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Tap: A Novel Cellular Protein That Interacts with Tip of Herpesvirus Saimiri and Induces Lymphocyte Aggregation

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Summary

Tip of herpesvirus saimiri associates with Lck and down-regulates Lck-mediated activation. We identified a novel cellular Tip-associated protein (Tap) by a yeast two-hybrid screen. Tap associated with Tip following transient expression in COS-1 cells and stable expression in human Jurkat-T cells. Expression of Tip and Tap in Jurkat-T cells induced dramatic cell aggregation. Aggregation was likely caused by the up-regulated surface expression of adhesion molecules including integrin α, L-selectin, ICAM-3, and H-CAM. Furthermore, NF-κB transcriptional factor of aggregated cells had approximately 40-fold higher activity than that of parental cells. Thus, Tap is likely to be an important cellular mediator of Tip function in T cell transformation by herpesvirus saimiri.

Introduction

Herpesvirus saimiri (HVS), a member of the γ2 subgroup of herpesviruses, naturally infects squirrel monkeys (Saimiri sciureus) of South America. Recently, Kaposi's sarcoma–associated herpesvirus has been shown to have high homology to HVS (Chang et al., 1994). HVS persists in T lymphocytes of the natural host without any apparent disease, but infection of other species of New World primates results in fulminant lymphomas, lymphosarcomas, and leukemias of T cell origin (Jung and Desrosiers, 1994). A pronounced divergence among different strains of HVS has been localized to the left end of viral genomic DNA, and this has led to classification into three subgroups A, B, and C (Desrosiers and Falk, 1982; Medveczky et al., 1984). Strains from subgroups A and C are highly oncogenic and are able to immortalize common marmoset T lymphocytes in vitro to interleukin 2 (IL-2)–independent growth (Desrosiers et al., 1986; Szomolanyi et al., 1987). Subgroup C strains are further capable of immortalizing human and rhesus monkey lymphocytes into continuously proliferating T cell lines (Biesinger et al., 1992). Human T cell clones transformed by HVS strain C488 retain the ability to respond to their specific antigens with increased proliferation, cytokine production, and cell killing (Berend et al., 1993; Bröker et al., 1993; Mittrücker et al., 1995).

Nucleotide sequence analysis of the entire HVS genome has revealed a number of genes with homology to cellular proteins, some of which may possibly to contribute to T cell transformation (Albrecht et al., 1992a). These include the superantigen homolog (Thomson and Nicholas, 1991; Yao et al., 1996), IL-8 receptor homolog (Ahuja and Murphy, 1996), CD59 homolog (Albrecht et al., 1992b; Rother et al., 1994), bcl-2 homolog, virus-encoded IL-17 (Yao et al., 1995), and virus-encoded cyclin (Nicholas et al., 1992; Jung et al., 1994). Mutational analyses have demonstrated that the left-most STP gene is required for immortalization of primary T lymphocytes but not for replication of the virus (Desrosiers et al., 1985, 1986). STP-C488 has been shown to associate with cellular Ras in transformed cells (Jung and Desrosiers, 1995). Mutations that disrupted this association with Ras disrupted the transforming ability of the STP-C488 oncogene. Binding assays showed that STP-C488 was capable of competing with Raf-1 for binding to Ras. Expression of STP-C488 activates the Ras signaling pathway, as evidenced by a 2- to 4-fold increase in the ratio of Ras-GTP to Ras-GDP and by the constitutive activation of mitogen-activated protein kinase. Consistent with activation of signaling through Ras, STP-C488 expression induced Ras-dependent neurite outgrowth in PC12 cells (Jung and Desrosiers, 1995). Thus, STP-C488 is the first virus-encoded protein shown to achieve oncogenic transformation via association with cellular Ras.

A protein called Tip (tyrosine kinase–interacting protein), encoded by the gene adjacent to STP-C488 at the left end of the viral genome, has been identified in virus-transformed T cells (Biesinger et al., 1990). Tip does not show transforming activity in rodent fibroblast cells (Jung et al., 1991), but the protein has been found to be associated with the major T cell tyrosine kinase Lck and to be phosphorylated on tyrosine residues by purified Lck in several cell-free assay systems (Biesinger et al., 1995). Mutational analysis of a GST–Tip fusion protein has revealed that binding to Lck requires a putative SH3-binding (SH3B) sequence and a sequence homologous to the carboxyl terminus of Src-related kinases (Jung et al., 1995a). These sequences are referred to as SH3B and carboxy-terminal Src-related kinase homology (CSKH) elements. Peptide fragments as short as 37 amino acids containing CSKH, a spacer sequence, and SH3B motifs are sufficient to form a stable complex with Lck in vitro. Furthermore, these same sequences of Tip are necessary for in vivo association with Lck when Tip and Lck are expressed transiently in COS-1 cells or stably in Rat-1 cell lines (Jung et al., 1995a). These results demonstrate that the CSKH element of Tip participates in the binding of sequences within Lck. Tip of HVS has apparently acquired such CSKH and SH3B elements for the purpose of targeting cellular protein kinases.

Previously, we have shown that expression of Tip in Jurkat-T cells dramatically suppressed cellular tyrosine phosphorylation and surface expression of lymphocyte antigens (Jung et al., 1995b). Expression of Tip also blocked the induction of tyrosine phosphorylation by

anti-CD3 stimulation. Furthermore, the expression of Tip in fibroblast cells suppressed the transforming activity of oncogenic F505 Lck (Jung et al., 1995b). Therefore, Tip can act at an early stage of the T cell signal-transduction cascade by associating with Lck and down-regulating 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 (EBV).

While LMP2A of EBV is not required for B cell transformation, we have found that Tip is essential for primary T cell immortalization and for lymphoma induction in vivo (unpublished data). This suggests that Tip may have functions in addition to the blocking of Lck-mediated signal transduction. To study the events elicited by Tip other than the association with Lck, we have exploited a yeast system for detecting protein interactions and have identified a novel cellular Tip-associated protein (Tap). Our results suggest that the association between Tip and Tap is an important cellular event for biological properties of the virus.

Results

Identification of cDNA Encoding Protein That Interacts with a Truncated Form of Tip

Previously, we have shown that binding of Tip to Lck requires a proline-rich SH3B sequence and a sequence homologous to the carboxyl terminus of Src-related kinases (CSKH) (Jung et al., 1995a). Specially, proline-rich SH3B was essential to form a stable complex with Lck in vitro and in vivo (Jung et al., 1995a). To identify specific cellular proteins other than Lck that associate with Tip, we deleted the carboxy-terminal region of Tip containing the SH3B motif and hydrophobic domain for a yeast two-hybrid system. DNA sequences encoding amino acid residues 1–226 of Tip were fused in frame to the LexA DNA-binding domain for use as bait in a yeast two-hybrid screen in order to identify cDNAs encoding interactive proteins. A yeast expression plasmid containing this fusion gene was introduced into a reporter strain harboring the Saccharomyces cerevisiae Leu2 and β-galactosidase coding sequences under the control of a synthetic promoter bearing LexA-binding sites. The resulting yeast strain was transformed with a transcriptional activator fusion protein library, which was prepared from mRNA isolated from human Jurkat-T cells and had the *GAL1* promoter to direct transcription of the library insert (Choi et al., 1996). Transformants were plated on selective (Leu-deficient) plates containing galactose, which induces the *GAL1* promoter. Leu prototrophs were transferred to plates containing X-Gal and galactose, and colonies yielding a dark blue color were recovered and analyzed further.

Of 1×10^6 transformants that were tested for growth in the absence of leucine, histidine, and tryptophan and in the presence of X-Gal, 80 colonies showed moderate growth and were positive for β-galactosidase expression. Seventy-seven of 80 clones were found to contain an identical insert by restriction enzyme digestion and partial DNA sequence analysis of the cDNA inserts of the candidate clones. DNA sequence analysis showed that the 0.5 kb insert from candidate clones encoded a total of 51 amino acids. To test the specificity of the interaction between the candidate interaction partners and Tip, the library plasmids were reintroduced into a second strain harboring a LexA-SIV-nef fusion gene that is heterologous to Tip. None of the candidate plasmids showing evidence of strong interaction with Lex–Tip were capable of interacting with LexA-SIV-nef (data not shown). Thus, we designated this clone as Tip-associated protein (Tap).

The clone recovered in the yeast-two hybrid system contains 51 amino acids corresponding to the carboxyl terminus of Tap. This suggests that the 51–amino acid sequence of the carboxyl terminus of Tap is sufficient to bind to Tip. To demonstrate this directly, a GST fusion

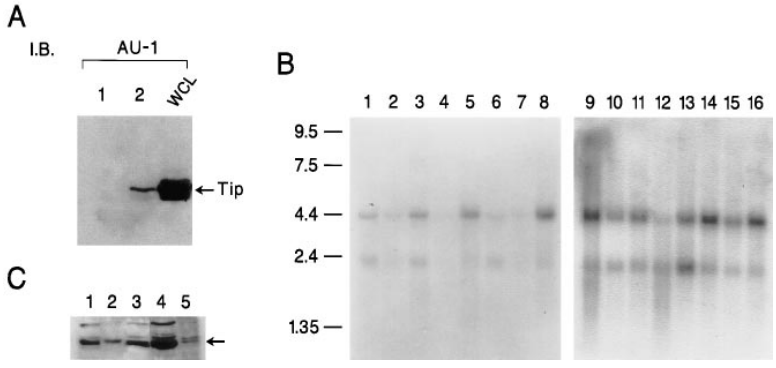

Figure 1. In Vitro Binding of GST-Tap Fusion Protein to Tip and Northern Blot Analysis

(A) Carboxyl region of Tap is sufficient for binding to Tip in vitro. Glutathione Sepharose beads containing 5 μg of GST or GST-Tap-c fusion proteins were mixed with COS-1 cell lysates containing Tip followed by three washing steps with lysis buffer. Whole-cell lysates (WCL) of COS-1 cells containing Tip were used for control. Associated proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and immunoblotted with anti-Tip antibody as described previously (Jung et al., 1995a).

(B) Northern blot analysis of *tap* mRNA expression. Two micrograms of poly(A)$^+$ RNA from the indicated human tissues was separated on a 1.2% agarose gel, transferred to a charge-modified nylon membrane, and probed with a ^{32}P-labeled, nick-translated Tap full-length DNA. Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas; lane 9, spleen; lane 10, thymus; lane 11, prostate; lane 12, testis; lane 13, ovary; lane 14, small intestine; lane 15, colon; lane 16, peripheral blood lymphocyte.

(C) Immunoblot detection of Tap protein. Cell lysates were resolved by SDS–PAGE, transferred to nitrocellulose and immunoblotted with anti-Tap antibody. Lane 1, COS-1 cells; lane 2, 293 cells; lane 3, Raji cells; lane 4, Jurkat-T cells; lane 5, IL-2 stimulated common marmoset peripheral blood mononuclear cells.

A

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                                                                        80
MSDAQDGPRVRYNPYTTRPNRRGDTWHDRDRIHVTVRRDRAPPERGGAGTSQDGTSKNCFKITIPYGRKYDKAWLLSMIQ

                                                                       160
SKCSVPFTPIEFHYENTRAQFFVEDASTASALKAVNYKILDRENRRISIIINSSAPPHTILNELKPEQVEQLKLIMSKRY

                                                                       240
DGSQQALDLKGLRSDPDLVAQNIDVVLNRRSCMAATLRIIEENIPELLSLNLSNNRLYRLDDMSSIVQKAPNLKILNLSG

                                                                       320
NELKSERELDKIKGLKLEELWLDGNSLCDTFRDQSTYISAIRERFPKLLRLDGHELPPPIAFDVEAPTTLPPCKGSYFGT

                                                                       400
ENLKSLVLHFLQQYYAIYDSGDRQGLLDAYHDGACCSLSIPFIPQNPARSSLAEYFKDSRNVKKLKDPTLRFRLLKHTRL

                                                                       480
NVVAFLNELPKTQHDVNSFVVDISAQTSTLLCFSVNGVFKEVDGKSRDSLRAFTRTFIAVPASNSGLCIVNDELFVRNAS

                                                                      560
SEEIQRAFAMPAPTPSSSPVPTLSPEQQEMLQAFSTQSGMNLEWSQKCLQDNNWDYTRSAQAFTHLKAKGEIPEVAFMK*
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B

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Tap          100 QFFVEDASTASALKAVNYKILDREN 124  143 ELKPEQVEQLKLIMSKRYDGSQQALDLKGLRSDPDLVA 170
                    +++V D  TAS ++A N +++ +E+        +LK E++E +  ++ KR++   + LDL     D +  A
C15H11.e     141 EWYVRDEDTASTIRANNRRVVHKES 165  182 KLKREEIEIIHRVVDKRHNAENRVLDLSNFHEDEEFKA 209

171 QNIDVVLNRRSCMAATLRIIEENIPELLSLNLSNNRLYRLDDMSSIVQKAPNLKILNLSGNELKSERELDKIKGLKLEELWLD 263
    +++ + L + + M   L  I++    +++L+LSNNR+  LD  S++V  A  +  L+LS N + +E+EL+K  GL +E  + +
210 KDMMMNLTKGNVMLTVLDHIDDKYGNIVALSLSNNRIRHLDYASALVSIAKFVMELDLSHNHISTEKELEKFAGLPVERFFFE 302

264 GNSLCDTFRDQSTYISAIRERFPKLLRLDGHELPPPIAFDVEAPTTLPPCKGSYFGTENLKSLVLHFLQQYYAIYDSGDRQLN 344
    GN + ++F  ++ YIS I + FP+   LDG E+ P +           P +  Y+    ++ LV  F+  Y+  YD  D Q++
303 GNPVVESFTQRAAYISYIHQSFPRCNMLDGVEVQPLVVGPDLDIHDAMPFRAGYYPNPQIRVLVEQFVTSYFDFYDGPDGQMD 383

402 VVAFLNELPKTQHDVNSFVVDISAQTSTLLCFSVNGVFKEVDGKSRDSLRAFTRTFIAVPASNSGLCIVNDELFVRNASSEEIQR 486
    +   L++LP + H  ++F+VD+  Q++ LL F+V G+F + D     S   F+R+F+  P  N  + +++D+LF+  AS + +++
452 IAVALSKLPTSSHMRDTFIVDVFLQSNDLLGFTVQGLFCDGDLTQTPSPSFFSRSFLVSPRENDSVAVISDQLFITVASLDRLEK 536

505 PEQQEMLQAFSTQSGMNLEWSQKCLQDNNWDYTRSAQAFTHLKA 548
    P ++EM++A    SGM   +S+KCL D  W++  + Q F  +K+
576 PIREEMIKAMCQFSGMIPPFSEKCLADCAWNFDFACQKFNEIKS 619
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Figure 2. Predicted Amino Acid Sequences of Human Tap and Regional Homology with of the C. elegans C15H11.e Gene

(A) The amino acid sequence of human Tap deduced from the cDNA sequence is shown. The carboxyl terminal region of Tap obtained from the yeast two-hybrid screen is shown in bold letters. The cDNA nucleotide sequences are deposited in GenBank under accession number U80037.

(B) Regional homology of Tap with the C. elegans C15H11.e gene.

protein containing the carboxy-terminal 51 amino acids of Tap was used in in vitro binding assay. As shown in Figure 1A, the carboxy-terminal 51–amino acid sequence was sufficient to bind to Tip in vitro. In contrast, a control GST protein was not able to bind to Tip under the same conditions.

Cloning and Structure of Cellular Tap

To obtain the full length of the predominant positive clone, the 0.5 kb cDNA insert from the yeast-two hybrid system was used to identify clones from a human lymphocyte library. One clone with a 2.5 kb insert contained a 1650 nt open reading frame beginning with a translational initiation consensus Kozak sequence; the predicted polypeptide was 560 amino acids in length and had a predicted molecular weight of 70,177 Da (Figure 2A).

Northern blot analysis indicated that although the 2.5 kb *tap* mRNA is expressed in all human tissues, expression was slightly higher in the heart, placenta, skeletal muscle, and ovary than in the brain, lung, liver, kidney, pancreas, spleen, thymus, prostate, testis, small intestine, colon, and peripheral blood lymphocyte (Figure 1B). In addition, an approximately 4.4 kb *tap* transcript was detected in all human tissues. In contrast, it was most abundant in heart, placenta, liver, pancreas, spleen, prostate, ovary, small intestine, and peripheral blood lymphocyte (Figure 1B). We also examined the expression of Tap protein in a variety of cell lines with anti-Tap antibody. Although Tap was detected ubiquitously in cell lines tested, the level of its expression varied (Figure 1C).

Database searches found that Tap has homology with sequences from Caenorhabditis elegans. The homology of Tap reaches 30% identity and 54% similarity at an amino acid level with the C. elegans cDNA C15H11.e

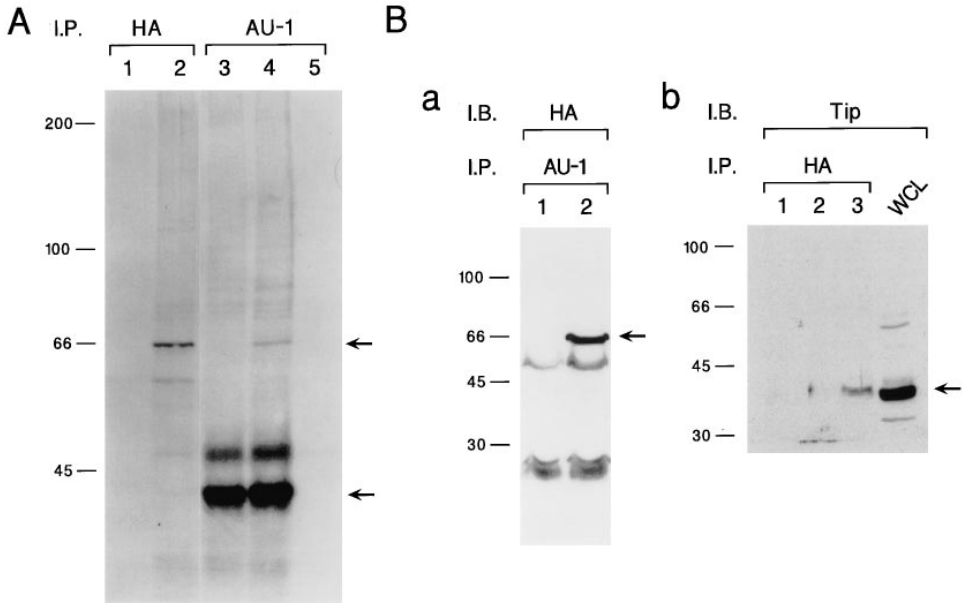

Figure 3. Association of Tip with Tap in COS-1 Cells

(A) Immunoprecipitation of ^{35}S-labeled protein with HA and AU1 antibodies. Lanes 1 and 5, COS-1 cells transfected with pFJ vector; lane 2, COS-1 cells transfected with pcDNA3-HA-Tap; lane 3, COS-1 cells transfected with pFJ-AU1-Tip; lane 4, COS-1 cells transfected with pcDNA3-HA-Tap and pFJ-AU1-Tip.

(B) Identification of an association between Tip and Tap in COS-1 cells. (a) Identification of Tap in anti-AU1-Tip immune complexes by using HA antibody. Lane 1, COS-1 cells transfected with pFJ-AU1-Tip; lane 2, COS-1 cells transfected with pFJ-AU1-Tip and pcDNA3-HA-Tap. (b) Identification of Tip in anti-HA-Tap immune complexes by using anti-Tip antibody. Lane 1, COS-1 cells transfected with pFJ vector; lane 2, COS-1 cells transfected with pcDNA-HA-Tap; lane 3, COS-1 cells transfected with pcDNA-HA-Tap and pFJ-AU1-Tip.

After transfection, cells with (A) or without (B) radioactive labeling were lysed with lysis buffer. Proteins for SDS–PAGE were prepared by immunoprecipitation (I.P.) with indicated antibody. Precipitated proteins were detected by autoradiography of the dried gel (A) or immunoblot (I.B.) analysis with anti-HA antibody (B, a) or anti-Tip antibody (B, b). Whole cell lysate (WCL) containing Tap or Tip were used for control. Arrows indicate the locations of Tap and Tip. The molecular markers are lysozyme (14 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (30 kDa), ovalbumin (66 kDa), bovine serum albumin (66 kDa), phosphorylase b (97.4 kDa), and myosin (200 kDa).

clone (Figure 2B). This homology suggests that Tap is likely to be the mammalian homolog of C. elegans C15H11.e gene (Wilson et al., 1994). Since the C. elegans C15H11.e clone was obtained by random cDNA sequence, a functional role of this gene product remains to be elucidated. In addition to homology with the C. elegans C15H11.e clone, several other features are evident from the Tap sequence. First, Tap contains charged amino acids, which constitute approximately 26% of the total amino acid sequence. Second, the carboxyl terminus of Tap that was initially isolated from the yeast-two hybrid system has a glutamine-rich motif similar to those that have been shown to be involved in transcriptional activation (Colgan et al., 1995; Li and Green, 1996). Third, a leucine- and isoleucine-rich region was found in the central region of the Tap sequence from amino acids 111–410. Finally, a possible integrin binding motif (RGD) was found in amino acid residues 22–24 of Tap.

Association of Tap with Tip in COS-1 Cells

To demonstrate complex formation between Tap and Tip, cells were transfected with expression vectors containing *tip* or *tap* and incubated with ^{35}S-radioactive amino acids. To detect the proteins, the amino termini of both Tip and Tap were tagged with AU1 and hemagglutinin (HA), respectively. ^{35}S-labeled proteins from transfected COS-1 cells were incubated with AU1 and HA antibodies. As shown in Figure 3A, HA-tagged Tap protein migrated as a 65 kDa molecule on SDS polyacrylamide gel electrophoresis (PAGE). AU1 antibody precipitated 40 kDa Tip protein as well as 65 kDa protein only from COS-1 cells expressing Tip and Tap and not from COS-1 cells expressing only Tip (Figure 3A). This 65 kDa protein comigrated with Tap protein, as demonstrated in Figure 3A, lane 2. To show that the 65 kDa protein associated with Tip was indeed Tap, polypeptides present in anti-AU1 immune complexes were separated by SDS–PAGE, transferred to nitrocellulose, and reacted with HA antibody. The 65 kDa protein associated with Tip was indeed detected by the HA antibody (Figure 3B, a). By these same procedures, a Tap-specific rabbit polyclonal antibody also recognized the 65 kDa protein associated with Tip (data not shown). Conversely, polypeptides present in HA immune complexes were separated by SDS–PAGE, transferred to nitrocellulose, and reacted with anti-Tip rabbit polyclonal antibody. The 40 kDa protein associated with Tap was detected by the anti-Tip antibody (Figure 3B, b). These tests demonstrate an association of Tip with Tap in transient expression of COS-1 cells.

Association of Tip with Tap in Stable T Cell Lines

To understand the role of Tip association with cellular Tap, Jurkat-T and Jurkat-Tip cells, which have been described previously (Jung et al., 1995b), were used to

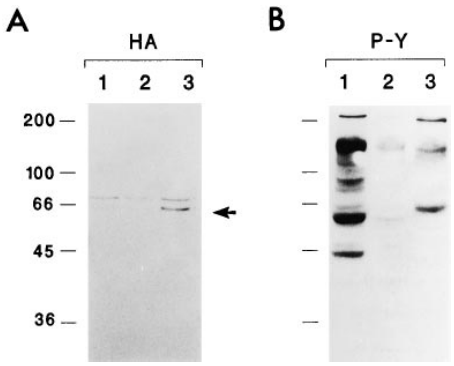

Figure 4. Expression of Tap and Cellular Tyrosine Phosphorylation in Jurkat-T Cells

(A) Expression of Tap.

(B) Detection of tyrosine phosphorylations by anti-phosphotyrosine antibody. Whole cell lysates of Jurkat-T cells (lane 1), Jurkat-Tip cells (lane 2), and Jurkat-Tip/Tap (lane 3) were resolved by SDS–PAGE, transferred to nitrocellulose, and reacted with HA antibody (A) or anti-phosphotyrosine P-Y antibody (B). The arrow indicates the Tap protein.

establish stable cell lines expressing the *tap* gene. As described above, the full-length *tap* gene was modified to encode an HA epitope tag at the amino terminus and was cloned into the expression vector pcDNA3. After electroporation of the HA-Tap expression vector DNA into Jurkat-T and Jurkat-Tip cells, cells were selected by growth in medium containing 1 mg/ml G418. To demonstrate expression of the *tap* gene in G418- resistant cells, whole-cell lysates were immunoblotted with an HA antibody. The 65 kDa HA-tagged Tap was detected only from Jurkat-Tip cells containing the HA-tagged *tap* gene (Figure 4A). To verify an association of Tap with Tip, Tip was precipitated with anti-AU1 antibody from Jurkat-T, Jurkat-Tip, and Jurkat-Tip/Tap cell lysates prepared with 0.5% NP-40 detergent. Anti-Tip immunoprecipitates were incubated with [γ-^{32}P]ATP and analyzed by SDS–PAGE. Strong phosphorylations of 44 kDa Tip and 56 kDa Lck were observed from Jurkat-Tip and Jurkat-Tip/Tap cells, while a 65 kDa phosphorylated protein was detected only from Jurkat-Tip/Tap cells (Figure 5A). Interestingly, an additional 50 kDa phosphorylated protein was detected from Jurkat-Tip/Tap cells. In parallel, the same cell lysates were used for immunoprecipitation by HA antibody followed by an in vitro kinase assay. A 65 kDa phosphorylated Tap and a 50 kDa phosphorylated protein were strongly detected only from Jurkat-Tip/Tap cells and not from Jurkat-T and Jurkat-Tip cells (Figure 5A). In addition, a phosphorylated Tip was weakly detected only in the HA-Tap complexes from Jurkat-Tip/Tap cells (Figure 5A). To demonstrate that the approximately 44 kDa protein associated with Tap was indeed Tip, polypeptides present in anti-HA immune complexes were separated by SDS–PAGE, transferred to nitrocellulose, and reacted with anti-Tip antibody. The 44 kDa protein associated with Tap was

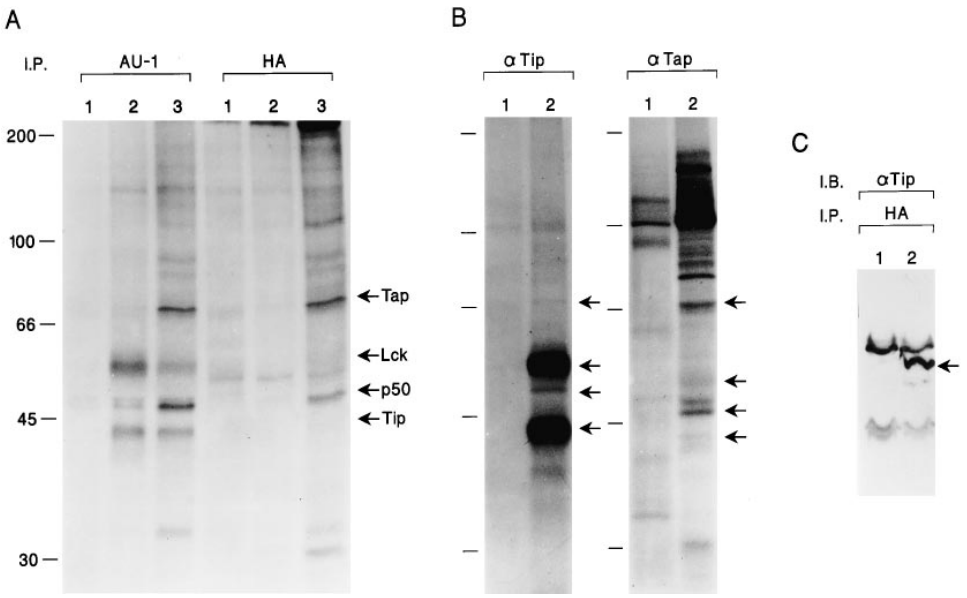

Figure 5. Association of Tap with Tip in Jurkat-T Cells and HVS- Transformed Common Marmoset T Cells

Association of Tip with Tap was examined in Jurkat-T cells (A) and HVS-transformed common marmoset T cells (B). Approximately 2×10^7 cells were used for immunoprecipitation with anti-AU1 and anti-HA antibodies (A) or anti-Tip and anti-Tap antibodies (B). Immune complexes were assayed for kinase activity with [γ-^{32}P]ATP.

(A) lane 1, Jurkat-T cells; lane 2, Jurkat-Tip; lane 3, Jurkat-Tip/Tap.

(B) Lane 1, IL-2 stimulated common marmoset peripheral blood mononuclear cells; lane 2, HVS-transformed common marmoset T cells. Arrows indicate the locations of Tap, Tip, Lck, and p50.

(C) Identification of Tip in anti-HA-Tap immune complexes by using anti-Tip antibody. Cell lysates of Jurkat-T cells (lane 1) and Jurkat-Tip/Tap cells (lane 2) were used for immunoprecipitation (I.P.) with HA antibody. Precipitated proteins were detected by immunoblot (I.B.) analysis with anti-Tip antibody.

The position of molecular mass standards (in kilodaltons) are shown at the left of each gel.

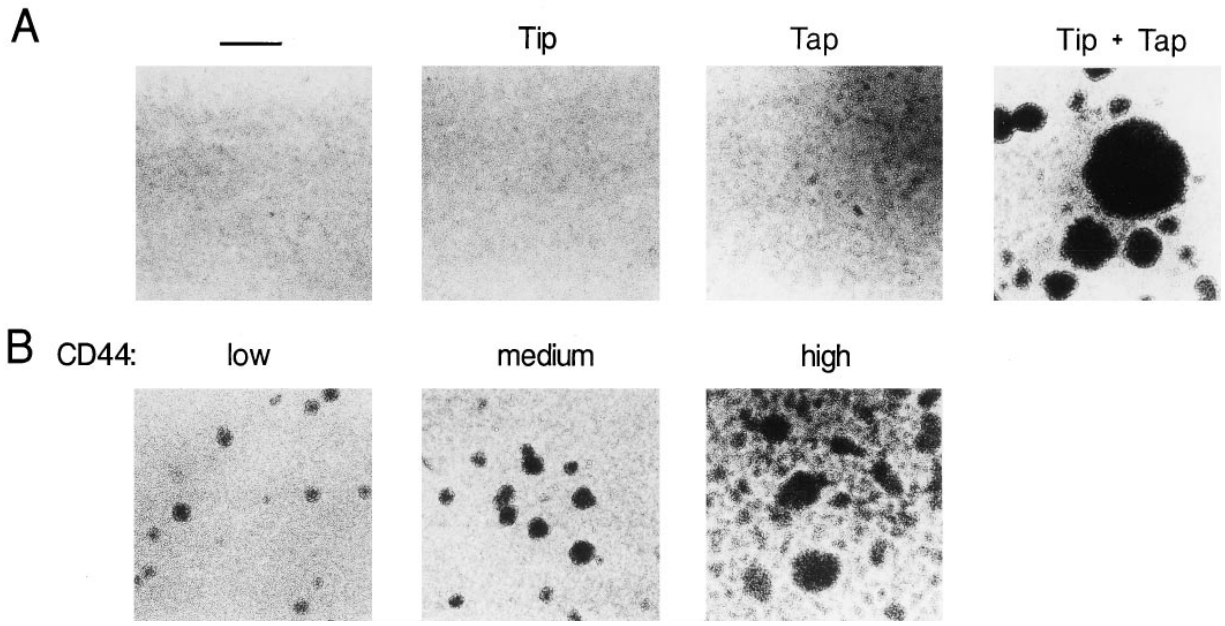

Figure 6. Increased Aggregation of Jurkat-T Cells Induced by Tip and Tap Expression

(A) Coexpression of Tip and Tap in Jurkat-T cells induces a marked cellular aggregation. Jurkat-T cells (−), Jurkat-T cells expressing Tip (Tip), Jurkat-T cells expressing Tap (Tap), and Jurkat-T cells expressing Tip and Tap (Tip + Tap) were photographed at a magnification of ×40 with an inverted microscope.

(B) The level of aggregation correlates with the level of CD44 surface expression. Jurkat-T cells expressing Tip and Tap were sorted by FACS based on CD44 surface expression into low, medium, and high CD44 subtypes. Sorted cells were collected and photographed at a magnification of ×40 with an inverted microscope.

indeed detected by the anti-Tip antibody (Figure 5C). Finally, we examined the association between Tip and Tap in IL-2 independent common marmoset T cells transformed by HVS C488. Anti-Tip and anti-Tap immunoprecipitates were incubated with [γ-^{32}P]ATP and analyzed by SDS–PAGE. Analysis of immunoprecipitates from HVS-transformed common marmoset T cells yielded essentially the same results as those from Jurkat-T cells with the exception of a 110 kDa phosphorylated protein that was strongly detected in HVS-transformed common marmoset T cells (Figure 5B).

We have shown previously that the overall level of cellular tyrosine phosphorylation was dramatically decreased by the expression of Tip in Jurkat-T cells (Jung et al., 1995b). Since tyrosine phosphorylation is a major indicator for T cell activation, cellular tyrosine phosphorylation was examined in Jurkat-T cells expressing the *tip* and *tap* genes. Control Jurkat-T cells contained many tyrosine phosphorylated proteins, while tyrosine phosphorylation of these proteins was drastically decreased in Jurkat-Tip and Jurkat-Tip/Tap cells (Figure 4B). Interestingly, the 65 kDa protein, which is about the same molecular size as Tap, was found to be highly tyrosine phosphorylated from Jurkat-Tip/Tap cells (Figure 4B).

Alterations in Surface Expression of Adhesion Molecules by Expression of Tip and Tap

Interestingly, expression of Tip and Tap in Jurkat-T cells induced overt aggregation of cells in culture (Figure 6A). While expression of Tip alone in Jurkat-T cells did not induce aggregation, the expression of both Tip and Tap dramatically induced aggregation of Jurkat-T cells. In addition, the overexpression of Tap in Jurkat-T cells induced weak aggregation (Figure 6A). This dramatic induction of the cellular aggregation suggested that the surface expression of adhesion molecules may be altered by the coexpression of Tip and Tap. We therefore examined the surface expression of adhesion molecules, including CD11a, CD29, CD44, CD50, and CD62L. The levels of surface expression of CD3, CD4, and CD45 were also tested in these cells. Jurkat-T and Jurkat-Tip cells had similar surface expression of these lymphocyte surface antigens except for the CD4 antigen, which has previously been shown to be down-regulated in Jurkat-T cells as result of Tip expression (Jung et al., 1995b). In contrast, approximately 10% of polyclonal Jurkat-Tip/Tap cells showed significantly high surface levels of CD11a, CD44, CD50, and CD62L adhesion molecules (Figure 7).

To investigate whether increased surface expression of adhesion molecules correlated with the aggregation, cells were sorted based on the level of surface expression of the adhesion molecule CD44. Whereas there was almost no CD44 surface expression on the Jurkat-T and Jurkat-Tip cells, CD44 was expressed on the surface of the polyclonal Jurkat-Tip/Tap cells. The CD44 adhesion molecule was thus selected for cell sorting. Jurkat-Tip/Tap cells were stained with CD44 antibody and by fluorescence-activated cell sorting (FACS) were sorted into three populations: CD44-low, CD44-medium, and CD44-high. Figure 6B shows that the degree of cellular aggregation correlates with the level of CD44 surface expression. Cells containing high surface expression of CD44 antigen demonstrated the most marked aggregation,

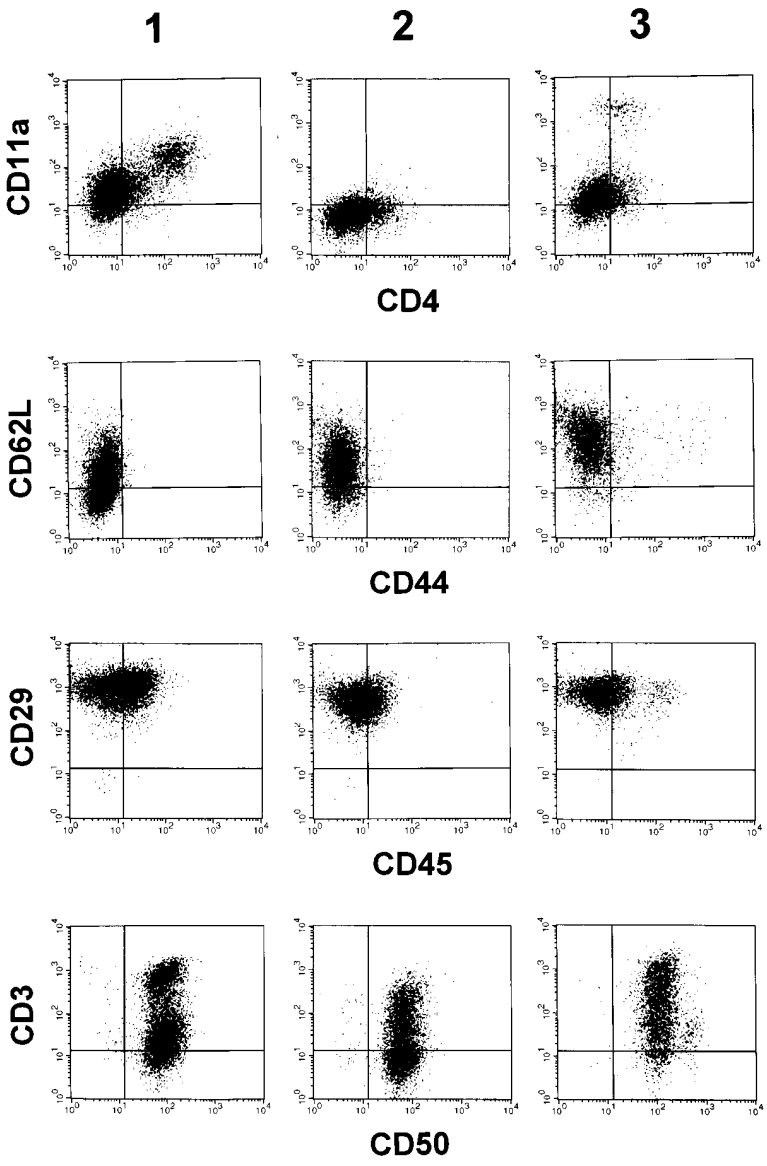

Figure 7. FACS Analysis of Jurkat-T Cells Expressing the *tip* and/or *tap* Gene

Surface phenotypes were determined by flow cytometry. Positive gates were established using matched isotype control antibody. Column 1, Jurkat-T; column 2, Jurkat-Tip; column 3, Jurkat-Tip/Tap.

while cells containing low to moderate surface expression of CD44 antigen demonstrated weak aggregation (Figure 6B). We subsequently measured the surface expression of adhesion molecules on FACS-sorted cells after they had been in culture. Jurkat-Tip/Tap CD44-low and -medium cells had levels of surface expression of adhesion molecules similar to those of the parental Jurkat-T cells (Figure 8). In contrast, Jurkat-Tip/Tap CD44 high cells had a dramatic induction of expression of adhesion molecules including CD11a, CD44, CD50, and CD62L (Figure 8, column 5). Specifically, the surface expression of the CD11a integrin receptor α chain was induced at levels greater than 100-fold as compared to the parental Jurkat-T cells. In addition, surface expression of CD50 and CD62L was also markedly increased in Jurkat-Tip/Tap CD44-high cells. In contrast, CD29 was not altered or slightly reduced in CD44-high cells. After more than 6 months of cell culture, sorted cells still maintained the phenotype described above. Finally, the level of Tap and Tip expression was measured by immunoblot with anti-Tap and anti-AU1 antibody in these three cell lines. The expression of Tap was higher in CD44-high cells than in CD44-low and -medium cells, whereas the expression of Tip was similar in all three cell lines (Figure 9A). These results suggest that the high expression of Tap in human Jurkat-Tip/Tap cells correlates with up-regulation of the surface expression of various cellular adhesion molecules and thereby induces cellular aggregation.

Activation of NF-κB Transcription Factor Activity in Markedly Aggregated Jurkat-Tip/Tap Cells

Adhesion of lymphocytes or monocytes to extracellular matrix proteins results in a rapid increase in message levels of inflammatory mediator genes including IL-1β, tumor necrosis factor α, and IL-8 as well as transcription-associated factors including IκB, A20, c-Fos, and c-Jun, most of which contain consensus NF-κB elements in their 5′ regulatory regions (Rosales and Juliano,

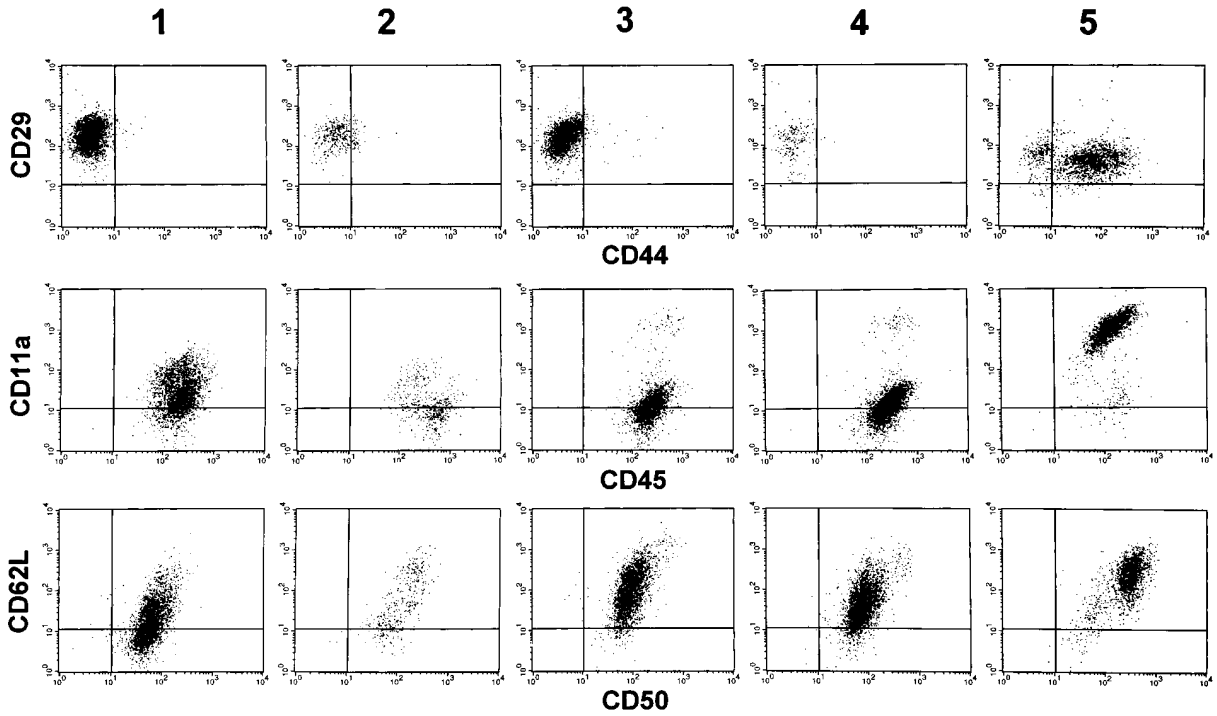

Figure 8. Flow Cytometry of Highly Aggregated Jurkat-T Cells Expressing Tip and/or Tap

Cells were sorted by FACS based on surface CD44 expression into CD44-low, -medium, and -high cells. Cells were cultured and reanalyzed for the surface expression of adhesion molecules. Column 1, Jurkat-T; column 2, Jurkat-Tap; column 3, Jurkat-Tip/Tap CD44 low; column 4, Jurkat-Tip/Tap CD44 medium; column 5, Jurkat-Tip/Tap CD44 high.

1996). To determine whether an increased aggregation of Jurkat-Tip/Tap cells has an effect on NF-κB activity, we measured NF-κB activity using a reporter construct. Parental Jurkat-T, Jurkat-Tip, and Jurkat-Tip/Tap CD44 high cells were transfected with NF-κB–driven reporter plasmid 3X-κB-L and control β-galactosidase plasmid pGKβgal. NF-κB activity in Jurkat-Tip/Tap CD44-high cells was approximately 40-fold higher than that in the parental Jurkat-T (Figure 9B). Jurkat-Tip cells also showed approximately 6-fold higher NF-κB activity than parental Jurkat-T cells. This result shows that expression of Tip and Tap in Jurkat-T cells activates NF-κB activity.

Discussion

We have previously shown that the association of Tip with Lck has dramatic effects on the tyrosine phosphorylation of cellular proteins and, in addition, that this association blocks the induction of tyrosine phosphorylation after anti-CD3 stimulation (Jung et al., 1995b). In the current report, we identify a novel cellular Tip-associated protein (Tap). Tap was associated with Tip in vivo when Tap and Tip were expressed transiently in COS-1 cells or stably in Jurkat-T cell lines. Expression of Tip and Tap in Jurkat-T cells induced a marked increase in the surface expression of lymphocyte adhesion molecules and subsequent cellular aggregation. Also, expression of Tip and Tap induced NF-κB transcription factor activity in these cells. These results suggest that in addition to Lck, Tap is an important cellular mediator for Tip function in HVS transformation.

We obtained 80 clones from a yeast-two hybrid system using Tip as a bait to isolate the Tip-interacting protein. Surprisingly, 77 of 80 clones were found to have the identical sequence, which comprises the 51 amino acids of the carboxyl terminus of Tap. This high frequency of the same clone suggests that the carboxy-terminal 51 amino acids of Tap may have a higher binding affinity than the full-length Tap in the yeast-two hybrid system. Along with yeast two-hybrid test, a GST fusion binding assay showed that the carboxy-terminal 51–amino acid sequence efficiently bound to Tip in vitro. Interestingly, the carboxy-terminal Tip-binding sequence of Tap contains glutamine-repeat motifs, $Q(X)_4Q$. Glutamine-rich sequences have been shown to function as activation domains of numerous transcriptional factors (Colgan et al., 1995; Li and Green, 1996).

Another significant feature of the Tap sequence is the leucine-rich sequence in the central region. This central leucine-rich sequence of Tap shows a regional homology with the leucine-rich repeat (LRR) of potent heat-stable protein phosphatase 2A inhibitor (Li et al., 1996). More than 30 proteins have been identified as members of the LRR superfamily (Ohsumi et al., 1993). These proteins occur in a wide range of organisms, from yeast to humans. One example of this superfamily is yeast adenylate cyclase, in which the LRR motif serves as a site of interaction with Ras protein, a process important for adenylate cyclase activation (Field et al., 1990). Likewise, the repeat motif is thought to be the binding site for the von Willebrand factor and thrombin in human platelet glycoprotein Ibα (Lopez et al., 1988; Titani et

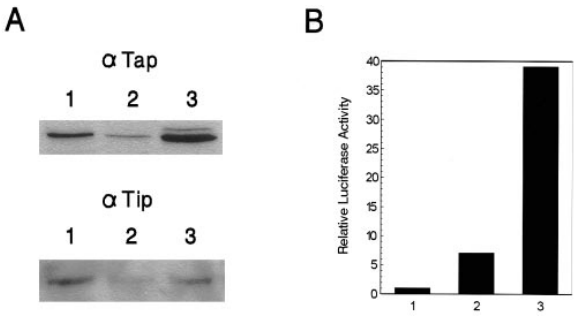

Figure 9. Tap and Tip Expression and NF-κB Activity in Markedly Aggregated Cells

(A) The level of Tap expression correlates with the level of cell aggregation. After FACS sorting, whole-cell lysates of Jurkat-Tip/Tap CD44 medium cells (lane 1), Jurkat-Tip/Tap CD44 low cells (lane 2), and Jurkat-Tip/Tap CD44 high cells (lane 3) were resolved by SDS–PAGE, transferred to nitrocellulose, and reacted with anti-Tap and anti-Tip antibodies.

(B) Activation of NF-κB activity in markedly aggregated cells. Cells were transfected with 30 μg of an NF-κB–driven reporter plasmid along with pGKβgal plasmid. Forty-eight hours after transfection, cell lysates were used for luciferase and β-galactosidase assays. Luciferase activities were determined and normalized on the basis of β-galactosidase activities. The fold activation represents normalized luciferase activity relative to that of the Jurkat-T cells. Bar 1, Jurkat-T cells; bar 2, Jurkat-Tip cells; bar 3, Jurkat-Tip/Tap CD44-high cells.

al., 1996) and for the target hormones in the lutropon-choriogonadotropin receptor (McFarland et al., 1989). Thus, despite the broad distribution of this superfamily, most are related to signal transduction pathways, and the LRR motif appears to play a role in mediating protein–protein interactions.

We demonstrate that the coexpression of Tap and Tip in human Jurkat-T cells up-regulates the surface expression of cellular adhesion molecules and thereby induces cellular aggregation. We examined the surface expression of adhesion molecules including CD11a, CD44, CD50, and CD62L. Specially, the surface expression of CD11a integrin receptor α chain was induced at levels greater than 100-fold compared with parental Jurkat-T cells. Marked increase in aggregation of Jurkat-T cells expressing Tap and Tip is a similar phenotype that has been associated with HVS transformation of primary T lymphocytes and EBV transformation of primary B lymphocytes. Lymphocyte adhesion facilitates cell growth because proliferating lymphocytes secrete autocrine growth factors. When growing at low cell density, HVS-transformed T cells thus tend to exhibit retarded cell growth (unpublished data). This suggests that cellular aggregation may induce intracellular signals required for transformed cell growth and that this type of signal transduction may be initiated by the association between Tip and Tap in HVS-transformed T cells.

LMP1 of EBV induces many of changes associated with EBV infection of primary B lymphocytes or with antigen activation of primary B lymphocytes (Wang et al., 1990). These include cellular aggregation; increased vimentin expression; and increased cell surface expression of CD23, CD39, CD40, class II major histocompatibility complex (MHCII), and the cell adhesion molecules CD11a, CD44, CD54, and CD58 (Wang et al., 1990). LMP1 not only increases surface expression of adhesion molecules but also functionally activates CD11a gene expression (Wang et al., 1988). At least a part of transcriptional activation by LMP1 is mediated by NF-κB activation (Mosialos et al., 1995). In common with LMP1 of EBV, expression of Tap and Tip in Jurkat-T cells induces NF-κB activity approximately 40-fold when compared to a parental Jurkat-T cells. These results suggest that association of Tip and Tap is likely to transduce the signal up-regulating surface expression of adhesion molecules as well as activating NF-κB activity. Recent reports demonstrate that the activation of NF-κB transcription factor protects lymphocytes from programmed cell death (apoptosis) (Beg and Baltimore, 1996; VanAntwerp et al., 1996; Wang et al., 1996). Thus, these suggest that the association of Tip with cellular Tap may introduce intracellular signals to induce NF-κB activity, which in turn may protect the HVS-infected T cells from apoptosis.

We have described intriguing similarities between the STP and Tip genes of HVS and the LMP1 and LMP2A genes of EBV (Jung et al., 1995b). 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 discernible sequence homology in the corresponding genes, similarity in size, or similarity in the organization of structural motifs in the gene products. LMP2A of EBV is analogous to Tip in that it associates with the major B cell tyrosine kinases Lyn and Syk and that this association blocks the activation of B cells by surface immunoglobulin cross-linking. However, whereas LMP2A is not required for B cell transformation by EBV (Longnecker et al., 1993), Tip is required for in vitro T cell transformation and in vivo T cell lymphoma induction by HVS (unpublished data). Also, association of Tip with Lck is not required for in vitro and in vivo oncogenicity of HVS (unpublished data). This demonstrates that unlike LMP2A of EBV, Tip has functions in addition to the blocking of lymphocyte activation by association with Lck. Thus, the association of Tip and Tap may be an important cellular event for T cell transformation induced by HVS. Finally, it will be interesting to investigate whether Kaposi's sarcoma–associated herpesvirus, which is extensively homologous to HVS and EBV, harbors gene products with functions similar to those of STP/Tip of HVS and LMP1/LMP2A of EBV.

Experimental Procedures

Cell Culture and Transfection

Jurkat-T cells were grown in RPMI supplemented with 10% fetal calf serum. Common marmoset T lymphocytes immortalized by HVS were grown in RPMI supplemented with 20% fetal calf serum. Primary common marmoset peripheral blood lymphocytes were purified using lymphocyte separation medium (Organon Teknika, Malvern, PA), washed, activated with 1 μg of phytohemagglutinin per ml for 48 hr, and then grown in RPMI with 20% fetal calf serum supplemented with 10% IL-2. COS-1 cells were grown in DME medium supplemented with 10% fetal calf serum. A diethylaminoethyl-dextran transfection procedure was used for transient expression in COS-1 cells. The pDNA3-Tap (20 μg) was introduced into Jurkat-T cells by electroporation (BioRad) at 250 V and 960 μF in serum-free DME medium. After a 48 hr incubation, the cells were cultured

with selection medium containing 1 mg/ml of G418 for the next 5 weeks.

Yeast Strains and Library Screen

Yeast transformation with library DNA was performed by the method of Schiesti and Gietz (1989), as follows. EGY 48/pRB1840 (Gyuris et al., 1993) bearing LexA fusion protein plasmids were grown overnight in yeast extract peptone dextrose (YPAD) medium to a density of approximately 10^7 cells/ml and then diluted in 100 ml of warmed YPAD to a density of 2×10^6 cells/ml and regrown to 10^7 cells/ml. The cells were harvested and washed in water, resuspended in 1 ml of water, transferred to a sterile microcentrifuge tube, and pelleted. The pellet was resuspended in 0.5 ml of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M Li-acetate (LiOAc). Fifty microliters of the resulting suspension was mixed with 1 μg of transforming DNA and 50 μg of single-stranded salmon sperm DNA, after which 0.3 ml of a solution of 40% polyethyleneglycol-4000 in Tris-EDTA-LiOAc was added and mixed thoroughly, followed by incubation at 30°C with agitation for 30 min. After a heat pulse at 42°C for 15 min, the cells were pelleted in a microcentrifuge, and pellets were resuspended in 1 ml of Tris-EDTA and plated in selective medium. Library screening and recovery of plasmids were performed as described by Gyuris et al. (1993).

To assess the interaction of Tap with Tip, cells of the yeast strain EGY48/pSH18–34 were transformed with the indicated bait construct and selected on $Ura^-His^-Trp^-$ glucose plates. Several colonies from each bait–interactor combination were picked and plated on $Ura^-His^-Trp^-$ X-Gal plates containing either 2% glucose or 2% galactose.

cDNA Cloning

Clones overlapping the primary Tap isolates were sought with a commercially available human lymphocyte plasmid library (GIBCO, Grand Island, NY). The library was screened by filter replica hybridization, using radioactive probes derived from the insert isolated by interaction screening. Thirteen independent clones were isolated, and the coding sequence of Tap was obtained as a composite from the cDNA clones sequenced on both strands using an ABI PRISM 377 automatic DNA Sequencer.

Northern Blot Analysis

Human multiple tissue Northern blots were purchased from Clontech (Palo Alto, CA). Northern blots contained approximately 2 μg of poly(A)$^+$ RNA per lane from eight different human tissues. A 2.5 kb full-length *tap* cDNA was used to generate the radioactively labeled probe by nick translation with [^{32}P]dATP. Hybridization and washing were performed according to the protocol provided by the manufacturer.

Plasmid Construction

DNA containing the EcoRI and BamHI fragment of the *tip* gene containing amino acids 1–226 was subcloned into the pJG4-5 vector for yeast-two hybrid screening (Gyuris et al., 1993). For stable expression, the EcoRI and XbaI DNA or BamHI and EcoRI DNA containing HA-tagged *tap* gene was cloned into the EcoRI and XbaI or BamHI and EcoRI sites of pcDNA3 (Invitrogen, San Diego, CA).

Expression and Purification of Glutathione S-Transferase (GST) Fusion and Production of Tap Antibody

An EcoRI–XhoI fragment containing the coding sequences for amino acid residues from 1–522 of Tap (GST-Tap) or an EcoRI–SalI fragment containing the 51 amino acids of the carboxyl terminus of Tap (GST-Tap-c) was inserted into the EcoRI and XhoI sites of the expression vector pGEX-4T (Pharmacia LKB, Piscataway, NJ). GST fusion protein expression and purification were performed essentially as described by Smith and Johnson (1988). For fusion protein recovery using glutathione Sepharose, bacterial cell pellets were frozen once, resuspended with 1/10 volume 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–-Tap protein was used to generate polyclonal antibody in New Zealand White rabbits. AU1 monoclonal antibody recognizing a DTYRYI epitope from bovine papilloma virus L1 capsid protein and HA monoclonal antibody recognizing a YPYDVPDYA epitope from the influenza hemagglutinin were purchased from Berkeley Antibody (Richmond, CA).

Immunoprecipitation and Immunoblot

COS-1 cells at 80%–90% confluence in a 25 cm^2 dish were rinsed three times with PBS, washed once with labeling medium (minimum essential medium minus methionine and cysteine plus 10% dialyzed fetal calf serum), and then incubated with 2 ml of the same medium containing 200 μCi of [^{35}S]methionine and [^{35}S]cysteine (New England Nuclear, Boston, MA) for 7 hr. Cells were incubated in labeling medium for 30 min prior to the addition of the radioisotopes. For immunoprecipitation, cells were harvested and lysed with lysis buffer (0.15 M NaCl, 0.5% Nonidet P-40, and 50 mM HEPES 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 SDS–PAGE and transferred to a nitrocellulose membrane filter. Immunoblot detection was performed with a 1:1000 or 1:3000 dilution of primary antibody for conventional color development or with an enhanced chemiluminescence system (Amersham, Chicago, IL).

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 (6000 Ci/mmol, New England Nuclear) for 15 min at room temperature, and separated through SDS–PAGE. Kinase activity was measured by Bio-Rad Molecular Imager model GS-250.

FACS Analysis

First, 5×10^5 cells 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 4°C. After washing, each sample was fixed with 1% formalin solution, and FACS analysis was performed with a FACS Scan (Becton-Dickinson, Mountain View, CA). For cell sorting, 2×10^7 cells were stained with FITC-conjugated CD44 antibody for 30 min at 4°C. Stained cells were separated into three different fractions based on CD44 surface expression by a FACS Vantage (Becton-Dickinson). After sorting, cells were washed twice with PBS and cultured with RPMI plus 10% FCS medium. Antibodies for CD3 (UCHT1, Pharmingen, San Diego, CA), CD4 (RPA-T4, Pharmingen), CD11a (HI111, Pharmingen), CD45 (HI30, Pharmingen), CD44 (Leu-44, Becton-Dickinson), CD50 (Antigenx), CD29 (MAR4, Becton-Dickinson), and CD62L (Leu8, Becton-Dickinson) were commercially purchased.

In Vitro Binding of GST Fusion Proteins to Tip

Five micrograms of purified GST fusion proteins noncovalently coupled to glutathione Sepharose beads were mixed with precleared COS-1 cell lysates containing Tip for 1 hr at 4°C and washed four times in lysis buffer. Bound proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and immunoblotted with anti-Tip antibody.

NF-κB Assays

Approximately 1×10^7 cells were electroporated at 960 μF and 200 V and were harvested after 48 hr. All transfections included 30 μg of pGKβgal, which expresses β-galactosidase from a phosphoglucokinase promoter, and 30 μg of 3X-κB-L, which has three copies of the NF-κB binding site from the murine major histocompatibility complex class I promoter upstream of a minimal *fos* promoter and a luciferase gene. At 48 hr after transfection, cells were washed once in PBS and lysed in 200 μl of reporter lysis buffer (Promega, Madison, WI). Assays for luciferase or β-galactosidase activity were

performed with a Luminometer using luciferase assay reagent (Promega). Values were normalized by β-galactosidase activity.

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