The Epstein–Barr Virus Transforming Protein LMP1 Engages Signaling Proteins for the Tumor Necrosis Factor Receptor Family

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Summary

The cytoplasmic C-terminus of Epstein–Barr virus (EBV) latent infection membrane protein 1 (LMP1) is essential for B lymphocyte growth transformation and is now shown to interact with a novel human protein (LMP1-associated protein 1 [LAP1]). LAP1 is homologous to a murine protein, tumor necrosis factor receptor-associated factor 2 (TRAF2), implicated in growth signaling from the p80 TNFR. A second novel protein (EBI6), induced by EBV infection, is the human homolog of a second murine TNFR-associated protein (TRAF1). LMP1 expression causes LAP1 and EBI6 to localize to LMP1 clusters in lymphoblast plasma membranes, and LMP1 coimmunoprecipitates with these proteins. LAP1 binds to the p80 TNFR, CD40, and the lymphotoxin-β receptor, while EBI6 associates with the p80 TNFR. The interaction of LMP1 with these TNFR family–associated proteins is further evidence for their role in signaling and links LMP1-mediated transformation to signal transduction from the TNFR family.

Introduction

Epstein–Barr virus (EBV) causes infectious mononucleosis and lymphoproliferative diseases in immune-suppressed patients. EBV is also an important etiologic factor in endemic Burkitt's lymphoma, AIDS-associated central nervous system lymphomas, Hodgkin's disease, and nasopharyngeal carcinoma (Kieff and Liebowitz, 1990; Miller, 1990). EBV infection of B lymphocytes is primarily nonlytic, resulting in the expression of EBV-encoded nuclear (EBNAs) and integral membrane proteins (latent infection membrane proteins [LMPs]) as well as perpetual cell proliferation (Kieff and Liebowitz, 1990). EBV recombinant-based molecular genetic analyses have demonstrated that LMP1 and EBNA2, -LP, -3A, and -3C are critical for B lymphocyte transformation (Cohen et al., 1989; Hammerschmidt and Sugden, 1989; Kieff and Liebowitz, 1990; Mannick et al., 1991; Kaye et al., 1993; Tomkinson et al.,

1993), while LMP2, EBNA3B, the EBV-encoded small RNAs and most of the rest of the genome are dispensable (Marchini et al., 1991; Swaminathan et al., 1991; Longnecker et al., 1993; Tomkinson and Kieff, 1992; Robertson et al., 1994). EBNA1 is also important for transformation since it enhances transcription and replication from EBV episomes (Yates et al., 1984; Reisman and Sugden, 1986).

LMP1 is not only essential for primary B lymphocyte growth transformation but is also the only EBV gene that has transforming effects in nonlymphoid cells. In Rat1 cells, LMP1 induces growth in lower serum, loss of contact inhibition, anchorage independence, and nude mouse tumorigenicity, while in BALB/c 3T3 cells, LMP1 induces anchorage-independent growth (Wang et al., 1985, 1988a; Baichwal and Sugden, 1988; Moorthy and Thorley-Lawson, 1993). In B lymphoblasts, LMP1 induces most of the phenotypic effects of EBV infection, including induced expression of activation markers and adhesion molecules, altered growth, and NF-κB activation (Wang et al., 1988b, 1990; Hammarskjold and Simurda, 1992; Laherty et al., 1992; Rowe et al., 1994). In epithelial cells, LMP1 blocks differentiation (Dawson et al., 1990; Fahraeus et al., 1990; Wilson et al., 1990). These activities are probably relevant to tumorigenicity in humans since LMP1 is expressed not only in EBV-induced lymphoproliferative disease but also in EBV-associated Hodgkin's disease tumor cells and in most anaplastic nasopharyngeal carcinoma cells (Herbst et al., 1991; Pallesen et al., 1991; Brooks et al., 1992; Busson et al., 1992).

LMP1 is an integral membrane protein that may transform cells by constitutively activating a growth factor receptor pathway common to many cell types (Wang et al., 1985). LMP1 consists of a 23 amino acid amino-terminal cytoplasmic domain, 6 markedly hydrophobic transmembrane domains separated by short reverse turns, and a 200 amino acid carboxy-terminal cytoplasmic domain (Fennewald et al., 1984; Hennessy et al., 1984). The transmembrane domains enable LMP1 to posttranslationally insert into membranes and to accumulate in aggregates in the plasma membrane (Hennessy et al., 1984; Liebowitz et al., 1986).

Recent genetic analyses in primary B lymphocyte growth transformation indicate that the amino terminus is important for tethering of the first transmembrane domain, and the transmembrane domains are important for conferring plasma membrane aggregation (Kaye et al., 1993, 1995; Izumi et al., 1994). EBV recombinants deleted for the entire 200 or for the last 155 amino acids of the LMP1 carboxy-terminal cytoplasmic domain are incapable of transforming primary B lymphocytes (Kaye et al., 1995). While the nontransforming phenotype of EBV recombinants deleted for the last 155 amino acids of LMP1 can be transcomplemented by the growth of infected lymphocytes on fibroblast feeders, to date it has not been possible to similarly rescue primary B lymphocytes infected with EBV recombinants deleted for the entire 200 amino acid carboxy-terminal cytoplasmic domain (Kaye et al., 1995).
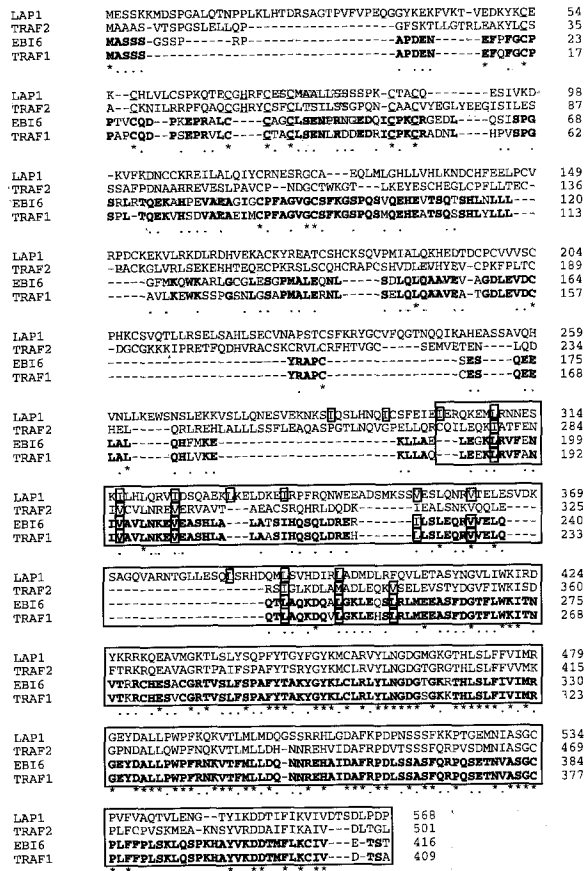
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LAP1    MESSKKMDSPGALQTNPPLKLHTDRSAGTPVFVPEQGGYKEKFVKT-VEDKYKCE      54
TRAF2   MAAAS-VTSPGSLELLQP-----------------GFSKTLLGTRLEAKYLCS        35
EBI6    MASSS-GSSP------RP-----------------APDEN------EFPFGCP       23
TRAF1   MASSS--------------------------------APDEN------EFQFGCP     17
               *....                                  ....    .  ..

LAP1    K--CHLVLCSPKQTECGHRFCESCMAALLSSSSPK-CTACQ-------ESIVKD-      98
TRAF2   A--CKNILRRPFQAQCGHRYCSFCLTSILSSGPQN-CAACVYEGLYEEGISILES     87
EBI6    PTVCQD--PKEPRALC----CAGCLSENPRNGEDQICPKCRGEDL----QSISPG     68
TRAF1   PAPCQD--PSEPRVLC----CTACLSENLRDDEDRICPKCRADNL----HPVSPG     62
            *.      .   * .  *.*...... ..  .  .     .   *

LAP1    -KVPKDNCCKREILALQIYCRNESRGCA---EQLMLGHLLVHLKNDCHFEELPCV     149
TRAF2   SSAFPDNAAHREVESLPAVCP--NDGCTWKGT---LKEYESCHEGLCPFLLTEC-     136
EBI6    SRLRTQEKAHPEVAEAGICGPFAGVGCSFKGSPQSVQEHEVTSQTSHLNLLL---     120
TRAF1   SPL-TQEKVHSDVAEAEIMCPFAGVGCSFKGSPQSMQEHEATSQSSHLYLLL---     113
             *..        ..    *  .*.      ..    .

LAP1    RPDCKEKVLRKDLRDHVEKACKYREATCSHCKSQVPMIALQKHEDTDCPCVVVSC     204
TRAF2   -BACKGLVRLSEKEHHTEQECPKRSLSCQHCRAPCSHVDLEVHYEV-CPKFPLTC     189
EBI6    -----GFMKQWKARLGCGLESGPMALEQNL-----SDLQLQAAVEV-AGDLEVDC     164
TRAF1   -----AVLKEWKSSPGSNLGSAPMALERNL-----SELQLQAAVEA-TGDLEVDC     157
                              .   ..*.          ..*.

LAP1    PHKCSVQTLLRSELSAHLSECVNAPSTCSFKRYGCVFQGTNQQIKAHEASSAVQH     259
TRAF2   -DGCGKKKIPRETFQDHVRACSKCRVLCRFHTVGC-----SEMVETEN----LQD     234
EBI6    --------------------YRAPC-----------------SES----QEE      175
TRAF1   --------------------YRAPC-----------------CES----QEE      168
                               *                      ..   ..

LAP1    VNLLKEWSNSLEKKVSLLQNESVEKNKSIQSLHNQICSFEIEERQKEMLRNNES     314
TRAF2   HEL-----QRLREHLALLLSSFLEAQASPGTLNQVGPELLQRCQILEQKIATFEN     284
EBI6    LAL-----QHFMKE--------------------KLLAE---LEGKLRVFEN       199
TRAF1   LAL-----QHLVKE--------------------KLLAQ---LEENLRVFAN       192
            .*   . ...                       ...

LAP1    KILHLQRVIDSQAEKIKELDKEIRPFRQNWEEADSMKSSVESLQNRVFELESVDK     369
TRAF2   IVCVLNREMERVAVT---AEACSRQHRLDQDK-------IEALSNKVQQLE----     325
EBI6    IVAVLNKEVEASHLA---LATSIHQSQLDRER-------ILSLEQHVVELQ----     240
TRAF1   IVAVLNKEVEASHLA---LAASIHQSQLDREH-------LLSLEQHVVELQ----     233
           .*.  ...            ...              ...

LAP1    SAGQVARNTGLLESQEISRHDQMLSVHDIRLADMDLRFQVLETASYNGVLIWKIRD     424
TRAF2   -----------------RSILGLKDLAMADLEQKVSELEVSTYDGVFIWKISD     360
EBI6    -------------------QTLAQKDQALGKLEQSLRLMEEASFDGTFLWKITN     275
TRAF1   -------------------QTLAQKDQVLGKLEHSLRLMEEASFDGTFLWKITN     268
                            .   ..  . ......     ........

LAP1    YKRRKQEAVMGKTLSLYSQPFYTGYFGYKMCARVYLNGDMGKGTHLSLFFVIMR     479
TRAF2   FTRKRQEAVAGRTPAIFSPAFYTSRYGYKMCLRVYLNGDGTGRGTHLSLFFVVMK     415
EBI6    VTRRCHESACGRTVSLFSPAFYTAKYGYKLCLRLYLNGDGTGKRTHLSLFIVIMR     330
TRAF1   VTKRCHESVCGRTVSLFSPAFYTAKYGYKLCLRLYLNGDGSGKKTHLSLFIVIMR     323
             .*. ...  .  ***.    .***.* .******.*.**..

LAP1    GEYDALLPWPFKQKVTLMLMDQGSSRRHLGDAFKPDPNSSSFKKPTGEMNIASGC     534
TRAF2   GPNDALLQWPFNQKVTLMLLDH-NNREHVIDAFRPDVTSSSFQRPVSDMNIASGC     469
EBI6    GEYDALLPWPFRNKVTFMLLDQ-NNREHAIDAFRPDLSSASFQRPQSETNVASGC     384
TRAF1   GEYDALLPWPFRNKVTFMLLDQ-NNREHAIDAFRPDLSSASFQRPQSETNVASGC     377
           ****.****.**. **.. ..*  *****.*..**  ** *   *******

LAP1    PVFVAQTVLENG--TYIKDDTIFIKVIVDTSDLPDP     568
TRAF2   PLFCPVSKMEA-KNSYVRDDAIFIKAIV---DLTGL     501
EBI6    PLFPPLSKLQSPKHAYVKDDTMFLKCIV---E-TST     416
TRAF1   PLFPPLSKLQSPKHAYVKDDTMFLKCIV---D-TSA     409
          *.*  .  ...   .*.**.* * **     .  .
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Figure 1. Amino Acid Alignment of Human EBI6 and LAP1 and Murine TRAF1 and TRAF2

The four polypeptide sequences were aligned using the CLUSTAL program (PCGene [IntelliGenetics]). Identical (asterisks) and homologous (dots) amino acids are shown. Pairwise alignment of EBI6 and TRAF1 is also shown, with identical amino acids designated by bold faced characters. Amino acids that form the RING finger motif in LAP1 and TRAF2 and the zinc finger structure in EBI6 and TRAF1 are underlined. Amino acids that form putative coiled-coil structures are boxed. The TRAF domain is shown by large boxes.

Table 1. β-Gal Assay of Protein–Protein Interactions in the Yeast Two-Hybrid System

Transformant	β-Gal Units
G4DBDLMP1(187–386)–G4TADLAP1(183–568)	56
G4DBDLMP1(187–386)–G4TADLAP1(346–568)	8
G4DBDLMP1(187–231)–G4TADLAP1(183–568)	5
G4DBDLMP1(187–231)–G4TADLAP1(346–568)	5
G4DBDLMP1(187–386)–G4TADEBI6(53–416)	0.07
G4DBDLAP1(12–568)–G4TADEBI6(53–416)	0.07
G4DBDLMP1(187–386)	0.04
G4DBDLMP1(187–231)	0.09
G4DBDLAP1(183–568)	0.04
G4TADLAP1(187–568)	0.1
G4TADEBI6(53–416)	0.04
G4DBDLAP1(12–568)	0.05
G4DBDSNF1–G4TADSNF4	0.8

The yeast strain Y190 was transformed with the indicated plasmids, and transformants were selected on appropriate selective-defined media. Isolated colonies were grown to mid to late log density and assayed for β-gal activity as described in Experimental Procedures. Four individual transformants were assayed in each case (except for the interaction between G4DBDSNF1 and G4TADSNF4, for which two transformants were tested), and the average values of β-gal units are shown. The interaction between G4DBDSNF1 and G4TADSNF4 was used as a positive control (Harper et al., 1993) and scored 0.8 β-gal units or higher in different assays.

These results implicate the LMP1 carboxyl terminus in cell growth transformation and suggest that the first 44 amino acids may be stringently required for transformed cell growth (Kaye et al., 1995). We have now discovered that the first 44 amino acids of the LMP1 carboxy-terminal cytoplasmic domain interact with a human protein related to the very recently described putative effectors of tumor necrosis factor receptor (TNFR) signaling (Rothe et al., 1994). The interaction of this human protein with LMP1 and with the cytoplasmic domains of the TNFR family members is evidence for a central role of this protein as an effector of cell growth or death signaling pathways.

Results

A Yeast Two-Hybrid Screen Reveals Proteins That Interact with the LMP1 Carboxy-Terminal Cytoplasmic Domain

DNA encoding the 200 amino acid LMP1 carboxy-terminal cytoplasmic domain was fused in frame to the GAL4 DNA-binding domain for use as bait in a yeast two-hybrid screen for cDNAs that encode interactive proteins. The GAL4-activating domain was fused to cDNAs made from RNA from EBV-transformed B lymphocytes (Durfee et al., 1993). Of 5 × 10⁵ transformants that were tested for growth in the absence of tryptophane, leucine, and histidine and in the presence of 25 mM 3-aminotriazole, 147 colonies showed at least moderate growth and were analyzed for β-galactosidase (β-gal) expression. Two clones were strongly positive for β-gal, scoring higher than 8 U in a standard β-gal assay, whereas the rest of the clones had nearly background levels of β-gal activity (less than 0.04 U). The GAL4-activating domain–fusion proteins made from these two clones did not interact with GAL4 DNA-binding domain fusions to p53, pRB, lamin, or the yeast protein kinase SNF1, indicating specificity for the LMP1 cytoplasmic carboxyl terminus. The two clones were 3′ coterminal cDNAs from the same mRNA. The clones have a single long open reading frame predicted to encode part of a novel protein provisionally designated LMP1-associated protein 1 (LAP1). To obtain the full-length LAP1 open reading frame, the insert from the longer clone was used to identify clones from a λgt10 library of cDNAs (Birkenbach et al., 1993) prepared from RNA from an EBV-infected B lymphoblast cell line, BL41/B95-8. One clone has the beginning and most of the 568 codon LAP1 open reading frame, whereas two other clones were derived from an alternatively spliced mRNA that lacks 1061 bases, including the first 320 codons of LAP1.

From the sequence of the complete open reading frame, full-length LAP1 is predicted to have an amino-terminal RING finger metal-binding motif and a carboxy-terminal domain that begins with an extended coiled-coil motif (Fig-
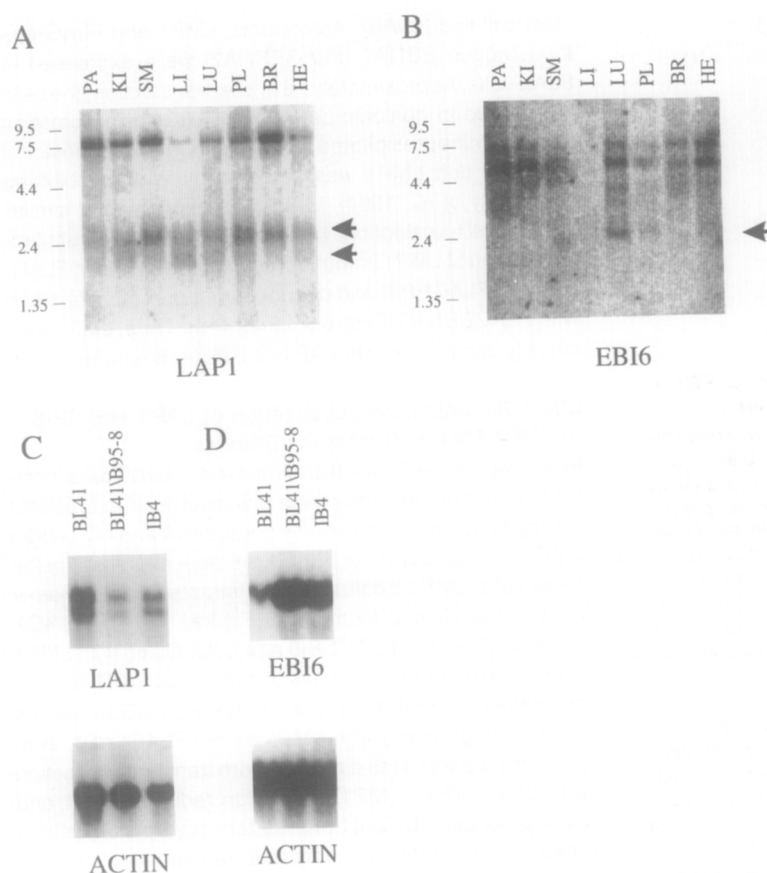
Figure 2. *LAP1* and *EBI6* mRNA Abundance

Northern blot of RNA from human tissues (A and B, both poly(A)$^+$ RNA) or cell lines (C and D, poly(A)$^+$ RNA and total RNA, respectively) hybridized with *LAP1* (A and C) or *EBI6* (B and D) probes. The probe is shown below the blot, and the origin of RNA is shown above each lane. Size markers are to the left of each blot, and arrows indicate the position of specifically and consistently detected mRNAs. The *LAP1* probe detected 2.8 kb and 1.8 kb RNAs, whereas the *EBI6* probe detected a 2.6 kb RNA. The high molecular weight bands were not consistently detected in other Northern blots with these probes. *LAP1* (C) and *EBI6* (D) mRNAs were also detected in RNA from EBV-infected BL41 (BL41/B95-8), EBV-negative BL41 (BL41), and EBV-transformed (IB4) cells. An actin probe (ACTIN) indicates the relative amounts of RNA in (C) and (D).

Abbreviations: PA, pancreas, KI, kidney, SM, skeletal muscle, LI, liver, LU, lung, PL, placenta, BR, brain, and HE, heart.

ure 1). The carboxy-terminal LAP1 domain (amino acids 302–568) has 45% colinear amino acid identity to the TNFR–associated factor (TRAF) homology domain of the recently identified murine TRAF1 or -2 (Rothe et al., 1994). LAP1 is similar to TRAF2 in having an amino-terminal RING finger motif, but is only 27% identical to TRAF2 overall. The longest open reading frame identified in the alternatively spliced *LAP1* mRNA encodes for a polypeptide that initiates at methionine codon 350 within the coiled-coil motif of full-length LAP1 and includes the rest of the TRAF domain. Since amino acids 345–568 of LAP1 interact strongly with the LMP1 carboxy-terminal cytoplasmic domain (Table 1), the protein encoded by the spliced *LAP1* mRNA is likely to interact also with the LMP1 carboxy-terminal cytoplasmic domain and could modulate interactions of LAP1 with LMP1.

A Carboxy-Terminal LAP1 Domain Interacts with a LMP1 Membrane Proximal Cytoplasmic Domain

The interaction of the full-length LMP1 cytoplasmic carboxyl terminus (amino acids 187–386) with the LAP1 carboxy-terminal 386 or 223 amino acid polypeptide scored higher than the positive control in the yeast two-hybrid dependent β-gal assay (Table 1). The interaction of the membrane proximal 44 amino acids of the LMP1 cytoplasmic carboxyl terminus (amino acids 187–231) with the LAP1 carboxy-terminal 386 or 223 amino acids also generated higher β-gal activity than did the positive control (Table 1). Thus, the membrane proximal 44 amino acids of

the LMP1 cytoplasmic carboxyl terminus and the LAP1 carboxy-terminal 223 amino acid–TRAF homology domain are sufficient for high level interaction in the yeast two-hybrid assay. The EBV recombinant molecular genetic evidence, which indicates that the LMP1 carboxy-terminal cytoplasmic domain is essential for transformation, provides a linkage between this biochemical interaction and transformation.

An EBV-Induced Cell Protein Is a Human TRAF1 Homolog

LAP1 is 32% colinearly homologous to another novel human protein that had been identified because its encoding mRNA is more abundant in the EBV-infected B lymphoblast line BL41/B95-8 than in the uninfected control BL41 cells (Figure 1). This EBV-induced mRNA 6 (EBI6) is predicted to encode for a 416 amino acid protein that is 86% colinearly identical to the murine TRAF1 (Rothe et al., 1994) (Figure 1). EBI6 is probably the human homolog of murine TRAF1 since these proteins are nearly identical throughout their entire sequence (Figure 1). EBI6 and TRAF1 have similar amino-terminal zinc finger motifs and are 95% identical in their TRAF domains (Figure 1). When fused to the GAL4-activating domain, EBI6 amino acids 53–416 do not interact in yeast with the LMP1 carboxy-terminal cytoplasmic domain or with amino acids 12–568 of LAP1 fused to the GAL4 DNA-binding domain (Table 1). The failure of EBI6 to interact with LAP1 distinguishes LAP1 from the murine RING finger protein TRAF2, which

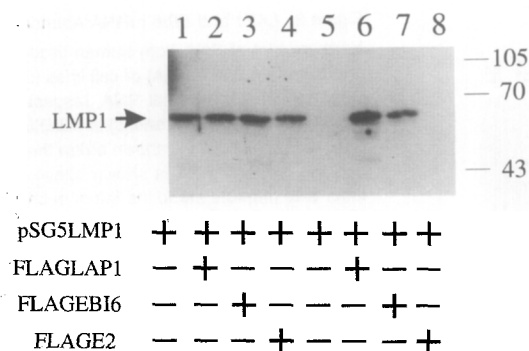|	1	2	3	4	5	6	7	8	
pSG5LMP1	+	+	+	+	+	+	+	+	
FLAGLAP1	−	+	−	−	−	+	−	−	
FLAGEBI6	−	−	+	−	−	−	+	−	
FLAGE2	−	−	−	+	−	−	−	+	

Figure 3. Intracellular Association of LMP1 with LAP1 or EBI6 in Transfected BJAB, Non-EBV-Infected, B Lymphoma Cells

BJAB cells (10 × 10⁶ cells per transfection) were electroporated with plasmids expressing the proteins indicated by a plus sign at the bottom of the figure. Approximately 20 hr posttransfection, 4 × 10⁶ cells from each transfection were lysed and subjected to immunoprecipitation with 10 μg of M2 anti-FLAG monoclonal antibody (Kodak). Equivalent cell lysates obtained before immunoprecipitation (lanes 1–4) and immunoprecipitated material (lanes 5–8) were analyzed by SDS–PAGE on a 7.5 % gel, transferred onto nitrocellulose, and subjected to Western blot analysis using rabbit anti-LMP1 polyclonal antisera (Hennessy et al., 1984) and ¹²⁵I-labeled protein A, followed by autoradiography. The position of LMP1 is shown by the arrow, and molecular weight markers are shown on the right side of the panel. LMP1 was readily coimmunoprecipitated with FLAGLAP1 or FLAGEBI6 (lanes 6 and 7). No detectable LMP1 was seen in anti-FLAG immunoprecipitations from cells cotransfected with pSG5LMP1 and either vector control (pSG5) or a construct expressing a FLAG-tagged EBNA2 (FLAGE2, lanes 5 and 8).

scored positive in the yeast two-hybrid system with TRAF1 (Rothe et al., 1994). While the failure could be due to differences between the human RING finger (LAP1) or zinc finger (EBI6) TRAFs and murine TRAF1 and -2, the lower level of homology of LAP1 to TRAF2 is more likely to account for this discrepancy given the extensive identity between EBI6 and LAP1.

Expression of LAP1 and EBI6 in Human Tissues and Cell Lines

The full-length 2.8 kb and alternatively spliced 1.8 kb *LAP1* mRNAs are expressed in all tissues examined (Figure 2A). In contrast, the 2.6 kb *EBI6* mRNA is readily detected in lung, spleen, and tonsil, is barely detected in placenta, and is not detectable in pancreas, kidney, smooth muscle, liver, brain, or heart (Figure 2B; data not shown). To evaluate the effects of EBV infection on *LAP1* and *EBI6* mRNA abundance, *LAP1* or *EBI6* probes were hybridized to RNAs from EBV-negative BL41 B lymphoblasts, from EBV-infected BL41 B lymphoblasts (BL41/B95-8), or from an EBV-transformed B lymphoblast cell line (IB4) (Figures 2C and 2D). *LAP1* mRNAs were similarly abundant in EBV-positive or -negative cells, whereas the *EBI6* message was at least 8-fold more abundant in the EBV-infected BL41/B95-8 or IB4 cells than in noninfected BL41 cells.

LMP1 Associates with LAP1 and EBI6 in B Lymphoblasts

LMP1 and FLAG epitope–tagged LAP1 (FLAGLAP1) or FLAG-tagged EBI6 (FLAGEBI6) were expressed in an efficiently transfectable non-EBV-infected human B lympho-

blast cell line (BJAB). As controls, LMP1 and FLAG epitope–tagged EBNA2 (FLAGEBNA2) were expressed in BJAB cells. Approximately 18 hr posttransfection, the cells were lysed in nonionic detergent; FLAG fusion proteins were immunoprecipitated with an anti-FLAG monoclonal antibody; and LMP1 was detected by Western blotting (Hennessy et al., 1984). LMP1 was expressed at similar levels in all transfections (Figure 3, lanes 1–4). A substantial fraction of LMP1 immunoprecipitated along with FLAG-LAP1 or FLAGEBI6 and did not immunoprecipitate along with FLAGEBNA2 (Figure 3, lanes 4–8). Thus, LMP1 specifically associates with LAP1 or EBI6 in B lymphoblasts.

LMP1 Redirects the Localization of LAP1 and EBI6 to LMP1 Plasma Membrane Patches

FLAG-tagged LAP1 or EBI6 expressed in BJAB cells localizes throughout the cytoplasm to punctate structures that resemble cytoplasmic vesicles (Figures 4A and 4C). When LMP1 and FLAGLAP1 or FLAGEBI6 were expressed in the same cells, LMP1 localized to its characteristic patches or caps in the plasma membrane (Figures 4F, 4I, 4L, 4O), and FLAGLAP1 or FLAGEBI6 now colocalized with LMP1 in plasma membrane patches or caps (Figures 4E and 4H or Figures 4K and 4N, respectively). FLAGEBI6 did not localize as precisely with LMP1 as did FLAGLAP1, suggesting a weaker, less direct, or more transient interaction with LMP1. Thus, LMP1 expression redirects LAP1 and (to a lesser extent) EBI6 from scattered cytoplasmic structures to LMP1 plasma membrane patches.

LAP1 and EBI6 Associate with TNFR Family Cytoplasmic Domains

The homology of LAP1 and EBI6 to murine TRAF2 and TRAF1 prompted us to examine whether LAP1 and EBI6 can interact with members of the TNFR family, including CD40, lymphotoxin-β receptor (LTβR), and Fas (Banchereau et al., 1994; Nagata, 1994; Smith et al., 1994; Ware et al., 1995), using receptor cytoplasmic domains constructed as fusion proteins with glutathione S-transferase (GST). In vitro translated LMP1 did not interact with GST or GST fusions to TNFRs (Figure 5A, lanes 1–3). However, in vitro translated LAP1 bound specifically (112-fold over GST background) to the p80 cytoplasmic domain and less well (14-fold over GST background) to the p60 cytoplasmic domain (Figure 5A, lanes 4–6; Figure 5B, lanes 3 and 4; data not shown). LAP1 also showed nearly quantitative binding to the cytoplasmic domains of TNFR-related proteins CD40 and LTβR and less efficient binding to Fas (Figure 5B). EBI6 bound less efficiently than did LAP1 to p80 and showed weak binding to p60 (Figure 5A, lanes 7–9).

LAP1 and EBI6 also associate with the p80 receptor in vivo. The p80 TNFR was coexpressed with FLAGLAP1, FLAGEBI6, or FLAGEBNA2 in BJAB cells that do not express any surface p80 receptor (data not shown). FLAG-tagged proteins were immunoprecipitated from cell lysates using M2 anti-FLAG antibody, and the immune complexes were analyzed for the presence of p80 TNFR by Western blotting (Figure 5C). The M2 anti-FLAG antibody coimmunoprecipitated the p80 receptor from cells
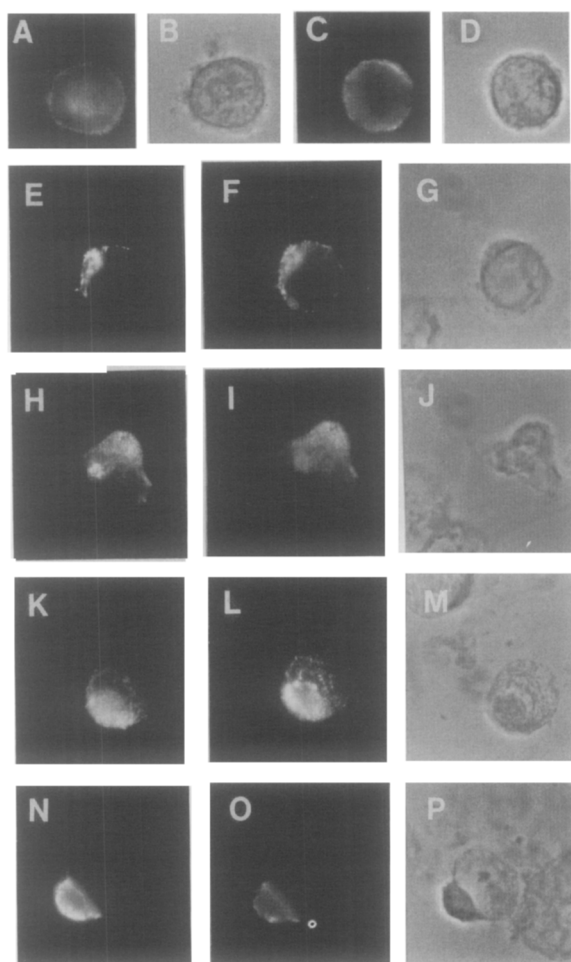
Figure 4. Subcellular Localization of LAP1 and EBI6 in the Presence or Absence of LMP1

The intracellular distribution of FLAG-tagged LAP1 and EBI6 was determined by indirect immunofluorescence using M2 anti-FLAG monoclonal antibody and rabbit anti-LMP1 polyclonal antisera. BJAB cells were transfected with FLAGLAP1 (A, B, and E–J) or FLAGEBI6 (C, D, and K–P) expressing constructs in the presence of vector pSG5 (A–D) or pSG5LMP1 (E–P). M2 anti-FLAG reactivity was visualized with a fluorescein isothiocyanate–conjugated goat anti-mouse secondary antibody (A, C, E, H, K, N). LMP1 was detected with a Texas Red–conjugated goat anti-rabbit secondary antibody (F, I, L, O). Phase-contrast pictures are shown in (B), (D), (G), (J), (M), and (P). M2 and anti-LMP1 antibodies did not show any reactivity in untransfected cells. No cross-reactivity was observed between M2 and the goat anti-rabbit secondary antibody or between the rabbit anti-LMP1 and goat anti-mouse secondary antibody (data not shown).

Figure 5. Association of LAP1 and EBI6 with TNFR-Related Proteins

(A) Cytoplasmic domains of the p60 or p80 TNFR constructed as fusion proteins with GST and bound to glutathione beads were incubated with [^{35}S]methionine-labeled LMP1, LAP1, or EBI6 translated in vitro (5 μl reaction mix), and the fraction bound to glutathione beads was analyzed on a 8.5% SDS–PAGE and processed by a phosphorimager. Lanes 1, 4, and 7 are pull downs with GST–p80; lanes 2, 5, and 8 are those with GST–p60; and lanes 3, 6, and 9 are those with GST–control. Coomassie blue staining of the gel demonstrated the presence of approximately equivalent amounts of GST or GST–fusion proteins.

(B) Glutathione beads containing the cytoplasmic domains of p60 (lane 3), p80 (lane 4), Fas (lane 5), CD40 (lane 6), and LTβR (lane 7) expressed as GST–fusion proteins or GST (lane 2) were incubated with [^{35}S]methionine-labeled LAP1 (2 μl of in vitro translation reaction mix were used per reaction) as in (A) and were analyzed by SDS–PAGE and autoradiography. In vitro translated LAP1 (2 μl) was analyzed in lane 1.

(C) Coimmunoprecipitation of LAP1 and EBI6 with p80 TNFR in cotransfected cells. BJAB cells were cotransfected with plasmids expressing the FLAG-tagged proteins indicated by a plus sign at the bottom of the figure or were left untransfected (lane 7). Approximately 20 hr posttransfection, the cells were lysed, and lysates from 10 × 10⁶ cells were subjected to immunoprecipitation with M2 anti-FLAG monoclonal antibody. Equivalent cell lysates obtained before immunoprecipitation (lanes 4–7) and immunoprecipitated complexes (lanes 1–3) were analyzed by Western blotting using an anti-p80 TNFR antibody (VanArsdale and Ware, 1994). The positions of mature p80 TNFR and the immunoglobulin heavy chain (Ig) are shown by arrows. The asterisk shows the position of a precursor form of the p80 TNFR. The p80 receptor was readily coimmunoprecipitated with FLAGLAP1 or FLAGEBI6 (lanes 1 and 2). No detectable p80 receptor was immunoprecipitated with anti-FLAG antibody from cells cotransfected with plasmids expressing p80TNFR and FLAGEBNA2 (FLAGE2, lane 3).

expressing FLAGLAP1 or FLAGEBI6 (Figure 5C, lanes 1 and 2), but not from cells expressing FLAGEBNA2 (Figure 5C, lane 3). FLAGLAP1 and FLAGEBI6 associated with both the ~70 kDa precursor and the mature p80 TNFR (Ware et al., 1991), indicating that the association with LAP-1 occurs prior to receptor expression at the cell surface. These studies indicate the potential of LAP1 and EBI6 to participate in TNFR signaling and also provide evidence that LAP1 may be a component common to the CD40, LTβR, and TNFR signal transduction pathways.

Discussion

We have identified two human TRAFs and confirmed their association with the human p80 TNFR. The TRAF domain was initially defined because of more than 50% colinear primary sequence identity through the carboxy-terminal 230 amino acids of murine TRAF1 and -2 (Rothe et al., 1994). Genetic and biochemical data link TRAF1 and -2 to the growth and NF-κB-transducing effects of a domain near the carboxyl terminus of the p80 TNFR cytoplasmic tail (Rothe et al., 1994). EBI6 is almost certainly the human TRAF1, based on the ability of both proteins to associate with the p80 TNFR, similar tissue restricted expression, similar amino-terminal zinc finger motifs, 95% primary sequence identity in the TRAF domains, and an overall 86% colinear primary sequence identity. LAP1 is probably not the human TRAF2, but rather a human RING finger analog. If so, there may be one or more human RING finger TRAFs as yet unidentified. Although LAP1 and TRAF2 are similar in size, have an amino-terminal RING finger domain, and are constitutively expressed in most tissues, LAP1 is 45% identical to either TRAF2 or TRAF1 in the TRAF domain and is quite divergent from TRAF2 outside of the TRAF domain. Overall, LAP1 and TRAF2 are only 27% identical. LAP1 also appears to be functionally distinct from TRAF2 in not directly interacting with EBI6, the human TRAF1.

LAP1 and EBI6 interact with the cytoplasmic domain of not only p80 TNFR but also with p60 TNFR, albeit less well than with p80. This is the first evidence of interaction beyond p80 between these putative effectors and the TNFR family. Indeed, LAP1 also interacts strongly with the LTβR and CD40 cytoplasmic domains. These findings provide a new molecular basis for understanding the common effects of activation of these members of the TNFR family. Heterogeneity among TRAFs or other cell proteins may provide components to the receptor–TRAF complexes that determine the specific phenotypic outcome(s) of receptor activation.

The identification of these human TRAFs is in the context of an investigation into the mechanisms by which LMP1 transforms cells. These biochemical and genetic experiments and previous EBV recombinant molecular genetic analysis establish a connection between the role of LMP1 in B lymphocyte growth transformation and TNFR signaling pathways. As briefly reviewed in the introduction, LMP1 is a dominant oncogene that has multiple downstream effects on cell growth and gene expression, at least some of which are NF-κB mediated. LMP1 interacts strongly and directly with LAP1 and also associates with EBI6 in human lymphoblasts. Our genetic, biochemical, and intracellular localization data on the association of LMP1 with LAP1 and EBI6, taken together with the previous genetic and biochemical linkage of TRAF1 and -2 to p80 TNFR signaling, reinforce a role for the TRAFs as mediators of cell growth or death and NF-κB responses (for schematic model, see Figure 6).

The six markedly hydrophobic transmembrane domains of LMP1 enable it to aggregate in the plasma membrane and to present aggregated cytoplasmic domains to the
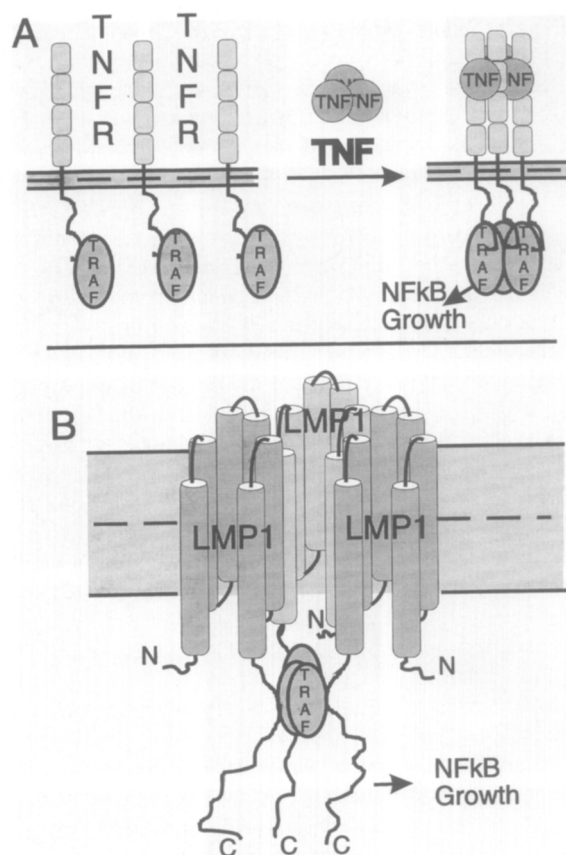

Figure 6. Schematic Representation of p80 TNFR Activation and LMP1 Complexes at the Plasma Membrane

(A) Model for the activation of the p80 TNFR is shown. The extracellular region of the TNFR is composed of four domains with characteristic cysteine patterns. The cytoplasmic domain of the receptor is known to associate with complexes of TRAF molecules (TRAF), including TRAF1, TRAF2, and LAP1. Upon binding of TNF (shown here as a trimer), the extracellular domains of several receptor molecules are believed to be cross-linked, causing aggregation of intracellular domains and their associated TRAF molecules. Clustering of receptor molecules and their intracellular domains results in signal transduction as manifested by a number of phenotypic alterations, including the activation of transcription factor NF-κB (NFkB) and cell growth.

(B) Three LMP1 molecules are shown to form a constitutive complex at the plasma membrane (depicted by the stippled area between the two solid horizontal lines). The amino-terminal (N) and carboxy-terminal (C) cytoplasmic regions of LMP1 are shown by short and long lines, respectively. The transmembrane domains of LMP1 are depicted by vertical cylinders, which are joined by short reverse turns (short curved lines). Aggregation of LMP1 molecules at the plasma membrane brings together LMP1-associated TRAF molecules (TRAF) in a complex, thus, generating a constitutive signal that results in pleiotropic effects, including activation of NF-κB (NFkB) and cell growth.

TRAFs (Figure 6B). In presenting aggregated TRAF-interacting domains, LMP1 mimics TNFR aggregation, which appears to be essential for signal transduction (Engelmann et al., 1990; Loetscher et al., 1991; Pennica et al., 1992; Tartaglia and Goeddel, 1992). Receptor cross-linking probably locally aggregates TRAFs and associated molecules, creating a second messenger signal perhaps mediated by a receptor-associated serine/threonine kinase (Figure 6A) (Darnay et al., 1994a, 1994b; VanArsdale and Ware, 1994). Since LMP1 constitutively aggregates

LAP1 and EBI6 in oligomeric complexes at plasma membrane patches, these complexes could constitutively activate growth signals and NF-κB in the absence of extracellular stimuli (Figure 6B). LMP1 signaling via TRAF molecules may thus proceed independently of TNFR molecules. Alternatively, LMP1-TRAF aggregates may nucleate larger, more diverse, or more stable TNFR family-TRAF complexes through interactions among the extended coiled-coils of the TRAF domains. In fact, some evidence favors the latter alternative in that lymphotoxin-α (LTα) is an autocrine growth factor for EBV-transformed lymphoblastoid cell lines (Estrov et al., 1993; Gibbons et al., 1994). Furthermore, expression of the full range of EBV latent infection–associated proteins in Burkitt lymphoma cell lines induces LTα and the p80 receptor (Gibbons et al., 1994). Moreover, antagonistic antibodies to the p60 TNFR have a negative growth effect in such cells (Gibbons et al., 1994). The LMP1 cytoplasmic carboxy-terminal domain and TNFR family members could even interact with different domains of the same LAP1 molecule since there is no obvious homology between the LMP1 cytoplasmic carboxyl terminus and the cytoplasmic domains of TNFR family members.

The induction of EBI6 by latent EBV infection and the association of EBI6 with LMP1 in B lymphoblasts are also evidence of an important role for EBI6 in EBV-mediated B lymphocyte growth transformation. The interaction appears to be less direct than with LAP1 and may be mediated by another as yet unidentified human RING finger TRAF.

TNF and CD40 ligand are well known mediators of growth of B lymphocytes and of other cell types that are targets for LMP1-transforming effects (Noelle et al., 1992; Boussiotis et al., 1994). In fact, CD40 ligation and interleukin-4 treatment are sufficient to sustain the proliferation of primary B lymphocytes in vitro for several months, and the cells are phenotypically similar to EBV-transformed lymphocytes (Saeland et al., 1993; Banchereau et al., 1994; Galibert et al., 1994). The LTβR is expressed on epithelial cells (C. W. and J. Browning, unpublished data), while basal epithelial cells and anaplastic nasopharyngeal carcinoma cells also express high levels of CD40 (Busson et al., 1988; Young et al., 1989). LMP1, through constitutive direct interaction with LAP1, may amplify or usurp LTβR and CD40 signal transduction and constitutively promote cell growth. Nasopharyngeal carcinoma is tightly associated with EBV, and LMP1 is frequently expressed in the tumor cells (Brooks et al., 1992). Hodgkin's disease is another EBV-associated malignancy in which LMP1 is expressed (Herbst et al., 1991). CD40, TNFRs, and the related receptor CD30 are up–regulated in Hodgkin's disease cells (Froese et al., 1987; Pfreundschuh et al., 1989; Carde et al., 1990; O'Grady et al., 1994; Trumper et al., 1994). A potentially important consequence of the demonstrated interaction between LAP1 and LMP1 is that inhibitors of that interaction may affect the growth or development of these LMP1-associated malignancies.

In interacting with components of receptor signaling, LMP1 is reminiscent of bovine papilloma virus E5. Bovine papilloma virus E5 dimerizes in the plasma membrane, pre-sumably through hydrophobic interactions, and activates receptors for epidermal growth factor, platelet-derived growth factor, or colony-stimulating factor 1 (Martin et al., 1989; Petti et al., 1991; Petti and DiMaio, 1992). E5 binds a component of vacuolar H⁺-ATPases, and this may affect receptor recycling (Goldstein et al., 1991).

A curious aspect of our data is the finding that LAP1 or EBI6 localized to vesicle-like structures in the cytoplasm of B lymphoblasts. Furthermore, LAP1 is constitutively expressed in cells, and there may be another constitutively expressed RING finger protein that intermediates between LMP1 and EBI6. These constitutively expressed proteins may also have a role in vesicle biology.

The interaction of LMP1 with TNFR signaling pathways may also be important in enabling EBV-infected cells to evade cellular host defense mechanisms in latent or lytic EBV infection. LMP1 is one of the few EBV genes expressed in both phases of the virus life cycle (Mann et al., 1985; Rowe et al., 1992). Several virus families appear to specifically target the TNF/lymphotoxin pathways, presumably to avoid these immune cell mediators of cytotoxicity. Pox viruses produce soluble versions of the p80 TNFR (Smith et al., 1991; Massung et al., 1993); proteins encoded by the adenovirus E3 region block the apoptotic function of TNF (Gooding, 1992); and HIV utilizes NF-κB-activating signals induced by TNF signaling to enhance transcription (Poli et al., 1990). The binding of EBV LMP1 to LAP1 may effectively compete with normal LAP1 binding to the p60 TNFR, blocking the induction of cell death mediated by that receptor (Tartaglia et al., 1993b) or blocking other functions critical to host defense (Pfeffer et al., 1993; Rothe et al., 1993), while simultaneously usurping the growth-promoting signals of the p80 TNFR (Tartaglia et al., 1993a).

Experimental Procedures

Plasmid Construction

The GAL4 DNA-binding domain (G4DBD) fusions were constructed in vector pAS2 (Harper et al., 1993). G4DBDLMP1(187–386) was constructed by polymerase chain reaction–mediated (PCR-mediated) amplification of the *LMP1* cDNA fragment, encoding amino acids 187–386, using oligos L1-5PCR (5'-CGCGGATCCATGGACAACGACA-CAGTG-3') and L1-4PCR (5'-CGCGGATCCTTAGTCATAGTAGCT-TAG-3'), followed by cloning into the BamHI site of pAS2. G4D-BDLMP1(187–231) was constructed by PCR amplification of the *LMP1* cDNA fragment, encoding amino acids 187–231, using oligos L1-5PCR and LCA231 (5'-CGCGGATCCTTAGGCTCCACTCACGAG-CAG-3'), followed by cloning into the BamHI site of pAS2. G4DBDL-AP1(12–568) was constructed by isolating the BssHII–BamHI fragment of *LAP1* cDNA from pSG5LAP1, blunt-ending it using T4 DNA polymerase, and subcloning it into the SmaI site of pAS1. GAL4 transactivating domain (G4TAD) fusions were as follows. G4TA-DEBI6(53–416) was constructed by subcloning the BglII fragment of *EBI6* cDNA into the BamHI site of pACTII (a gift of S. Elledge). G4TA-DEBI6(53–416) encodes for an in-frame fusion of EBI6 amino acids 53–416 to the acidic transactivating domain of GAL4. Plasmids G4TADLAP1(183–568) and G4TADLAP1(345–568) were isolated from the yeast two-hybrid screening. Plasmids expressing SNF1 fused to the DNA-binding domain of GAL4 (G4DBDSNF1) or SNF4 fused to the activation domain of GAL4 (G4TADSNF4) were gifts of S. Elledge. SNF1 and SNF4 are two yeast proteins that are known to interact with each other, and they were used as a positive control in the yeast two-hybrid-dependent β-gal assay (Harper et al., 1993). *LAP1* cDNAs

were subcloned into the EcoRI site of plasmid pSG5 (Stratagene) for sequencing analysis. pSG5 subclones of the longest *LAP1* cDNAs were spliced at the NruI site to generate the full-length LAP1-expressing construct pSG5LAP1. The EcoRI insert of λgt10 clone *EBI6* was subcloned into the plasmid pBluescript for sequencing analysis. pSG5FLAGLAP1 and pSG5FLAGEBI6 were constructed in vector pSG5 by placing through PCR a FLAG-encoding DNA fragment just after the initiator AUG codon. Expression plasmids for TNFRs were previously described (Ware et al., 1991).

Subtractive Hybridization

Construction of the λgt10 cDNA library from the EBV-positive cell line BL41/B95-8 was previously described (Birkenbach et al., 1993). Subtractive hybridization and homology screening of the λgt10 library was done as described before (Birkenbach et al., 1993).

Yeast Two-Hybrid Screening

Yeast transformation was performed according to the method of Schiestl and Geitz (1989). The yeast strain Y190 (Durfee et al., 1993) was transformed with plasmid construct G4DBDLMP1(187–386), and transformants were plated on Trp⁻ selective-defined media. A single colony was picked, and the expression of the LMP1 fusion protein was verified by Western blotting using the S12 anti-LMP1 monoclonal antibody. The G4DBDLMP1(187–386) transformant was subsequently transformed with a cDNA library constructed previously from an EBV-transformed lymphoblastoid cell line (Durfee et al., 1993), and selection was done on Trp⁻ Leu⁻ His⁻ selective-defined media in the presence of 25 mM 3-aminotriazole (Sigma) as previously described (Durfee et al., 1993). Colonies that showed moderate to intense growth were streaked on Trp⁻ Leu⁻ His⁻ selective-defined media containing 50 mM 3-aminotriazole and tested for β-gal expression by a filter lift assay (Breeden and Nasmyth, 1985). For quantitation of β-gal expression, yeast clones were grown in appropriate selective media to OD_{600} of 0.5–1.2 and were assayed for β-gal activity using ONPG and standard conditions as previously described (Breeden and Nasmyth, 1985). β-Gal units were expressed as $(1000A_{415})/(assay$ time in minutes)(cell culture volume in milliliters)(OD_{600}). The interactions of G4DBD with G4TADLAP1(183–568), G4TADLAP1(346–568), or G4TADEBI6(53–416) and of G4TAD with G4DBDLMP1(187–386), G4DBDLMP1(187–231), or G4DBDLAP1(12–568) scored ≤0.1 U of β-gal. Library-derived plasmids were recovered by transformation of competent bacteria with total yeast DNA preps, followed by selection for ampicillin resistance as previously described (Ausubel et al., 1987).

Northern Blots

Northern blots containing poly(A)⁺ RNA (2 μg per lane) from eight human tissues were purchased from CLONTECH. RNA was prepared from EBV-positive (BL41/B95-8) or EBV-negative (BL41) Burkitt's lymphoma cell lines and a lymphoblastoid cell line (IB4) as previously described (Birkenbach et al., 1993). cDNA probes were labeled by random hexanucleotide priming (Stratagene) using [³²P]dCTP. The RNA blots were hybridized to ³²P-labeled cDNA probes under high stringency conditions as described (Mosialos et al., 1994). Northern blot filters were exposed to autoradiography film or were processed by phosphorimager analysis.

Immunoprecipitations, Western Blotting, and Immunofluorescence

BJAB cells were electroporated at 220 V and 960 μF in 400 μl of RPMI-1640 medium containing 10% fetal calf serum. Approximately 20 hr posttranfection, cells were lysed for 30 min on ice in 0.5% NP-40 lysis buffer containing 50 mM HEPES (pH 7.4), 250 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml pepstatin A, and 2 μg/ml leupeptin. Cell debris were removed by centrifugation at 10,000 × g for 10 min at 4°C. The cell lysates were precleared with protein G–Sepharose beads for 1 hr at 4°C. The primary antibody was then added for 1 hr at 4°C, and immunoglobulin complexes were collected on protein G–Sepharose beads for 1 hr at 4°C. The beads were then washed six times with 1 ml of lysis buffer each time, and protein complexes were recovered by boiling in SDS sample buffer and were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Western blotting was done using standard techniques as previously described (Mosialos et al., 1994).

Indirect immunofluorescence analysis on transfected cells was done approximately 18–20 hr posttransfection as previously described (Mosialos et al., 1994).

Production and Purification of GST–Fusion Proteins

The cytoplasmic domains of the p80 and p60 TNFRs were amplified from the corresponding cDNAs by PCR and were cloned in frame into the pGEX-4T-1 expression vector (Pharmacia) using the EcoRI and XhoI restriction sites for the p60 TNFR and the EcoRI and XhoI sites for the p80 TNFR. A similar strategy was employed for the construction of a GST fusion to the LTβR cytoplasmic domain. GST–fusion proteins of the cytoplasmic domains of CD40 and Fas were a gift of J. Reed (La Jolla Cancer Research Institute). Expression and purification of GST–fusion proteins were performed essentially as described previously (Smith and Johnson, 1988). Fusion protein concentrations of 3–5 mg/ml of glutathione–agarose beads (Pharmacia) were routinely obtained. In vitro translations were done using the rabbit reticulocyte-coupled in vitro transcription translation system (TNT, Promega) according to the protocol of the manufacturer. In vitro translated proteins were diluted with binding buffer (PBS containing 0.1% NP-40, 0.5 mM DTT, 10% glycerol, 1 mM PMSF, 2 μg/ml aprotinin) and were precleared with glutathione beads for 45 min at 4°C. GST or GST–fusion proteins bound to glutathione beads were then incubated with in vitro translated proteins for 1 hr at 4°C. The beads were subsequently washed five times with 0.5 ml of binding buffer each time, and bound proteins were recovered by boiling in SDS sample buffer and were analyzed by SDS–PAGE.

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GenBank Accession Numbers

The accession numbers for the *LAP1* and *EBI6* sequences reported in this study are U19260 and U19261, respectively.

Note Added in Proof

In the December 2, 1994 issue of the Journal of Biological Chemistry, Hu et al. reported the sequence of a protein that is nearly identical to that of LAP1. This protein became bound to wild type, but not to a signaling-defective mutant CD40 cytoplasmic domain.