JOURNAL OF VIROLOGY, Dec. 1995, p. 7814–7822 Vol. 69, No. 12
0022-538X/95/$04.00+0

Downregulation of Lck-Mediated Signal Transduction by tip of Herpesvirus Saimiri

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Received 6 July 1995/Accepted 15 September 1995

A protein, called tip, of herpesvirus saimiri associates with Lck in transformed T cells. To investigate the effects of complex formation on cellular signal transduction, we constructed human Jurkat-T-cell lines expressing tip. The expression of tip in Jurkat-T cells dramatically suppressed cellular tyrosine phosphorylation and surface expression of lymphocyte antigens. The expression of tip also blocked the induction of tyrosine phosphorylation by anti-CD3 stimulation. The expression of tip in fibroblast cells suppressed the transforming activity of oncogenic F505 Lck. Binding assays showed that the SH3 domain of Lck is sufficient to form a stable complex with tip in vitro. These results demonstrate that tip acts at an early stage of the T-cell signal transduction cascade by associating with Lck and downregulating Lck-mediated activation. Inhibition of Lck-mediated signal transduction by tip in T cells appears to be analogous to the inhibition of Lyn/Syk-mediated signal transduction in B cells by LMP2A of the B-cell-tropic Epstein-Barr virus.

Stimulation of the T-cell receptor (TCR) induces a signal transduction cascade leading to activation of mature T cells. One of the earliest biochemical events after TCR stimulation is the recruitment of protein tyrosine kinases resulting in enhanced tyrosine phosphorylation of a number of cellular proteins (24, 54). Some of the protein tyrosine kinase targets identified to date are CD3 ε, γ, and δ subunits; TCR ζ subunit; phospholipase C; valosin-containing protein; p95vav; CD5; CD6; CD28; mitogen-activated protein kinase; and ZAP-70 kinase (24, 26, 54). Accumulating data suggest that two members of the Src family kinases, Lck and Fyn, are involved in TCR signal transduction.

The *lck* gene product is a 56-kDa Src-related protein tyrosine kinase expressed exclusively in T lymphocytes (49). Lck is stably associated with the cytoplasmic tails of CD4 and CD8 T-cell surface antigens (42, 50). Activation mediated by CD4 and CD8 antibodies also rapidly stimulates the tyrosine kinase activity of Lck and causes a prompt increase in intracellular tyrosine phosphorylation, suggesting that Lck can transduce intracellular tyrosine phosphorylation signals from CD4 and CD8. Additionally, it has been suggested that Lck may participate in transducing signals from other membrane molecules, as evidenced by the finding of physical complex formations with the β chain of interleukin-2 (IL-2) receptor (18), glycophosphatidylinositol-anchored molecules (43), CD2 (3), CD5 (37), and phosphatidylinositol (PI) 3-kinase (35). Analysis of JCaM1 cells, which are defective in the expression of functional Lck tyrosine kinase, has indicated that Lck is required for appropriate signal transduction through the TCR (46). Iwashima et al. (19) have demonstrated that TCR signal transduction is initiated by the sequential interaction of Lck and ZAP-70 with TCR antigen recognition activation motifs or immune receptor tyrosine-activating motifs.

Herpesvirus saimiri (HVS) is a member of the gamma subfamily of herpesviruses (*Gammaherpesvirinae*). Some members of this group, e.g., Epstein-Barr virus (EBV), HVS, herpesvirus ateles, and herpesvirus sylvilagus, are capable of inducing lymphoproliferative disorders in natural or experimental hosts. Recently, sequences with high homology to HVS have been found in AIDS-associated Kaposi's sarcoma (10). HVS is apparently not associated with any disease in its natural host, the squirrel monkey (*Saimiri sciureus*). However, it does induce rapidly progressing fatal lymphomas, leukemia, and lymphosarcomas in several other species of New World primates (20). Strains of HVS have been divided into three subgroups (A, B, and C) on the basis of the extent of DNA sequence divergence at the left end of L-DNA (13, 30). Strains from subgroups A and C are highly oncogenic and able to immortalize common marmoset peripheral blood lymphocytes in vitro to IL-2-independent growth (14, 47). Subgroup C strains are further capable of immortalizing human and rhesus monkey lymphocytes into continuously proliferating T-cell lines (4) (unpublished results).

A protein, called tip (tyrosine kinase-interacting protein), encoded by the left end of the viral genome was previously identified in virus-transformed T cells (6). tip did not show transforming activity in rodent fibroblast cells (23), but the protein was found to be associated with the major T-cell tyrosine kinase Lck and phosphorylated on tyrosine residues by purified Lck in several cell-free assay systems (6). Mutational analysis of a glutathione *S*-transferase (GST)–tip fusion protein revealed that binding to Lck requires putative SH3-binding (SH3B) sequence and a sequence homologous to the carboxy terminus of Src-related kinases (22). These sequences are referred to as SH3B and C-terminal Src-related kinase homology (CSKH) elements. Peptide fragments as short as 37 amino acids containing CSKH, spacer sequence, and SH3B motifs are sufficient to form a stable complex with Lck in vitro. Furthermore, the same sequences of tip are necessary for in vivo

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association with Lck. tip of HVS has apparently acquired CSKH and SH3B elements for the purpose of targeting cellular protein kinases.

In this report, we show that HVS tip downregulation of Lck-mediated signal transduction in T cells is analogous to EBV LMP2A downregulation of Lyn/Syk-mediated signal transduction in B cells. Striking parallels in the genomic location and functional role of corresponding HVS and EBV proteins occur in the complete absence of any sequence similarity.

MATERIALS AND METHODS

Cell culture and transfection. Jurkat-T and JCaM1 cells were grown in RPMI medium supplemented with 10% fetal calf serum. Common marmoset T lymphocytes immortalized by HVS were grown in RPMI medium supplemented with 20% fetal calf serum. Primary common marmoset peripheral blood lymphocytes were purified with lymphocyte separation medium (Organon Teknika Corp., Malvern, Pa.), washed, activated with 1 μg of phytohemagglutinin per ml for 48 h, and then grown in RPMI medium with 20% fetal calf serum supplemented with 10% IL-2. Sf9 cells were maintained at 27°C in Grace's medium containing 10% fetal calf serum, yeastolate, and lactalbumin hydrolysate. COS-1 and NIH 3T3 cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. A DEAE-dextran transfection procedure was used for transient expression in COS-1 cells. pBabe and pBabe-tip DNA (20 μg) was introduced into Jurkat-T and JCaM1 cells by electroporation (Bio-Rad) at 250 V and 960 μF in serum-free Dulbecco modified Eagle medium or into NIH 3T3 cells by $CaPO_4$ transfection. After incubation for 48 h, the cells were cultured with selective medium containing 5 μg of puromycin per ml for the next 5 weeks.

Plasmid constructions. DNA containing the *tip* open reading frame was amplified from HVS strain C488 virion DNA by PCR using primers containing *Eco*RI and *Xho*I recognition sequences at the ends (5). Amplified DNA was ligated into the *Eco*RI and *Xho*I cloning sites of the pSP72 vector (Promega Biotech, Madison, Wis.). For AU-1 tagging, 5′ primer CGC GGA TCC ATG GAC ACC TAT CGC TAT ATA GCA AAT GAA GGA GAA was used for PCR amplification, and AU-1-tagged *tip* DNA was subcloned into pSP72 vector. AU-1-tagged tip DNAs were completely sequenced to verify 100% agreement with the original sequence. For stable expression, *Eco*RI and *Xho*I DNA containing the *tip* gene from pSP72 was cloned into the *Eco*RI and *Sal*I sites of pBabe-puro retroviral vector (34).

Expression and purification of GST fusion proteins and antibody. For the construction of GST-tip, an *Eco*RI-*Xho*I fragment containing the coding sequences for amino acid residues from 1 to 226 of tip was inserted into the *Eco*RI and *Xho*I sites of expression vector pGEX-4T (Pharmacia LKB, Piscataway, N.J.). GST fusion protein expression and purification were performed essentially as described by Smith and Johnson (44). For fusion protein recovery using glutathione-Sepharose, bacterial cell pellets were frozen once, resuspended with 1/10 volume of lysis buffer (1% Triton X-100, 0.1% sarcosinate in phosphate-buffered saline [PBS]) containing protease inhibitors, and disrupted by sonication. After centrifugation to remove cell debris, supernatant fluids were mixed with preequilibrated glutathione-Sepharose for 30 min at 4°C. The beads were then washed three times with PBS and once with buffer (10 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM Tris [pH 7.0]). The purified recombinant GST-tip protein was used to generate polyclonal antibody in New Zealand White rabbits. AU-1 monoclonal antibody recognizing the DTYRYI epitope from bovine papillomavirus L1 capsid protein was purchased from Berkeley Antibody (Richmond, Calif.). Rabbit polyclonal anti-Lck antibody generated by synthetic peptide based upon residues 22 to 51 of the amino terminus of human Lck was purchased from Upstate Biotechnology Inc. (Lake Placid, N.Y.). Antibodies to ZAP-70, Fyn, Fgr, Hck, Lyn, Yes, and Src were purchased commercially from UBI and Santa Cruz Biotech (Santa Cruz, Calif.).

Immunoprecipitation and immunoblot. Cells were harvested and lysed with lysis buffer (0.15 M NaCl, 0.5% Nonidet P-40, 50 mM HEPES [*N*-2-hydroxyethylpiperazone-*N*′-2-ethanesulfonic acid] buffer [pH 8.0]) containing 1 mM Na_2VO_3, 1 mM NaF, and protease inhibitors (leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and bestatin). Immunoprecipitated proteins from cleared cell lysates were used for immunoblot or in vitro kinase assay. For protein immunoblots, polypeptides in cell lysates corresponding to 10^5 cells were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane filter. Immunoblot detection was performed with a 1:1,000 or 1:3,000 dilution of primary antibody for conventional color development or with the enhanced chemiluminescence system (ECL; Amersham, Chicago, Ill.).

In vitro kinase assays. For in vitro protein kinase assays, complexes prepared as described above were washed once more with kinase buffer (10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM unlabeled ATP, 20 mM Tris [pH 7.0]), resuspended with 10 μl of the same buffer containing 5 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; NEN) for 15 min at room temperature, and separated through SDS-PAGE. Kinase activity was measured with a Bio-Rad Molecular Imager model GS-250.

Fluorescence-activated cell sorter (FACS) analysis. Cells (5×10^5) were washed with RPMI medium containing 10% fetal calf serum and incubated with fluorescein isothiocyanate-conjugated or phycoerythrin-conjugated monoclonal antibody for 30 min at room temperature. After being washed, each sample was fixed with 1% formalin solution and cytofluorographic analysis of cell populations was performed with a FACS-1 (Becton Dickson and Co., Mountainview, Calif.). Antibodies for CD2 (RPA-2.10), CD3 (UCHT1), CD4 (RPA-T4), CD5 (UCHT2), CD8 (RPA-T8), CD11a (HI111), and CD45 (HI30) were purchased from PharMingen (San Diego, Calif.), and antibody for TCR αβ (BW242/412) was purchased from T Cell Diagnostics (Cambridge, Mass.).

Anti-CD3 stimulation. Cells (10^7) were incubated with anti-CD3 (OKT3) antibody at 37°C for the indicated times. After stimulation, cells were immediately lysed with cold lysis buffer containing 1 mM Na_2VO_3, 1 mM NaF, and protease inhibitors (leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and bestatin). Precleared cell lysates were used for immunoblot or for immunoprecipitation.

In vitro binding of GST-Lck fusion proteins to tip. Five micrograms of purified GST-Lck fusion protein noncovalently coupled to glutathione-Sepharose beads was mixed with precleared ^{35}S-labeled insect cell lysates for 1 h at 4°C and washed four times with lysis buffer. Bound proteins were resolved by SDS-PAGE and visualized by autoradiography.

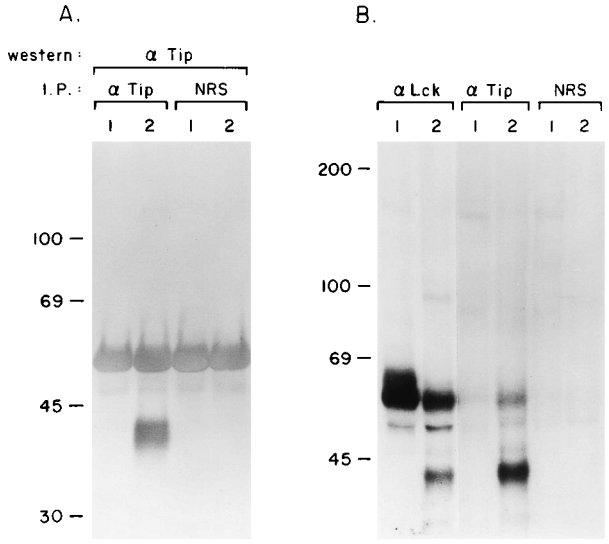

FIG. 1. Association of tip with Lck in common marmoset T cells transformed by HVS. (A) Identification of tip protein. A total of 5×10^7 primary common marmoset peripheral blood lymphocytes (lanes 1) and common marmoset T lymphocytes immortalized by HVS (lanes 2) were used for immunoprecipitations (IP). Polypeptides precipitated by anti-tip or normal rabbit serum (NRS) were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with anti-tip antibody. The 55-kDa protein present in all lanes is the immunoglobulin heavy chain. (B) In vitro kinase assays of immune complexes using anti-tip and anti-Lck antibodies. Immunoprecipitations were performed with anti-Lck, anti-tip, or NRS as described above. These immune complexes were assayed for kinase activity with [γ-^{32}P]ATP. Overnight exposure was used. The positions of molecular mass standards (in kilodaltons) are on the left of each gel.

RESULTS

Association of tip with Lck in stable T-cell lines. It has been shown that the 40-kDa tip protein is associated with Lck in human T cells transformed by HVS C488 (6). Since Lck is a major tyrosine kinase in T cells and many viral proteins from tumor viruses exert their effects through specific interactions with cellular proteins, an association between Lck and tip seems likely to play an important physiological role. We generated rabbit polyclonal antibody against recombinant bacterial GST-tip protein to examine the expression of the *tip* gene in IL-2-independent common marmoset T cells transformed by HVS C488. As shown in Fig. 1A, rabbit polyclonal antibody against tip reacted with a protein having an apparent molecular size of about 40 kDa on immunoblots from transformed common marmoset T cells, which is the same size as that

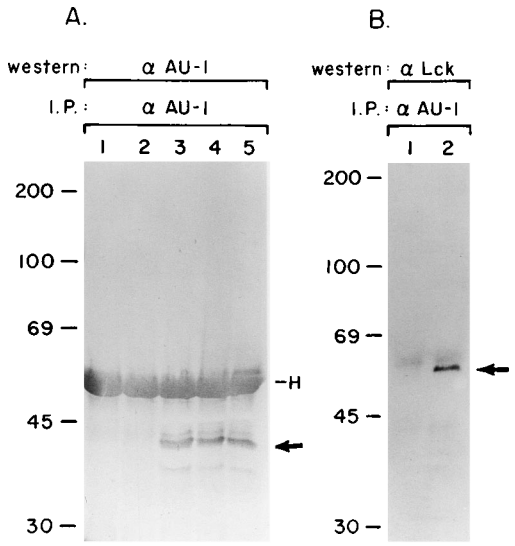

FIG. 2. Expression and association of tip with Lck in T-cell lines. (A) Immunoblot detection of tip protein in T cell lines. A total of 2×10^7 cells were used for the preparation of extracts used for immunoprecipitations (IP). Polypeptides in AU-1 immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with AU-1 antibody. Lane 1, Jurkat-babe; lane 2, JCaM1-babe; lane 3, Jurkat-babe-tip; lane 4, Jurkat-babe-tip clone 1; lane 5, JCaM1-babe-tip. The letter H indicates the position of the immunoglobulin heavy chain. An arrow indicates the position of tip protein. (B) Association of tip with Lck in Jurkat-T cells. Polypeptides in AU-1 immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with anti-Lck antibody. Lane 1, Jurkat-babe; lane 2, Jurkat-babe-tip. An arrow indicates the position of the Lck protein. The positions of molecular mass standards (in kilodaltons) are given on the left of each gel.

detected by others previously (6). In vitro kinase reactions showed that a physical association between tip and Lck was also present in these transformed cells (Fig. 1B).

Jurkat-T and JCaM1 cells were used to establish stable cell lines expressing the *tip* gene in the absence of the remainder of the HVS viral genome. The full-length *tip* gene was modified to encode an AU-1 epitope tag at the amino terminus and cloned into the retroviral vector pBabe-puro. After electroporation of retroviral vector into Jurkat-T and JCaM1 cells, polyclonal Jurkat-babe, Jurkat-babe-tip, JCaM1-babe, and JCaM1-babe-tip cell lines were selected by growth in medium containing 5 μg of puromycin per ml. Clonal cell lines were also established. To demonstrate expression of the *tip* gene in puromycin-resistant cells, tip protein was immunoprecipitated and blotted with AU-1 antibody. The 40-kDa tip was detected only from Jurkat-T and JCaM1 cells containing the *tip* gene (Fig. 2A). To verify an association with cellular Lck, Lck was precipitated with anti-Lck antibody from Jurkat-T-cell lysates prepared with 0.5% Nonidet P-40 detergent. Immunoprecipitates were incubated with [γ-^{32}P]ATP and analyzed by SDS-PAGE. Strong phosphorylation of 56-kDa Lck was observed from Jurkat-babe and Jurkat-babe-tip cells, while an additional 40-kDa phosphorylated protein was detected only from Jurkat-babe-tip cells (Fig. 3B). In parallel, immunoprecipitates from the same cell lysates with AU-1 antibody were used for in vitro kinase assay with [γ-^{32}P]ATP. The 40-kDa tip and 56-kDa Lck were detected only from Jurkat-babe-tip cells (Fig. 3A). In addition to these two proteins, 90- and 120-kDa proteins from Jurkat-babe-tip cells were associated with tip and phosphorylated. Analysis of immunoprecipitates from Jurkat-babe-tip cell lysates using rabbit polyclonal anti-tip antibody yielded essentially the same results (data not shown).

To show that the 56-kDa protein associated with tip was indeed cellular Lck, polypeptides in anti-AU-1 immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with anti-Lck antibody. The 56-kDa protein associated with tip was indeed detected by anti-Lck antibody (Fig. 2B). Thus, tip forms complexes with cellular Lck tyrosine kinase in Jurkat-T cells and tip can be phosphorylated in vitro by Lck immune complexes from Jurkat-T cells.

Downregulation of cellular tyrosine phosphorylation by expression of the *tip* gene. Lck tyrosine kinase is required for normal signal transduction by controlling the activity of ZAP-70 kinase, which in turn mediates signaling through the TCR (54). In vitro autophosphorylation of Lck was suppressed about three- to fivefold in Jurkat-babe-tip cells (Fig. 3B). Since Lck is a major tyrosine kinase in T cells, suppression of Lck kinase activity by the expression of tip may affect signal transduction in T cells. First, we examined the level of tyrosine phosphorylation in Jurkat-babe, Jurkat-babe-tip, and Jurkat-babe-tip clone 1 cells to show the effect of tip. Overall cellular tyrosine phosphorylation was dramatically decreased in Jurkat-babe-tip and Jurkat-babe-tip clone 1 cells compared with that in Jurkat-babe cells (Fig. 4). Control Jurkat-babe cells contained many tyrosine-phosphorylated proteins, while tyrosine phosphorylation of these proteins was drastically decreased in Jurkat-babe-tip and Jurkat-babe-tip clone 1 cells. Tests with four additional Jurkat-babe-tip clones showed the same results (data not shown). JCaM1 and JCaM1-babe-tip cells, which lack functional Lck because of a splicing defect (46), showed a very low level of tyrosine phosphorylation (Fig. 4). Cellular tyrosine phosphorylation in Jurkat-T cells is thus dramatically decreased by expression of the *tip* gene. The level of tyrosine phosphorylation in the presence of tip is not as low, however, as that in JCaM1 cells, which lack functional Lck.

We next examined the level of Lck expression in these Jurkat-T cells by immunoblot reactivity with anti-Lck antibody.

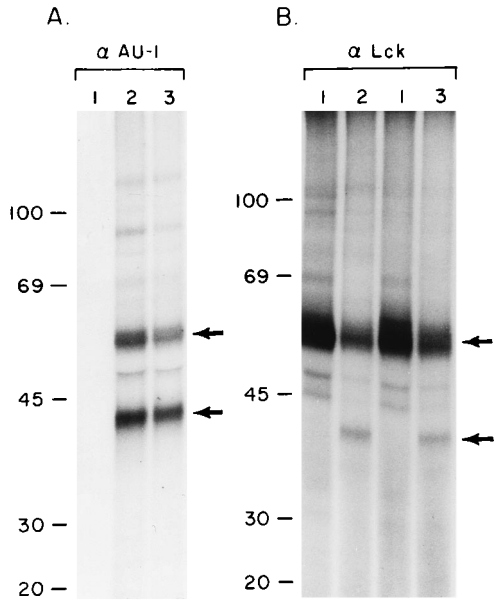

FIG. 3. In vitro kinase assays of AU-1–tip (A) and Lck (B) immune complexes. A total of 2×10^7 cells were the source of extracts for immunoprecipitations with AU-1 and anti-Lck antibodies. Each immune complex was subjected to in vitro kinase assay with [γ-^{32}P]ATP. Lanes 1, Jurkat-babe; lanes 2, Jurkat-babe-tip; lanes 3, Jurkat-babe-tip clone 1. The top arrows indicate the position of Lck, and the bottom arrows indicate the position of tip. The positions of molecular mass standards (in kilodaltons) are given on the left of each gel.

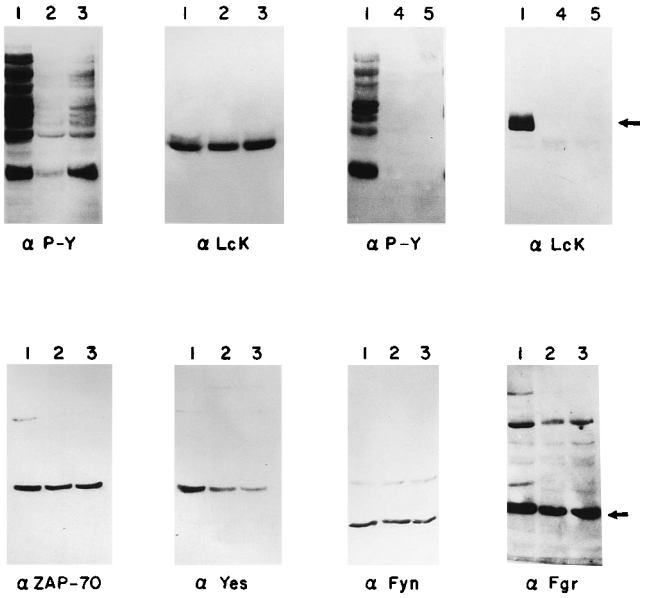

FIG. 4. Downregulation of tyrosine phosphorylation by the expression of tip in Jurkat-T cells. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and reacted with anti-phosphotyrosine antibody (αP-Y) or antigen-specific antibody as indicated below each gel. Reactivity was detected by ECL. Lanes 1, Jurkat-babe; lanes 2, Jurkat-babe-tip; lanes 3, Jurkat-babe-tip clone 1; lanes 4, JCaM1-babe; lanes 5, JCaM1-babe-tip. An arrow indicates the expected size of each protein.

Lysates from Jurkat-babe and Jurkat-babe-tip cells prepared with 0.5% Nonidet P-40 detergent were used for immunoblot or immunoprecipitation with specific tyrosine kinase antibodies. These tests showed that the level of Lck expression was approximately the same in Jurkat-babe, Jurkat-babe-tip, and Jurkat-babe-tip clone 1 cells (Fig. 4). We also examined the levels of expression of other tyrosine kinase proteins, including ZAP-70, Fyn, Yes, Fgr, Src, and Hck, by immunoblot. This also showed no specific, consistent change in the level of any of these tyrosine kinase proteins in repeated experiments (Fig. 4 and data not shown). Thus, the suppression of cellular tyrosine phosphorylation was not caused by a decrease in the expression of Lck or of other tyrosine kinases that were tested in Jurkat-babe-tip and Jurkat-babe-tip clone 1 cells.

Next, we measured the in vitro enzymatic activities of tyrosine kinases. Immune complexes precipitated by specific tyrosine kinase antibodies as described above were subjected to in vitro kinase assays in the presence of [γ-^{32}P]ATP. Similar levels of autokinase activity were consistently detected for Fyn, Yes, Fgr, Lyn, Src, and Hck from Jurkat-babe and Jurkat-babe-tip cells (Fig. 5 and data not shown). As described above, Lck kinase activity from Jurkat-babe-tip cells was consistently decreased about three- to fivefold compared with that from Jurkat-babe cells. Moreover, the autokinase activity of ZAP-70 from Jurkat-babe-tip cells was dramatically decreased compared with that in control Jurkat-babe cells (Fig. 5). Thus, these results demonstrate that the activities of Lck and ZAP-70 are specifically downregulated by the expression of tip.

tip inhibits the induction of protein tyrosine phosphorylation after anti-CD3 stimulation. The earliest biochemical event after TCR stimulation is the induction of tyrosine phosphorylation on a number of cellular proteins (24, 26). We thus examined the effects of tip expression on anti-CD3 stimulation of tyrosine phosphorylation. Jurkat-babe, Jurkat-babe-tip, JCaM1, and JCaM1-babe-tip cells were stimulated with an anti-CD3 (OKT3) antibody, and the time course of induced tyrosine phosphorylation was measured by immunoblot with anti-phosphotyrosine antibody (Fig. 6). Anti-CD3 stimulation of Jurkat-babe cells induced tyrosine phosphorylation of a number of proteins within 1 min (Fig. 6A). In contrast, the same stimulation resulted in a greatly reduced level of tyrosine phosphorylation in Jurkat-babe-tip cells; only a few proteins, 34- and 70-kDa proteins, were weakly tyrosine phosphorylated by 2 min or longer after stimulation (Fig. 6). Similar to what has been described previously (46), anti-CD3 stimulation of Lck-negative JCaM1 cells resulted in very weak induction of only a subset of tyrosine-phosphorylation products, 19- and 110-kDa proteins. The induction of tyrosine-phosphorylation proteins was not observed in JCaM1-babe-tip cells (Fig. 6B).

ZAP-70 tyrosine kinase protein has been shown to be phosphorylated at tyrosine residues and recruited to the ζ chain of TCR after anti-CD3 stimulation (9). The changes in tyrosine phosphorylation of ZAP-70 following anti-CD3 stimulation were assayed by immunoprecipitation with anti-ZAP-70 and immunoblotting with anti-phosphotyrosine antibody. Tyrosine phosphorylation of ZAP-70 in Jurkat-babe cells was strongly induced, and it was detectable within 30 s of stimulation (Fig. 6C). In contrast, tyrosine phosphorylation of ZAP-70 in Jurkat-babe-tip cells was very poorly induced and it was weakly detected only after 2 min of stimulation (Fig. 6C).

Alterations in surface expression of T-cell antigens by tip. JCaM1 cells, which are derived from Jurkat-T cells and lack Lck kinase activity, express a very low level of CD4 on the cell surface (46). Since the association of *tip* with Lck in Jurkat-T cells suppressed cellular tyrosine phosphorylation and blocked the induction of tyrosine phosphorylation through TCR stimulation, we examined the expression of lymphocyte surface markers in Jurkat-babe, Jurkat-babe-tip, and Jurkat-babe-tip clone 1 and 2 cells. FACS analysis of Jurkat-babe-tip cells and Jurkat-babe-tip clones 1 and 2 showed a dramatic reduction in the surface expression of CD2, CD4, and CD11a compared

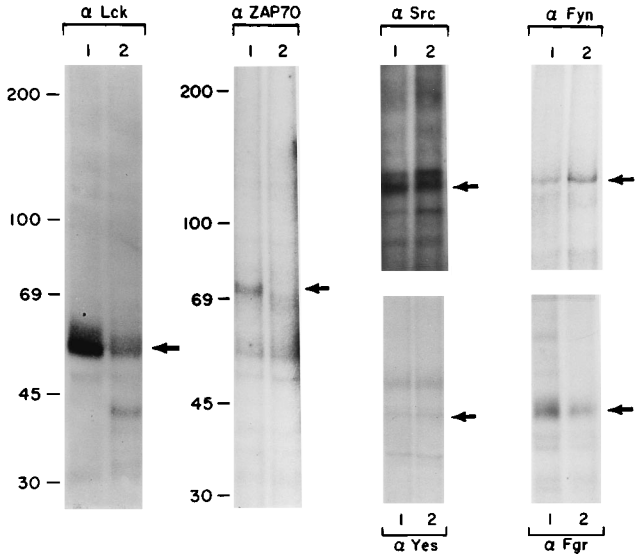

FIG. 5. Suppression of the autokinase activities of Lck and ZAP-70. A total of 2×10^7 cells were used for immunoprecipitations with antigen-specific antibodies. Each immune complex was subjected to in vitro kinase assay with [γ-^{32}P]ATP. Lanes 1, Jurkat-babe; lanes 2, Jurkat-babe-tip clone 1. Arrows indicate the positions of autophosphorylated proteins. The positions of molecular mass standards (in kilodaltons) are given on the left of the two gels on the far left.

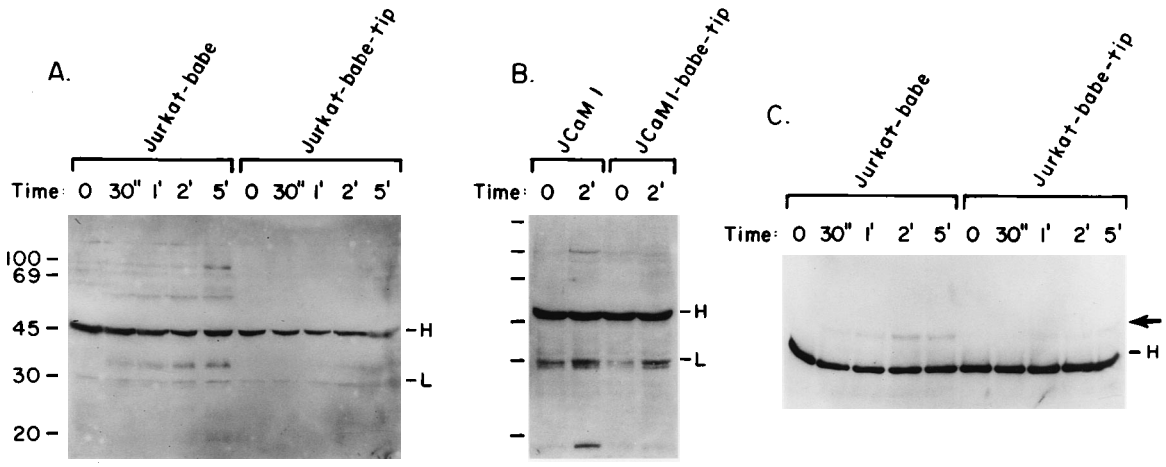

FIG. 6. tip blocks the induction of tyrosine phosphorylation after anti-CD3 stimulation. Cells (10^7) were incubated with anti-CD3 (OKT3) antibody at 37°C for the indicated times and lysed with lysis buffer. Precleared cell lysates were used for immunoblot with anti-phosphotyrosine antibody (A and B) or for immunoprecipitation with rabbit anti-ZAP-70 antibody (C). Cell lysates and immunoprecipitates with anti-ZAP-70 antibody were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with anti-phosphotyrosine antibody (1:3,000 dilution); reactivity was detected with ECL. The arrow indicates the position of ZAP-70 protein. The positions of the heavy chain (H) and the light chain (L) of immunoglobulin and those of molecular mass standards (in kilodaltons) are indicated on the right and left of gels, respectively.

with that in control Jurkat-babe cells (Fig. 7). Additionally, the surface expression of CD5 was more heterogeneous in Jurkat-babe-tip cells than in Jurkat-babe cells. Since JCaM1 cells express a very low level of CD4 on the surface, we measured the surface expression of cell surface markers on JCaM1 cells for comparison with that on Jurkat-babe-tip cells. The phenotype of JCaM1 cells was very similar to that of Jurkat-babe-tip cells (Fig. 7). These results demonstrate that the expression of *tip* in Jurkat-T cells drastically affects surface expression of several lymphocyte antigens and also suggest that this effect is derived from the association of tip with Lck kinase protein.

Suppression of the transforming activity of constitutively active F505 Lck by tip. We have also investigated the effects of tip on Lck transforming activity in fibroblast cells. Phosphorylation at Tyr-505 negatively regulates the enzymatic activity of Lck (52). Indeed, the F-505 substitution mutation results in a constitutively activated version of Lck which transforms fibroblasts to round neuronal-cell morphology and increases the level of intracellular tyrosine phosphorylation (52). NIH 3T3 cells expressing wild-type Lck or constitutively activated F505 Lck were used for transfection with the recombinant retroviral vector pBabe-tip, and cell lines were selected by growth in medium containing 5 μg of puromycin per ml. Equivalent levels of tip and Lck expression in these cells were confirmed by immunoblots with anti-AU-1 and anti-Lck antibodies (see bottom panels of Fig. 9). Expression of the *tip* gene in NIH 3T3 and NIH 3T3/Lck cells did not alter cellular morphology (Fig. 8). In contrast, the expression of the *tip* gene in NIH 3T3/F505 Lck cells resulted in partial conversion of the transformed neuronal-cell appearance to a more normal fibroblast cell appearance (Fig. 8).

The levels of protein tyrosine phosphorylation were examined by immunoblot with anti-phosphotyrosine antibody. Tyrosine-phosphorylated proteins of 56 (Lck) and 100 kDa were mainly detected in NIH 3T3/Lck and NIH 3T3/Lck/tip cells (Fig. 9, lanes 2 and 3, respectively). As described previously (52), the expression of F505 Lck, but not wild-type Lck, resulted in an elevated level of cellular tyrosine phosphorylation; a 36-kDa protein which has been suggested to be calpactin I was highly tyrosine phosphorylated in these cells (Fig. 9, lane 4) (52). The expression of tip in NIH 3T3/F505 Lck cells reduced the level of cellular tyrosine phosphorylation (Fig. 9, lane 5). Tyrosine phosphorylation of the 36-kDa putative calpactin I was dramatically decreased in NIH 3T3/F505 Lck/tip cells.

The SH3 domain of Lck is sufficient to form a stable complex with tip in vitro. A peptide fragment of tip as short as 37 amino acids containing CSKH, spacer sequence, and SH3B elements has been shown to be sufficient to form a stable complex with Lck in vitro (22). To investigate which regulatory domains of Lck are required for stable complex formation with tip, SH2 and/or SH3 regulatory domains of Lck were fused with GST to produce bacterial fusion proteins. Purified GST-SH2, GST-SH3, and GST-SH2/SH3 fusion proteins were mixed with precleared ^{35}S-labeled insect cell lysates containing tip, washed extensively, and resolved by SDS-PAGE for autoradiography. GST-SH2/SH3 and GST-SH3 fusion proteins efficiently bound to Lck in vitro, while GST and GST-SH2 proteins did not (Fig. 10). Thus, the SH3 domain of Lck appears to be sufficient for stable complex formation with tip in vitro.

DISCUSSION

While tip is likely to be important for the viral life cycle in T cells in vivo, its specific role is yet to be elucidated. Medveczky et al. (29) have generated a mutant of HVS strain C484 with the tip-equivalent gene deleted and found decreased levels of IL-2-independent human T-cell stimulation with this mutant. In this report, we have shown that the association of tip with Lck has dramatic effects on the tyrosine phosphorylation of proteins in the cell and on surface expression of lymphocyte markers. It also blocks the induction of tyrosine phosphorylation after anti-CD3 stimulation.

The SH3 domain of Lck is sufficient to form a stable complex with tip in vitro, and the association of tip with Lck dramatically reduces the in vivo cellular tyrosine phosphorylation in Jurkat-T cells. SH3 domains are small units of 55 to 70 amino acids found in nonreceptor tyrosine kinases and other signaling molecules, such as PLCγ, PI 3-kinase, and Grb-2 (2, 25). Binding assays with expression cloning, affinity chromatography, and biased recombinatorial peptide libraries show that SH3 domains bind to short proline-rich peptide motifs of 9 or 10 amino acids (11, 39, 55). Mutational analysis of tip shows that binding to Lck requires a proline-rich SH3B sequence and a sequence homologous to the carboxy terminus of Src family

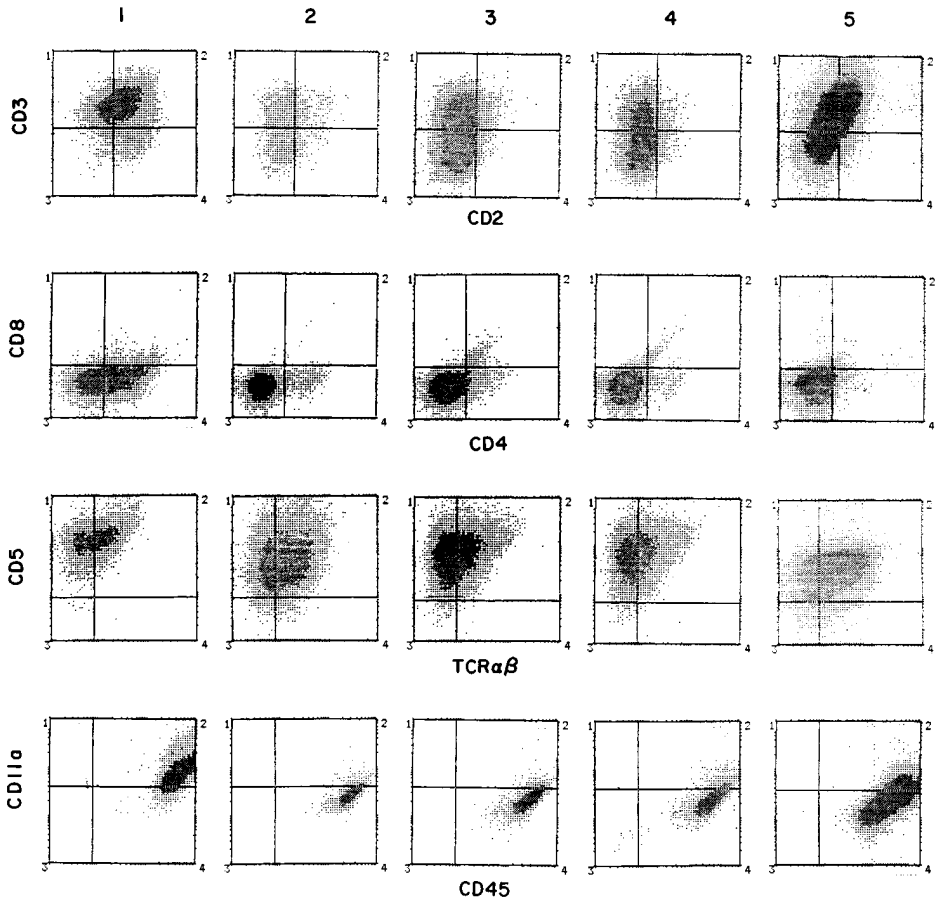

FIG. 7. Altered surface expression of lymphocyte antigens by the expression of tip. A total of 5×10^5 cells were washed with RPMI medium containing 10% fetal calf serum and incubated with fluorescein isothiocyanate-conjugated monoclonal antibody (horizontal axis) or phycoerythrin-conjugated monoclonal antibody (vertical axis) for 30 min at room temperature. After cells were washed, cytofluorometric analysis of cell populations was performed with an EPICSC flow cytometer. Panels 1, Jurkat-babe; panels 2, Jurkat-babe-tip; panels 3, Jurkat-babe-tip clone 1; panels 4, Jurkat-babe-tip clone 2; panels 5, JCaM1.

kinases (22). This suggests that tip associates with Lck through the SH3 domain and that this association blocks Lck-mediated signal transduction.

A number of cellular proteins have been found to associate with Lck. These include cell surface receptors like CD2, CD4, CD5, CD8, and IL-2 receptor (3, 18, 37, 42, 51); downstream effectors like glycophosphatidylinositol-anchored proteins (40, 43, 45), PI 3- and PI 4-kinases (35), p95vav (16, 17), rasGAP (1), and p120 (38); and protein kinases like raf-related protein (36), ZAP-70 (15, 19), and Syk (12). Among these, PI 3-kinase and p120 have been shown to associate with the SH3 domain of Lck (27, 38, 53). The amino-terminal proline-rich regions of the p85 regulatory subunit of PI 3-kinase have been shown to be important for binding to the SH3 domain of Src family kinases (27, 53). Additionally, p120 has been shown to be associated with the SH3 domain of Lck in unstimulated Jurkat-T cells and to be tyrosine phosphorylated within 10 s of stimulation with anti-CD3 antibody (38). These results provide evidence for a role of the SH3 domain of Lck in recruitment of substrates for Lck-mediated signal transduction. Complex formation of tip with Lck may block the interaction of Lck with cellular substrates which normally bind to its SH3 domain. Thus, alterations in complex formation between cellular proteins and Lck induced by tip may ultimately downregulate signal transduction through Lck in transformed T cells expressing tip.

The earliest biochemical signaling event following TCR stimulation is the induction of tyrosine phosphorylation on a number of proteins (54). TCR stimulation rapidly induces strong tyrosine phosphorylation in Jurkat-babe cells, while this response is almost completely blocked in Jurkat-babe-tip cells. The lack of induction of tyrosine phosphorylation by TCR stimulation is likely to be linked to the ability of tip to associate with Lck. In a mutant variant of the Jurkat-T-cell line, a two-fold reduction in Fyn activity has been shown to result in diminished CD3-mediated tyrosine phosphorylation and insufficient activation of ZAP-70 (48). Similarly, a three- to fivefold reduction of Lck kinase activity in Jurkat-babe-tip cells results in a severe reduction of kinase activity and tyrosine phosphorylation of ZAP-70 (Fig. 5) (unpublished results). We have also shown that tyrosine phosphorylation of ZAP-70 induced after CD3 stimulation is delayed and that the level of tyrosine phosphorylation is drastically reduced in tip-expressing cells. It has been suggested that tyrosine kinases, including Fyn and other Src family kinases, may replace Lck as an integral part of the signaling pathway (7, 8, 41). We have observed that tip protein associates with Src family kinase(s) other than Lck in Lck-negative JCaM1 cells (unpublished results). Our results suggest that an association of tip with Lck and possibly other kinases in T cells interferes with the early signaling events through the TCR.

Lck-deficient mice show a pronounced thymic atrophy with a dramatic reduction in the double-positive ($CD4^+$ $CD8^+$)

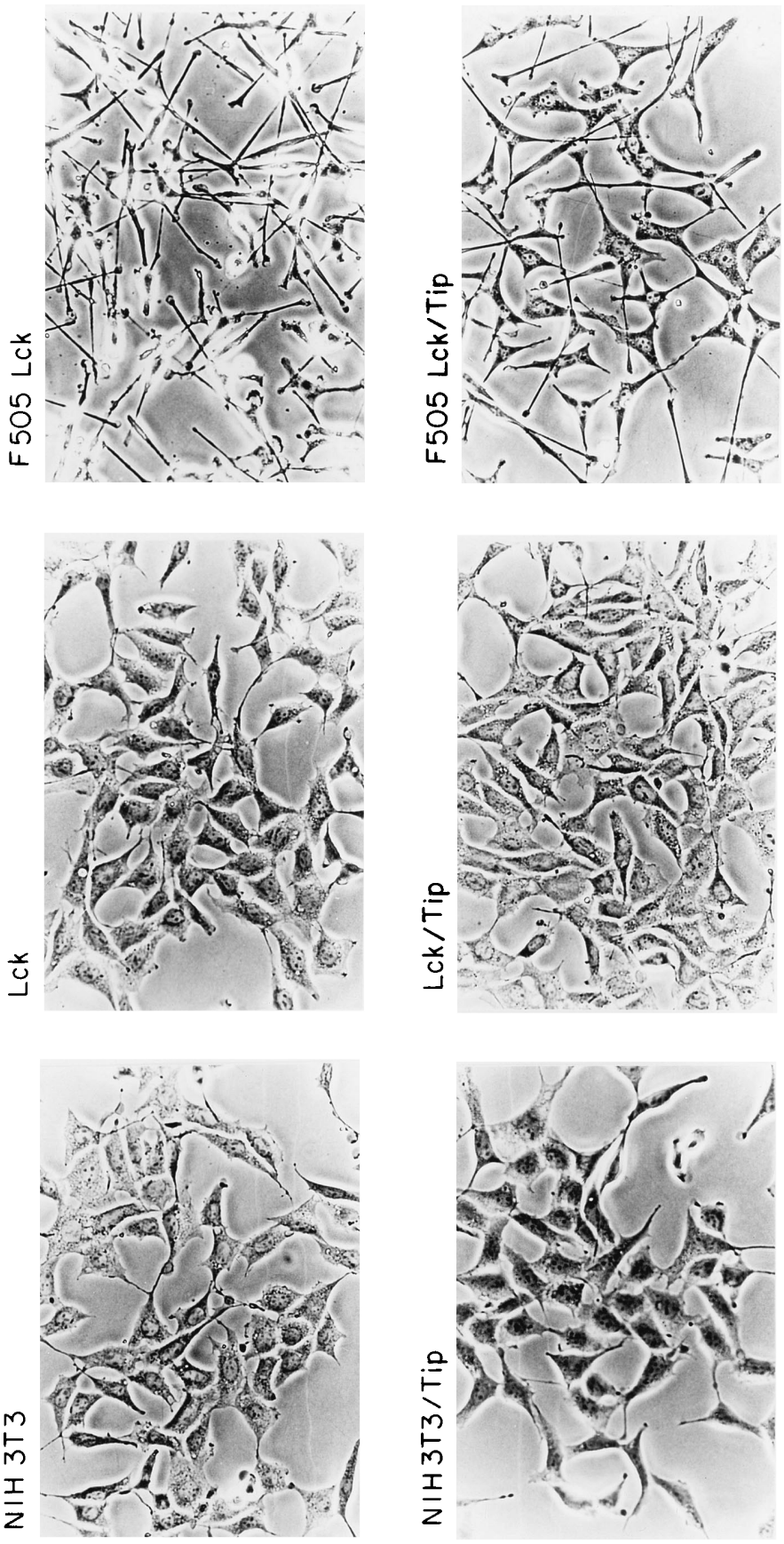

FIG. 8. Suppression of the transforming activity of oncogenic F505 Lck by tip in NIH 3T3 cells. NIH 3T3 fibroblasts expressing wild-type Lck or the F505 mutant form of Lck (F505 Lck) were transfected with retroviral vector pBabe-tip as described in Materials and Methods. Cells expressing tip were established by growth in medium containing 5 μg of puromycin per ml. Cells were photographed at a magnification of ×100 with a phase-contrast inverted microscope.

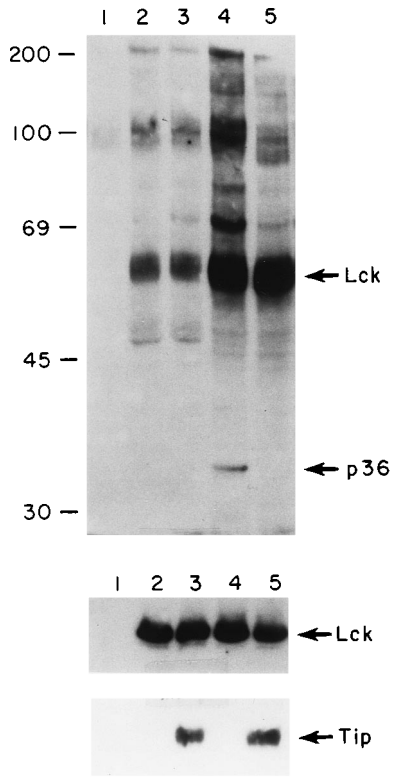

FIG. 9. Reduction of the level of tyrosine phosphorylation by the expression of tip. Equivalent amounts of whole-cell lysates were analyzed by anti-phosphotyrosine antibody (upper panel). The levels of Lck and tip expression were determined by immunoblot with anti-Lck (middle panel) and AU-1 antibody (lower panel). Lane 1, NIH 3T3; lane 2, NIH 3T3/Lck; lane 3, NIH 3T3/Lck/tip; lane 4, NIH 3T3/F505 Lck; lane 5, NIH 3T3/F505 Lck/tip. The position of Lck as well as that of the putative p36 calpactin I (p36) on the right of the upper panel. The arrows on the right of the middle and lower panels indicate the positions of the Lck and tip proteins. The positions of molecular mass standards (in kilodaltons) are given on the left of the upper panel.

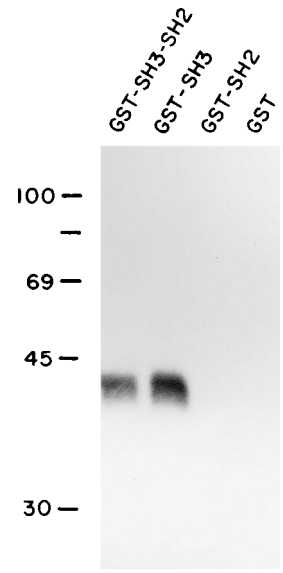

FIG. 10. The SH3 domain of Lck is sufficient to form a stable complex with tip in vitro. Glutathione-Sepharose beads containing 5 μg of GST, GST-SH2, GST-SH3, and GST-SH2/SH3 Lck fusion proteins were mixed with ^{35}S-labeled cell lysates containing tip from insect cells, with three subsequent washing steps with lysis buffer. Associated proteins were resolved by SDS-PAGE and autoradiographed. Overnight exposure was used. The positions of molecular mass standards (in kilodaltons) are given on the left.

thymocyte population (33). Mature, single-positive thymocytes were not detected in these mice, and peripheral T cells were very rare. In addition, Lck-negative JCaM1 cells express very low levels of CD4 (46). Reconstitution of Lck into JCaM1 cells induces CD4 surface expression, and these cells subsequently respond to TCR stimulation as do the CD4 parental Jurkat-T cells (46). Interestingly, cells expressing tip have a phenotype similar to that observed in Lck-negative JCaM1 cells. The expression of the *tip* gene in Jurkat-T cells induces a dramatic reduction in surface expression of CD2, CD4, and CD11a, and it also somewhat reduces surface expression of CD5. CD2 and CD4 surface antigens have been shown to associate with Lck in vivo (3, 50). CD11a (LFA-1) activity for antigen-independent T-cell adhesion has been shown to be regulated by CD4 and Lck function (28). Thus, cell surface expression of a number of lymphocyte antigens appears to be regulated by Lck.

HVS transforms T cells in vitro and in vivo to immortalized growth. The synthesis of a viral product that blocks Lck-mediated signal transduction and cellular activation may at first seem counterintuitive. However, this virus is known to contain another gene, STP, that is capable of functioning as an oncogene for rodent fibroblasts and is required for viral T-cell transformation (23, 29). Recent results suggest that the STP product binds to and activates ras (21). The expression of tip may allow cellular immortalization to proceed and at the same time prevent potential adverse effects that may be associated with Lck activation, e.g., apoptosis or untimely viral replication.

There are intriguing similarities between the STP and *tip* genes of HVS and the LMP1 and LMP2A genes of EBV. Both sets of genes are similarly located near the ends of their respective genomes. Like STP, LMP1 is a viral oncogene that transforms rodent fibroblasts in vitro and is required for viral transformation. LMP2A is expressed in latently infected B lymphocytes and, analogous to tip, associates with the major B-cell tyrosine kinases Lyn and Syk. LMP2A blocks the effects of surface immunoglobulin cross-linking on calcium mobilization and reactivation of EBV from latent infection in transformed human B lymphocytes (32). LMP2A is a constitutive dominant negative modulator of surface immunoglobulin receptor signaling through its effects on Lyn and Syk (31). The striking correspondence in genomic locations and functional roles of STP and tip of HVS and LMP1 and LMP2A of EBV occurs in the complete absence of any discernable sequence homology in the corresponding genes, similarity in size, or similarity in the organization of structural motifs in the gene products.

ACKNOWLEDGMENTS

We thank G. Voss for help with FACS analysis, A. Weiss for JCaM1 cells, and E. Reinherz for anti-CD3 antibody. We also thank J. Newton and T. Connors for manuscript preparation.

This work was supported by U.S. Public Health Service grants CA31363 and RR00168 and by a fellowship from the Deutsches Krebsforschungszentrum (S.M.L.).

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