model could affect HTLV-1 replication and whether p12^I would affect it. To test this, we used the replication-competent proviral HTLV-1 clone pACH (30), as well as an isogenic clone in which the initiating ATG codon for the p12^I protein was mutated (pACH-GTG) without altering the amino acid sequence of either the p30^{II} or HBZ protein, as both of these ORFs overlap in the HTLV-1 genome (Fig. 6D). The pACH and the isogenic p12^I KO pACH-GTG clones produce equivalent virus upon transfection in unstimulated Jurkat T cells, as demonstrated by equivalent Tax-driven luciferase levels (Fig. 6E) and by the production of equivalent p19 Gag levels in the supernatants of the transfected Jurkat T cells (Fig. 6F).

pACH was expressed in Jurkat T cells that were cocultured with Raji B cells transfected with an HTLV-1 LTR-luciferase reporter gene, and the cells were cocultivated or placed in a TransWell system. Luciferase activity was observed only when B cells were cocultured under conditions where cell-to-cell contact could occur (Fig. 7A). Prepulsing of B cells with the bacterial antigen SEE under normal coculture conditions resulted in a 4-fold increase in luciferase activity and a 2.5-fold increase in p19 Gag production (Fig. 7A and B). We excluded the possibility that extracellular Tax may activate LTR-driven luciferase expression in B cells because expression of Tax alone in Jurkat T cells, followed by cocultivation with prepulsed B cells, did not result in activation of the LTR-luciferase reporter gene in B cells (data not shown).

To discriminate between cell fusion and/or transmission of HTLV-1 particles to the B cells, we treated the Jurkat T-cell and SEE-prepulsed B-cell cocultures with the reverse transcriptase inhibitor Zerit. As demonstrated in Fig. 7C, Zerit decreased Tax-mediated luciferase activity in B cells, providing support to the notion that Tax activity is dependent on the reverse transcription of the viral genome.

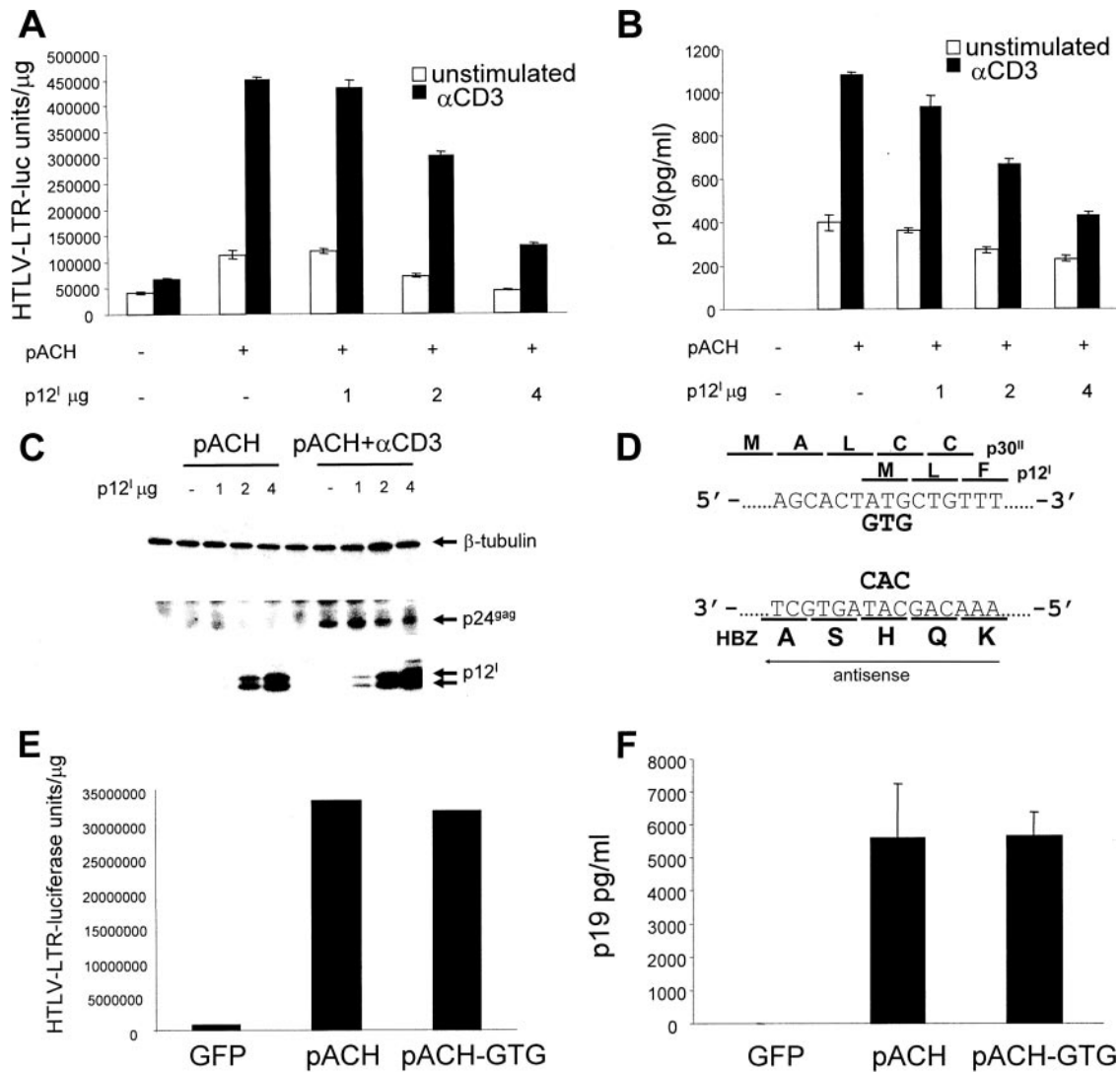


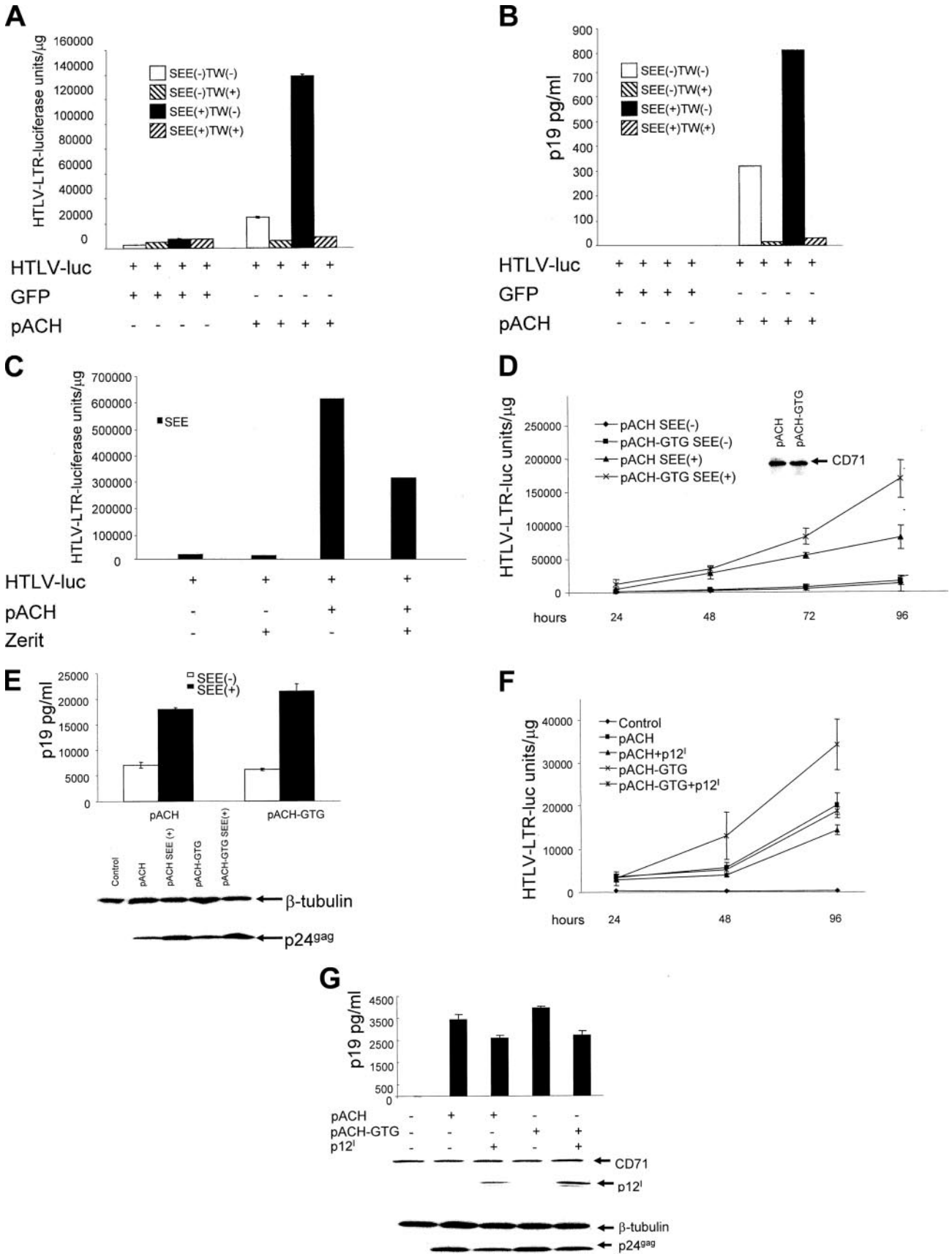
FIG. 6. p12^I suppresses viral expression. Jurkat T cells were cotransfected with the pACH clone or control plasmid, together with the HTLV-1 LTR-luciferase reporter gene and increasing amounts of p12^I (μg/10⁶ cells). The cells were stimulated or not with 1 μg/ml of anti-CD3 antibody for 50 h. (A) Luciferase activity/μg protein in cell lysates of transfected Jurkat T cells. (B) p19 Gag levels in the supernatants of the transfected Jurkat T cells. (C) Western blot of cell lysates using β-tubulin (to control for equal protein loading), the anti-p24 Gag antibody, or the anti-HA1 antibody. (D) Schematic representation of ORFs I and II and the HBZ ORF in the provirus. The initiation (ATG) codon of ORF I in pACH-GTG was mutagenized to GTG as depicted. (E) Jurkat T cells were transfected with equivalent amounts of pACH or pACH-GTG, together with the HTLV LTR-driven luciferase expression plasmid. The luciferase activity was measured from triplicate samples of cell lysates 44 h after transfection. All values were corrected by the protein content of the cell lysates, and mean values and standard deviations are shown. GFP, green fluorescent protein. (F) p19 Gag levels in the cell supernatants. The error bars indicate standard deviations.

Next, we transfected equivalent amounts of wild-type pACH and the pACH-GTG molecular clone in Jurkat T cells. Jurkat T cells were cocultivated with B cells transfected with the HTLV-1 LTR-luciferase reporter gene prepulsed or not with SEE. Measurement of Tax activity in the pACH or GTG clone over time demonstrated that the luciferase levels were higher in cocultures that expressed the pACH-GTG virus over time, suggesting that ablation of p12^I results in more efficient viral expression and therefore a higher level of transmission to B cells upon antigen stimulation (Fig. 7D). Accordingly, the levels of p19 Gag in the supernatants, as well as those of p24 Gag in cell lysates, were highest in cocultures producing the mutant pACH-GTG virus (Fig. 7E). Furthermore, coexpression of p12^I with either pACH

or the pACH-GTG clone resulted in a decrease in luciferase, p19 Gag, and p24 Gag production from both the pACH and the pACH-GTG transfectants (Fig. 7F and G), as expected. Thus, it appears that, in the absence of p12^I, more virus was expressed in T cells, and in turn, more virus was transmitted to B cells. Also, in this model of TCR engagement by MHC class II, the overexpression of p12^I decreased viral production.

DISCUSSION

The engagement of peptide MHC with the TCR triggers a cascade of events that leads to phosphorylation of the TCR, the TCR proximal signaling molecule, and LAT, which bridges



proximal-to-distal effector molecules for T-cell activation. These events also lead to the formation of a polarized cell-cell junction designated the immunological synapse (26). Although the biochemical events peak before the formation of a mature synapse, the stability and length of this contact are thought to be important in the determination of whether T cells enter the cell cycle following antigen stimulation. We observed in our study that the p12¹ protein encoded by HTLV-1, a virus associated with immune suppression and autoimmune and proliferative syndromes, perturbs TCR signaling in CD4⁺ T cells. We obtained evidence that p12¹ may interact directly or indirectly with a critical signaling molecule, LAT. However, the biological significance of this finding remains uncertain, as we were unable to demonstrate the interaction of p12¹ with endogenous LAT.

Here, we demonstrated that the HTLV-1 p12¹ protein upon antigen stimulation decreases TCR signaling by inhibiting LAT phosphorylation and, consequently, the phosphorylation of PLC- γ 1 and Vav. The result is a decrease in NFAT activation. The specificity of LAT targeting by p12¹ is suggested by the fact that ZAP70 phosphorylation, which is LAT independent, is not affected by p12¹ (data not shown). There is no contrast between our data and the positive effect of p12¹ on NFAT activation that has been previously reported to follow PMA stimulation. In those experiments, PMA stimulation bypassed TCR ligation, and we have reproduced these findings here (Fig. 1A). TCR stimulation drives T cells from a resting state to early G₁, whereas PKC activation may occur later in cell cycle progression, suggesting that p12¹ may have differential effects in different phases of the cell cycle. Indeed, p12¹ has also been demonstrated to increase STAT-5 activation and responsiveness to IL-2, and both events are associated with the S phase of the cell cycle.

The importance of the physical recruitment of p12¹ to the immunological synapse in TCR signaling remains unclear, but collectively, the data presented here indicate that HTLV-1 has evolved a function to decrease TCR signaling. There are, however, limitations in this model, as we overexpressed p12¹ in some of our experiments. However, the hypothesis that p12¹ may dampen TCR signaling and viral replication is also supported by the finding that selective ablation of p12¹ expression by mutation of the initiation methionine codon of ORF 1 results in an increase in viral production upon TCR stimulation, as demonstrated in Fig. 7E.

Other viruses that infect lymphocytes perturb TCR signaling and encode proteins that also associate with lipid rafts. These

proteins include the Epstein-Barr virus LMP1 protein (23), human immunodeficiency virus type 1 Nef (58), measles virus proteins (3), the human herpesvirus 8 K5 protein (11), and herpes simplex virus and herpesvirus saimiri (9, 47).

Interestingly, the HIV-1 Nef and p12¹ proteins have opposite effects on T-cell activation and viral production. Nef associates with the lipid rafts and is thought to transport cholesterol to them and to increase human immunodeficiency virus infectivity. p12¹ is also in the lipid rafts, but it decreases viral expression. Both Nef and p12¹ are recruited to the immunological synapse, but Nef potentiates TCR signaling (15) while p12¹ dampens it. Nef expression is associated with an increase in Cbl phosphorylation (54), whereas p12¹ decreases it (our unpublished data). The Nef effect is upstream of PKC activation, as it is abolished by PMA, a direct activator of PKC. The negative effect of p12¹ is not observed when T cells are treated with PMA (12). Nef physically interacts with CD3 ζ of the TCR complex (49, 53) and, through its proline-rich motif, mediates interaction with phosphatidylinositol 3-kinase, Vav, and Lck (4, 10, 14, 44). p12¹ likely interacts directly or indirectly with LAT and, by decreasing its phosphorylation, decreases Vav and PLC- γ 1 phosphorylation.

Measles virus also interferes with T-cell activation by directly interacting with the T-cell lipid rafts and causing alteration in the recruitment of proteins central to TCR activation, such as Vav and the AKT kinase (3). The K5 protein of Kaposi sarcoma virus affects T-cell activation by down-regulating ICAM-1 and B7-2 in B cells (11). Herpes simplex virus, like HTLV-1 p12¹, decreases LAT phosphorylation, whereas herpesvirus saimiri targets ZAP70. Thus, usurping the TCR signaling pathway, albeit by different mechanisms, is a shared feature of viruses that infect lymphocytes and probably reflects a successful adaptation to the host immune response.

The recent observation that HTLV-1 spreads to T cells through polarization of the cytoskeleton (27) at an interface between cells defined as the viral synapse raises questions about the possible role of p12¹ in cell-to-cell transmission of HTLV-1. The data presented here suggest that p12¹ reduces viral replication indirectly by affecting T-cell activation, as demonstrated by the fact that, in unstimulated Jurkat T cells, the wild type and p12¹ KO molecular clones produce equivalent amounts of virus. The experimental model of viral transmission used here (Jurkat T/B cells) is not suited to direct quantitation of viral transmission. The increased viral transmission to B cells when cocultured with antigen-stimulated Jurkat T cells expressing the p12¹ KO molecular clone could be

FIG. 7. TCR MHC class II antigen engagement increases HTLV-1 replication. (A and B) The HTLV-LTR-driven luciferase expression in Raji cells was used as a reporter of viral expression. Jurkat T cells expressing pACH or green fluorescent protein (GFP) were cocultured with Raji cells in the presence or absence of SEE under normal coculture conditions [TW(-)] or in the TransWell (TW) system. After 43 h, reporter cells and culture media were analyzed by triplicate dual-luciferase assays (A) and p19 Gag antigen capture assay (B), respectively. (C) Jurkat T cells were transfected with the pACH clone and cocultivated with prepulsed B cells transfected with the HTLV-1 LTR-luciferase control. The cells were cultured in the presence or absence of 35 μ M of Zerit for 72 h. Cell lysates were analyzed for the level of luciferase. (D and E) Equivalent numbers of Jurkat T cells transfected with the pACH or pACH-GTG clone were cocultured with Raji cells with or without SEE. Control for an equal input of cells was performed using the anti-CD71 antibody by Western blotting (D, inset). Cells, as well as culture media, were harvested at each time point up to 96 h, and the luciferase levels (D) and p19 Gag values at 48 h (E) from triplicate samples are shown with standard deviations. Expression of p24 Gag in cell lysates at 96 h is shown in the lower part of panel E. (F and G) pACH and pACH-GTG were expressed with or without p12¹ in Jurkat T cells, which in turn were cocultured with SEE-prepulsed B cells transfected with the LTR-luciferase reporter gene. p12¹ protein expression and intracellular p24 Gag were assessed by Western blotting at 96 h. Luciferase expression (F) and p19 Gag (G) in the supernatants were measured at 48 h from triplicate samples. Standard deviations are shown.

ascribed simply to an increased level of virus produced by T cells following antigen stimulation, rather than increased transmission per se.

HTLV-1 has been reported to also infect CD8⁺ T cells and to impair their function. As TCR signaling is simultaneously required for degranulation by cytotoxic T lymphocytes (45), p12^I could affect the cytolytic activity of infected CD8⁺ T cells by down-regulating TCR signaling. Thus, p12^I may play an essential role in viral persistence by down-regulating TCR signaling, viral production, and MHC class I presentation in infected CD4⁺ T cells (28) on one hand and, on the other, by impairing the function of infected virus-specific CD8⁺ cytotoxic T cells. Hopefully, further elucidation of the domains of p12^I and LAT involved in the perturbation of TCR signaling may provide rational approaches to limit or eliminate the vicious cycle that causes viral persistence in the host and to further minimize the chance for disease development.

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