The Epstein-Barr Virus Protein, Latent Membrane Protein 2A, Co-opts Tyrosine Kinases Used by the T Cell Receptor*

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Robert J. Ingham‡1, Judith Raaijmakers‡, Caesar S. H. Lim‡§, Geraldine Mbmamal°, Gerald Gish‡, Fu Chen‡, Liudmila Matskova‡2, Ingemar Ernberg‡, Gösta Winberg‡, and Tony Pawson‡§3

From the ‡Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada, §Karolinska Institutet, Microbiology and Tumor Biology Center, Stockholm SE-171 77, Sweden, and the ‡Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada

Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis and is associated with several human malignancies. The EBV protein latent membrane protein 2A (LMP2A) promotes viral latency in memory B cells by interfering with B cell receptor signaling and provides a survival signal for mature B cells that have lost expression of surface immunoglobulin. The latter function has suggested that LMP2A may enhance the survival of EBV-positive tumors. EBV is associated with several T cell malignancies and, since LMP2A has been detected in several of these disorders, we examined the ability of LMP2A to transmit signals and interfere with T cell receptor signaling in T cells. We show that LMP2A is tyrosine-phosphorylated in Jurkat TAg T cells, which requires expression of the Src family tyrosine kinases, Lck and Fyn. Lck and Fyn are recruited to the tyrosine-phosphorylated Tyr112 site in LMP2A, whereas phosphorylation of an ITAM motif in LMP2A creates a binding site for the ZAP-70/Syk tyrosine kinases. LMP2A also associates through its two PPPPY motifs with AIP4, a NEDD4 family E3 ubiquitin ligase; this interaction results in ubiquitylation of LMP2A and serves to regulate the stability of LMP2A and LMP2A-kinase complexes. Furthermore, stable expression of LMP2A in Jurkat T cells down-regulates T cell receptor levels and attenuated T cell receptor signaling. Thus, through recruiting tyrosine kinases involved in T cell receptor activation, LMP2A may provide a survival signal for EBV-positive T cell tumors, whereas LMP2A-associated NEDD4 E3 ligases probably titie the strength of this signal.

Epstein-Barr virus (EBV) is a γ-herpesvirus that causes infectious mononucleosis and is associated with a number of human cancers, including Burkitt lymphoma and nasopharyngeal carcinoma (reviewed in Refs. 1 and 2). Infection is common, and it is estimated that ~90% of the world’s population is latently infected with EBV (3). This latent infection is maintained in the body’s memory B cells through the expression of a number of latency-associated viral genes, one of which is latent membrane protein 2A (LMP2A) (4).

LMP2A is a 12-membrane-spanning polypeptide with intracellular amino- and carboxyl termini. It has been reported to aggregate into “cap-like” structures at the plasma membrane (5, 6) and specifically associate with lipid rafts (7, 8); however, LMP2A has also been shown to localize to the Golgi and endocytic vesicles (9, 10). Also, the C-terminal tail of LMP2A has been reported to possess a clustering signal (11).

LMP2A is thought to maintain B cells in the latent state by interfering with signaling through the B cell receptor (BCR) and thus prevent activation of the lytic promoting viral BZLF-1 gene (4, 12). The ability of LMP2A to interfere with BCR signaling and maintain viral latency is due to a number of protein-protein interaction motifs located within the amino-terminal tail (see Fig. 1). These include a YEEA site that, when phosphorylated on the tyrosine residue (Tyr112), can bind the Src homology 2 domain of the Src family tyrosine kinase Lyn (12–14). In addition, LMP2A possesses an immunoreceptor tyrosine-based activation motif (ITAM) motif with the consensus sequence YAX(L/V)XX6–8XXI(Y/V), which is found in a number of immunoreceptors including the BCR, the T cell receptor (TCR), and the Fce receptor that binds IgE (15). This motif in LMP2A, when phosphorylated on tyrosines 74 and 85, provides a binding site for the dual Src homology 2 domains of the tyrosine kinase Syk (16, 17). LMP2A also possesses two PPPPY (PY) motifs that can bind to the WW domains of the NEDD4 family of ubiquitin ligases and either the 26 S proteasome or a lysosomal pathway (21). LMP2A and the LMP2A-associated kinases are substrates for the NEDD4 family of ubiquitin ligases, including AIP4, NEDD4-2, and WWP2 (18–20). Binding to NEDD4 proteins is abrogated by mutation of both tyrosines in the PY motifs (Tyr60 and Tyr100). NEDD4 family proteins contain HECT (homologous to E6-associated protein carboxyl terminus) domains that catalyze the ubiquitylation of proteins such as those associated with the WW domains and target them for degradation via either the 26 S proteasome or a lysosomal pathway (21). LMP2A and the LMP2A-associated kinases are substrates for the NEDD4 family of proteins, suggesting that LMP2A may not only sequester tyrosine kinases away from the BCR but may also direct them to ubiquitin-mediated pathways such as degradation.

Somewhat paradoxically, LMP2A has also been shown to mimic BCR signaling. When expressed as a transgene specifically in the B-lineage in mice, LMP2A can both drive B cell development and promote the survival of mature B cells in the absence of surface Ig expression (22–24). Furthermore, this signal appears to be attenuated by the NEDD4 family protein, Itch, since crossing mice homozygous for a deficiency in Itch into the LMP2A transgenic background enhanced LMP2A-mediated signaling (25). Therefore, LMP2A may provide a survival factor for EBV-positive B cell tumors that have lost expression of surface Ig as well as preventing virus reactivation.

EBV can also infect T cells and is found associated with a wide range
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of peripheral T cell and NK-/T-cell lymphomas, particularly localized to the upper respiratory tract (26, 27). These lymphomas are particularly prevalent in Southeast Asia, overlapping with the high incidence region for nasopharyngeal carcinoma (28). EBV-positive T cell malignancies are also associated with Japanese patients with chronic active EBV infection and EBV-associated hemophagocytic syndrome (29–32). In these potentially fatal disorders, EBV-infected T cells produce large numbers of proinflammatory cytokines such as interferon-γ and tumor necrosis factor-α that cause fever, hepatomegaly, and splenomegaly, with patients often succumbing to hepatic or cardiac problems. Whereas LMP1 has been suggested to regulate cytokine production in these EBV-infected T cells (33), they also express LMP2A (34), but the function of LMP2A in these and other T cell malignancies (35) has not been established. In this regard, the similarities between BCR and TCR signaling raise the possibility that LMP2A may be able to manipulate TCR signaling as it does BCR signaling. We have therefore, examined the potential functions of LMP2A in T cells. Here we show that in the Jurkat T antigen (TAg) human T cell line, LMP2A is tyrosine-phosphorylated and associates with the tyrosine kinases Lck and ZAP-70 through the phosphorylated YEEA and ITAM motifs, respectively. Also, the two PY motifs in LMP2A bind the NEDD4 family E3 ubiquitin ligase AIP4 in T cells, which can consequently ubiquitylate LMP2A. Mutation of the PY motifs in LMP2A greatly stabilizes the protein and increases the stability of LMP2A-kinase complexes. This may have the consequence of titering signaling through LMP2A. In this model system, stable expression of LMP2A down-regulated TCR levels and moderately inhibited signaling events downstream of the TCR. Our demonstration that LMP2A interacts with key T cell signaling proteins suggests a role for this viral protein in EBV-positive T cell tumors.

EXPERIMENTAL PROCEDURES

Antibodies, Constructs, and Other Reagents—The M2 anti-FLAG monoclonal antibody (mAb) and anti-α-tubulin mAb, DM 1A, were purchased from Sigma. The anti-LMP2A rat mAb, 14B7, was purchased from ITN GmbH (Neuherberg, Germany). The anti-Lck, anti-Fyn, anti-Syk polyclonal, and anti-ZAP-70 antisera were provided by Dr. André Veillette (McGill University, Montreal, Canada). The anti-AIP4 polyclonal antisemur has been previously described (18). The 4G10 anti-phosphotyrosine (Tyr(P)) mAb and anti-Erk polyclonal antisemur were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The anti-Myc mAb, 9E10, and anti-HA antisemur were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-ZAP-70 mAb was purchased from BD Transduction Laboratories (Mississauga, Canada). The phosphospecific anti-phospho-Erk (Thr202, Tyr204) and phospho-ZAP-70 (Tyr319) antisera were purchased from Cell Signaling Technology, Beverly, MA. The phycoerythrin (PE)-conjugated anti-CD4 (UCHT1), anti-CD69 (H1.2F3), anti-CD28 (CD28.2), and isotype control mAbs were purchased from BD Biosciences (Mississauga, Canada). The Myc-tagged murine ZAP-70 was provided by Dr. Andrew Shaw (Washington University, St. Louis, MO). The HA-ubiquitin construct was a gift from Dr. Ivan Dikic (Goethe University Medical School, Frankfurt, Germany). The mutant LMP2A constructs (see Fig. 1) have either been previously described (18) or were generated by PCR and sequence-verified. The 4× FLAG constructs were generated by cloning singly FLAG-tagged LMP2A constructs into the pCMV 3× FLAG vector (Sigma). The resulting proteins consist of three FLAG epitopes, a spacer of 14 amino acids, an additional FLAG epitope, and a spacer of 6 amino acids, followed by the LMP2A protein. The doubly Myc-tagged AIP4 constructs were generated by PCR of our previously published constructs (18) with the amino terminus of the published AIP4 sequence (19) replacing the amino-terminal Itch portion of the fusion used in our earlier study. These constructs were cloned into the pCDNA3.1A expression vector (Invitrogen). The synthesis of the biotinylated peptides has been previously described (18) with peptide sequences indicated in Fig. 1.

Cell Lines and Tissue Culture—Jurkat, Jurkat TAg, and the Syk/ZAP-70-deficient (P116) T cells were obtained from Dr. André Veillette. The JCAM-1 cells (Lck-deficient) and JCAM-1 cells reconstituted with Lck were a gift from Dr. David Strauss (Virginia Commonwealth University, Richmond, VA). Cells were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen). The stable LMP2A-expressing Jurkat cell lines were generated using retrovirus-mediated gene transfer using the pLXPOP vector containing the LMP2A cDNA (18) and the primate-tropic packaging line, PG13 (36). Stably expressing cells were selected in 2 μg/ml puromycin, and LMP2A expression was verified by anti-LMP2A immunoblotting.

Transfection of Jurkat and Jurkat-derivative Cell Lines—Jurkat or derivative cell lines were electroporated with a BTX ECM 830 square wave electroporator (BTX, San Diego, CA) with three 225-V pulses of 8-mJ pulse length and waiting for 1 s between pulses. The total amount of DNA electroporated in each experiment, indicated in the figures and figure legends, was kept constant through the addition of empty vector. After electroporation, cells were resuspended in fresh RPMI 1640 medium and incubated for 24 h at 37 °C.

Cell Lysis and Precipitations—Cells were collected by centrifugation at ~500 × g at 24 h post-electroporation and lysed in 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) for 15 min on a rocker in the cold. Detergent-insoluble material was removed by centrifugation of the lysate at ~18,000 × g for 10 min. Cleared lysates were incubated with 2–4 μg of the indicated antibodies and either protein A- or G-Sepharose or anti-mouse Ig agarose for 1 h on a rocker at 4 °C. Immunoprecipitates were then washed three times with Nonidet P-40 lysis buffer, and bound proteins were eluted by boiling in SDS-PAGE sample buffer. For precipitations with the biotinylated peptides, 10 μl of peptide-saturated streptavidin-agarose beads, corresponding to ~1 nmol of peptide, was used. Dephosphorylation of the tyrosine-phosphorylated peptides was achieved by incubating the peptides on streptavidin-agarose with 1 unit of calf intestinal phosphatase (Roche Applied Science) for 1 h at 37 °C prior to performing precipitation experiments.

Western Blotting—Samples were run out on SDS-polyacrylamide gels, transferred to nitrocellulose, and blocked in 5% skim milk powder in Tris-buffered saline containing 0.05% Tween 20 (TBST) or 5% bovine serum albumin in TBST. Blots were incubated with primary Ab in TBST with 5% bovine serum albumin either overnight at 4 °C or 1 h at room temperature. Blots were then rinsed several times in TBST followed by incubation with a secondary Ab, either goat anti-mouse, goat anti-rabbit, or protein A-horseradish peroxidase-coupled reagent (Bio-Rad) for 1 h at room temperature. Blots were then rinsed several times in TBST and, finally, rinsed once in TBS before being exposed to enhanced chemiluminescence reagent (Pierce). Blots were stripped with TBST, pH 2, for 30 min before being reprobed as above.

Cycloheximide and Ubiquitylation Experiments—For cycloheximide experiments, Jurkat TAg cells were transfected with the indicated plasmids and incubated for 24 h at 37 °C. Cells were then either left untreated or treated for the indicated times with 10 μg/ml cycloheximide (Sigma) prior to lysing the cells as described above. For ubiquitylation experiments, transfected cells were grown for 24 h and then treated for 1 h with 50 μM of the proteasome inhibitor MG132 (BACHEM, King of Prussia, PA). Cells were then lysed as described
above with the exception that 50 μM MG132 and 20 mM N-ethylmaleimide (Sigma) was added to the lysis buffer.

Stimulation of Jurkat T Cells—Jurkat T cells were resuspended to $1 \times 10^6$ /ml in RPMI 1640 medium. 1 ml of the cell suspension was incubated with the anti-TCR CD3ε chain mAb, OKT3, anti-CD28 mAb (a gift from Dr. Philippe Poussier, University of Toronto, Toronto, Canada), or both at 10 μg/ml for 30 min on ice with occasional mixing. The cells were then pelleted and resuspended with serum-free RPMI. Cells were then resuspended in 100 μl of fresh RPMI 1640 medium (no serum) containing 20 μg/ml goat anti-mouse IgG (Bio-Can, Mississauga, Canada) for the indicated times at 37 °C. The reaction was stopped by adding 500 μl of cold phosphate-buffered saline containing 1 mM sodium vanadate. Cells were then spun down and lysed in 1% Nonidet P-40 lysis buffer as described above, and the protein concentration of the cleared lysate was determined by a BCA assay (Pierce).

Fluorescence-activated Cell Sorting—1 × 10^6 cells were pelleted and washed in FACS buffer (phosphate-buffered saline containing 1% serum and 0.02% NaN₃). Cells were resuspended in 50 μl of FACS buffer to which 20 μl of PE-conjugated Ab was added. Cells were incubated for 30 min on ice with occasional mixing and washed a final time with FACS buffer before being resuspended in 1 ml of FACS buffer and analyzed on a BD Biosciences FACSCalibur cell sorter. For stimulation of cells prior to sorting, 5 × 10^5 cells were incubated for 24 h with 2 μg of the anti-CD3 mAb (UCHT1) or 50 ng/ml phorbol 12-myristate 13-acetate in 24-well plates.

RESULTS

Expression of LMP2A and LMP2A Mutant Constructs in Jurkat TAg Cells—To explore if LMP2A is tyrosine-phosphorylated in T cells, associates with T cell tyrosine kinases, and affects TCR signaling, we expressed wild type (WT) LMP2A containing a quadruple FLAG tag at the amino terminus (4× FLAG-LMP2A) in Jurkat TAg cells (Fig. 2). In addition, we constructed mutants in the 4× FLAG background that lack the putative binding sites for Src family tyrosine kinases (Y112F; SFK−), the tandem Src homology 2 domains of the Syk/ZAP-70 tyrosine kinases (Y74/85F; ITAM−), or the WW domains of NEDD4 family E3 ubiquitin ligases (Y60/101A; PY−) (see Fig. 1). To first assess the relative expression level of the different 4× FLAG-tagged LMP2A proteins, expression vectors encoding WT or mutant LMP2A were electroporated into Jurkat TAg cells, and expression was assessed 24 h post-transfection (Fig. 2A, upper panel). The expression level of the PY− mutant with substitutions in both PPPPY motifs was consistently several-fold higher than either WT LMP2A or any of the other mutant proteins (Fig. 2A, upper panel). Titering the amount of transfected cDNA for the WT and PY− mutant showed that the mutation was expressed at 5–10 times the level of the WT protein (Fig. 2B, upper panel). Furthermore, incorporating substitutions in either the SFK (Y112F) binding site or the ITAM (Y74/85F) motif into the PY− mutant background (4× FLAG-PY−/SFK− LMP2A and 4× FLAG-PY−/ITAM− LMP2A, respectively) significantly increased the expression levels of both the SFK− and ITAM− mutants (Fig. 2C, upper panel). To determine whether the increased level of the PY− mutant was due to stabilization of the LMP2A protein, we treated Jurkat TAg cells with cycloheximide and compared the stability of the WT and PY− mutant proteins. The WT protein showed a significant decrease in expression over time in the cycloheximide-treated samples that was attenuated with the PY− mutant (Fig. 2D). This was especially evident at the 12-h time point. Taken together, these data suggest a role for the PY motifs in controlling the stability of LMP2A in Jurkat TAg cells.

LMP2A Associates with the NEDD4 Family Protein, AIP4, and Is Ubiquitylated in Jurkat TAg Cells—Since mutation of the PY motifs within LMP2A has previously been shown to abolish interaction with the NEDD4 family of E3 ubiquitin ligases in B cells (18, 20), the increased abundance and stability of this mutant protein in Jurkat TAg T cells may reflect an inability to bind NEDD4 family proteins and consequently a defect in ubiquitylation and degradation. We therefore tested whether WT LMP2A associates with the NEDD4 family member AIP4 in Jurkat TAg cells. Fig. 3A shows that endogenous AIP4 co-purifies with WT LMP2A (4× FLAG-LMP2A) and with the putative Src family kinase (4× FLAG-SFK− LMP2A) and ITAM (4× FLAG-ITAM− LMP2A) mutants, but not with the PY− mutant (4× FLAG-PY− LMP2A) in Jurkat TAg cells. Thus, LMP2A associates with NEDD4 family proteins through its PY motifs in Jurkat T cells, and the inability to bind this family of proteins correlates with an increase in LMP2A stability. We therefore next tested whether LMP2A was ubiquitylated in Jurkat T cells. Co-transfection of Jurkat TAg cells with 4× FLAG-tagged LMP2A and an HA-tagged ubiquitin construct showed that the HA-tagged ubiquitin was incorporated into LMP2A, with species probably corresponding to mono-, di-, and tri-ubiquitylated LMP2A predominating (Fig. 3B, upper panel; indicated with arrows). Co-transfection of a Myc-tagged AIP4 construct enhanced LMP2A ubiquitylation, whereas co-transfecting a Myc-tagged mutant AIP4 protein that had been rendered enzymatically inactive by mutation of the catalytic cysteine (Myc-C380A AIP4) did not augment (and in most experiments slightly inhibited) the ubiquitylation of LMP2A in Jurkat TAg cells. Since we could readily identify ubiquitylation of LMP2A in Jurkat TAg cells, we tested whether the PY− LMP2A mutant was detectably ubiquitylated. cDNAs encoding the 4× FLAG-LMP2A or 4× FLAG-PY− LMP2A were co-transfected with the HA-tagged ubiquitin into Jurkat TAg cells. To equalize for protein expression, one-fifth the amount of the cDNA was transfected for the PY− mutant. The results show that in contrast to the WT protein, the PY− LMP2A mutant that lacks binding sites for the WW domains of AIP4 was not appreciably ubiquitylated in these cells (Fig. 3C, upper panel). Therefore, LMP2A is ubiquitylated by NEDD4 family E3 protein-ubiquitin ligases in Jurkat TAg cells, and the lack of ubiquitylation of the PY− mutant probably explains the increased stability of this protein.
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LMP2A Is Tyrosine-phosphorylated in Jurkat TAg Cells, Which Requires T Cell Src Family Tyrosine Kinases—In B cells, LMP2A is highly tyrosine-phosphorylated (6). To determine whether this was also apparent in T cells, we transfected into the Jurkat TAg cell line either the 4× FLAG-LMP2A or PY− mutant and looked at the tyrosine phosphorylation of LMP2A. Again, less DNA was transfected for the PY− mutant to normalize for the increased stability of this mutant. As seen in Fig. 4A (upper panel), LMP2A is tyrosine-phosphorylated in Jurkat TAg cells. Interestingly, the PY− mutant was substantially hyperphosphorylated relative to the WT protein despite a similar level of protein expression (Fig. 4A, upper and lower panels, respectively). No additional tyrosine-phosphorylated proteins were seen in the immunoprecipitates of either the WT or PY− mutant LMP2A proteins. To determine whether mutation of the putative Src family kinase-binding site and ITAM motif affected the phosphorylation of LMP2A, we examined the phosphorylation of these mutants in the context of the PY− background (due to the increased phosphotyrosine signal of the PY− mutant). When compared with the 4× FLAG-PY− LMP2A protein, the 4× FLAG-PY+/SFK− LMP2A double mutant was severely reduced in phosphorylation, whereas the PY−/ITAM− mutant retained a significant level of phosphorylation (Fig. 4B, upper panel). Since LMP2A is tyrosine-phosphorylated in Jurkat TAg cells, we next explored which tyrosine kinases in T cells might be responsible for phosphorylating LMP2A. In B cells, LMP2A associates with both the Src family tyrosine kinase Lyn and the Syk tyrosine kinase (12–14, 16, 17). Therefore, we tested whether the analogous T cell kinases, the Src family tyrosine kinases Lck and Fyn on the one hand and Syk and the related kinase ZAP-70 on the other, were required for phosphorylation of LMP2A in T cells. To do this, we made use of variants of the Jurkat cell line, including P116 cells, which are deficient in both Syk and ZAP-70 expression (37) (Fig. 4C, lower panel) and JCAM-1 cells, which lack Lck and are reduced in Fyn expression (38) (Fig. 4C, lower panel). Whereas the level of tyrosine phosphorylation of the 4× FLAG PY− LMP2A in the P116 cells was indistinguishable from the parental cell line, tyrosine phosphorylation of the 4× FLAG-PY− LMP2A was virtually absent in JCAM-1 cells (Fig. 4C, upper panel). The importance of Lck for the phosphorylation of LMP2A was further investigated by looking at LMP2A phosphorylation in JCAM-1 cells that had been reconstituted with Lck. Fig. 4D (upper panel) shows that reconstitution of JCAM-1 cells with Lck was able to restore LMP2A tyrosine phosphorylation to levels seen in the parental Jurkat line. Thus, Src family kinases are required for phosphorylation of LMP2A in Jurkat T cells; we therefore examined whether Lck and other tyrosine kinases in T cells could associate with LMP2A.

LMP2A Associates with Several Tyrosine Kinases in Jurkat TAg Cells—Since Lck is important for LMP2A tyrosine phosphorylation in Jurkat cells, we first examined whether Lck associates with LMP2A in these cells. Fig. 5A (upper panel) shows that 4× FLAG-LMP2A co-immunoprecipitates with Lck in anti-Lck immunoprecipitates. Furthermore, the PY− LMP2A mutant was greatly enhanced in its ability to co-immunoprecipitate with Lck in immunoprecipitates of Jurkat TAg cell lysates (Fig. 5A). To assess which motifs in LMP2A are required for its binding to Lck, we transfected the PY−/SFK− and PY−/ITAM− mutants into Jurkat TAg cells and looked at the ability of these mutants to precipitate Lck from Jurkat TAg cells as compared with the PY− mutant. As expected, the PY−/SFK− mutant was unable to form a stable complex with Lck, whereas the PY−/ITAM− mutant co-precipitated with Lck to levels comparable with the PY− mutant (Fig. 5B, upper panel). Similar results were also obtained for the additional T cell Src family tyrosine kinase, Fyn (Fig. 5B, lower panels).
We also inspected the association of ZAP-70 with LMP2A in T cells. To do this, we used a Myc-tagged ZAP-70 construct due to high levels of nonspecific binding seen with the antibody used to precipitate the endogenous ZAP-70 protein. Co-expressing a Myc-tagged ZAP-70 construct with the 4X FLAG-tagged LMP2A or PY mutant showed that ZAP-70 and LMP2A co-precipitate in Jurkat TAg cells, with slightly increased ZAP-70 binding seen with the PY mutant (Fig. 5C, upper panel). Experiments performed to examine the requirements for ZAP-70 binding to LMP2A showed that mutation of either the Src kinase-binding site or the ITAM motif inhibited association with the Myc-tagged ZAP-70 (Fig. 5D, upper panel). To further characterize the association of ZAP-70 with LMP2A, we found that a biotinylated peptide of the doubly phosphorylated ITAM peptide (pITAM), but not the corresponding dephosphorylated peptide (ITAM), selectively precipitated ZAP-70 (Fig. 5E, upper panel) and Syk (Fig. 5E, lower panel) from Jurkat lysate. Thus, LMP2A recruits T cell tyrosine kinases in a manner similar to that seen in B cells.

**LMP2A Interferes with TCR Signaling in Jurkat T Cells—**Since recruitment of the Lyn and Syk tyrosine kinases to LMP2A in B cells is important for the LMP2A-mediated inhibition of BCR signaling, we investigated whether expression of LMP2A in T cells similarly inhibited TCR signaling. To this end, we generated Jurkat T cell lines that stably expressed LMP2A (clones 3 and 5). Fig. 6A shows that LMP2A is expressed and tyrosine-phosphorylated in these cell lines. Examination of TCR (CD3) cell surface expression by FACS analysis showed that there was a decrease in TCR surface expression in the LMP2A-expressing cells as compared with the parental line (Fig. 6B). Stimulation of the LMP2A-expressing cell lines and the parental cell line with antibodies directed against the CD3 portion of the TCR showed that TCR-mediated induction of tyrosine-phosphorylated proteins was slightly attenuated in the LMP2A-expressing cells relative to the parental cell line (Fig. 6C). Using antibodies that recognize the phosphorylated, active forms of ZAP-70 and the Erk1 and -2 serine/threonine kinases (Fig. 6D) showed that TCR-mediated activation of these kinases was reduced in LMP2A-expressing cells relative to the parental cell line. The effect of LMP2A on TCR signaling was further examined by investigating the expression of the early T cell activation marker, CD69. CD69 expression was reduced in both the unstimulated and TCR-stimulated LMP2A-expressing cell lines relative to the parental control (Fig. 6E). Thus, expression of LMP2A in Jurkat T cells attenuates TCR signaling.
also observed that the basal levels of cellular tyrosine phosphorylation and ZAP-70 and Erk1 and -2 activation were reduced in the LMP2A-expressing cells relative to the parental line (Fig. 6, C and D).

In order for T cells to become activated, they require additional signals from antigen presenting cells including those generated through CD28 (reviewed in Ref. 39). Since CD28 has been shown to recruit the Fyn tyrosine kinase (40–42), we examined whether CD28 and TCR co-stimulation was affected in LMP2A-expressing Jurkat T cells. Fig. 7A shows that the levels of CD28 were similar in both the LMP2A-expressing cells and the parental cell line. The co-ligation of CD3 with CD28 (reviewed in Ref. 39) overexpressed in EBV-positive T cell lymphomas. Although the virus carries genes involved in cell cycle control and apoptosis, it is largely unknown how EBV contributes to the pathogenesis of these cancers. Therefore, we have examined the role of the EBV protein LMP2A in T cells in order to understand the contribution of LMP2A to EBV-positive T cell lymphomagenesis.
Our data show that LMP2A expressed in T cells recruits a number of cytoplasmic proteins normally involved in TCR signaling in a similar fashion to LMP2A in B cells. The use of mutant forms of LMP2A and peptides representing LMP2A interaction motifs showed that the Src family tyrosine kinases Lck and Fyn are specifically recruited to the phosphorylated Tyr112 site of LMP2A (Fig. 5). Phosphorylation of Tyr<sup>112</sup> seems critical for LMP2A to recruit T cell tyrosine kinases, since mutation of this residue to phenylalanine abolished phosphorylation of LMP2A on tyrosine (Fig. 4B) and the subsequent recruitment of Lck, Fyn, and ZAP-70 (Fig. 5). Tyr<sup>112</sup> is probably the most prominent tyrosine phosphorylation site on LMP2A in T cells, since mutation of the Tyr<sup>112</sup> site abolished LMP2A phosphorylation, whereas mutation of the two tyrosines in the ITAM motif had little effect on the overall level of LMP2A tyrosine phosphorylation (Fig. 4B). Despite the prominence of Tyr<sup>112</sup> phosphorylation, our data indicate that LMP2A recruits ZAP-70, which, based on the analysis of peptide pull-downs and LMP2A mutants, requires phosphorylation of the ITAM motif (Fig. 5).

The importance of the Src family tyrosine kinases for phosphorylation of LMP2A in Jurkat T cells was further demonstrated by the lack of LMP2A tyrosine phosphorylation in the Lck-deficient JCAM-1 cells, which also have reduced levels of Fyn (Fig. 5C). Since some Fyn is expressed in JCAM-1 cells, it is possible that Lck is a more active kinase for LMP2A in vivo than Fyn. Alternatively, a threshold level of total Src family kinase activity may be necessary for phosphorylation of LMP2A in Jurkat T cells. The fact that tyrosine phosphorylation of LMP2A was not reduced in the ZAP-70/Syk-deficient P116 cells shows that although these kinases are recruited to LMP2A, they play no significant role in the phosphorylation of LMP2A itself.

Our results also show that LMP2A associates with the NEDD4 family E3 ubiquitin ligase AIP4 and that LMP2A is polyubiquitylated in Jurkat T cells. Furthermore, mutation of the two PY motifs abolished binding of the NEDD4 family of proteins (Fig. 3A) and led to a strong stabilization of LMP2A (Fig. 2). Mutation of the two PY motifs has previously been shown to increase the stability of LMP2A in lymphoblastoid cell lines (20). However, in that study, mutation of the two PY motifs showed an approximately 2-fold increase in the level of the PY<sup>−</sup> mutant as compared with the WT protein, which is significantly lower than the 5–10-fold increase that we observed (Fig. 2B). This difference could be
due to a difference in the intrinsic stability of LMP2A in lymphoblastoid cell line B cells and Jurkat TAg cells, or alternatively, it might be due to the nature of the constructs used in each study. Whereas Ikeda et al. (20) used untagged LMP2A, we used a 4× FLAG-tagged LMP2A. Unlike most proteins that are ubiquitylated on lysine residues, LMP2A has been proposed to be ubiquitylated at the amino terminus, since a mutant LMP2A protein lacking all 3 lysine residues was still efficiently ubiquitylated (43). Of the 3 lysine residues in LMP2A, none are in the amino-terminal tail, and only 2 are predicted to be intracellular. The addition of the 4× FLAG epitope to the amino terminus adds an additional 5 lysine residues, which may be more readily ubiquitylated than the amino terminus and thus lead to the decreased stability seen for any of the 4× FLAG-tagged with intact PY motifs (see Fig. 2).

NEDD4 family proteins not only regulate the stability of LMP2A but also modulate the tyrosine phosphorylation of LMP2A and the recruitment of T cell kinases. When comparing equal levels of protein, the PY− mutant was substantially hyperphosphorylated and precipitated much more Lck and slightly more ZAP-70 than the WT protein (Fig. 4 and 5, respectively). The simplest interpretation of these results is that the NEDD4 family proteins also target LMP2A-associated kinases and that the failure of the PY− mutant to down-regulate these kinases, particularly Lck, leads to increased Lck and ZAP-70 association and consequently increased phosphorylation of LMP2A.

The properties of the LMP2A PY− mutant revealed by these experiments have important implications for the role of NEDD4 family proteins in LMP2A function. For example, in B cells, LMP2A appears to inhibit BCR signaling to maintain viral latency and at the same time inhibit BCR signaling to drive B cell development and act as a survival signal for mature B cells. As illustrated in Fig. 8, our data suggest that positive tyrosine kinase signaling induced by LMP2A is effectively titrated through the recruitment of NEDD4 family E3 ubiquitin ligases to the LMP2A PY motifs. This may ensure that downstream signals generated by LMP2A are not strong enough to cause viral reactivation but at the same time are sufficient to provide a weaker survival signal, akin to the antigen-independent signal generated by surface Ig that is required for the survival of mature B cells (22, 23). This latter function has been
suggested to be important in EBV-positive Hodgkin B cell lymphomas that express LMP2A but have lost expression of surface Ig (22). TCR expression (44) and the Lck and Fyn tyrosine kinases (45) are required to maintain peripheral naive T cells, raising the possibility that LMP2A-mediated signals could provide a survival signal for EBV-positive T cell tumors. Our experiments indicate that when stably expressed in Jurkat T cells, LMP2A does abrogate TCR signaling (see Figs. 6 and 7). However, since TCR levels were decreased in the LMP2A-expressing cells, we are unable to distinguish whether the observed decrease in TCR signaling is due to lower levels of TCR, to the sequestering of tyrosine kinases by LMP2A, or to both of these events. A reduction in BCR surface expression has been observed in LMP2A-expressing BJAB cells (46) but not in LMP2A-expressing RAMOS B cells (47). We also found that surface expression of the TCR was down-regulated in Jurkat TAg cells transiently expressing LMP2A (data not shown). We are currently investigating how LMP2A inhibits the surface expression of the TCR.

FIGURE 7. LMP2A limits signaling through CD3 and CD28. A, Jurkat cells stably expressing LMP2A (clones 3 and 5) or the parental cell line were stained with a PE-conjugated anti-CD28 mAb, as described under “Experimental Procedures,” and cell surface expression of CD28 was assessed by FACS. The parental line is indicated in black, clone 3 is shown in green, clone 5 is shown in red, and the PE-conjugated, isotype control Ab is shown in blue. B, the cell lines indicated in A were either left unstimulated (−), stimulated with anti-CD3 (CD3) or anti-CD28 (CD28) mAbs alone, or stimulated with both anti-CD3 and anti-CD28 (CD3 + CD28) together as described under “Experimental Procedures.” Cell lysates were prepared and analyzed by anti-Tyr(P) immunoblotting (PTyr; upper panel), whereas LMP2A expression was examined by blotting lysates with the anti-LMP2A mAb (middle panel), and tubulin expression was examined to indicate equal protein loading (lower panel). The arrow indicates the LMP2A protein. C, lysates prepared in B were analyzed for Erk activation using phosphospecific Abs directed against the phosphorylated, activated form of Erk1 and -2 (upper panel). The lower panel was reprobed with the anti-Erk Ab. Molecular mass standards (in kDa) are indicated to the left of the anti-Tyr(P) blot.

FIGURE 8. LMP2A recruits kinases involved in TCR signaling. In T cells, LMP2A recruits Lck (Fyn) and ZAP-70 (Syk) to its tyrosine-phosphorylated Tyr112 and ITAM motifs, respectively. Recruitment of NEDD4 proteins to the two PPPPY motifs leads to ubiquitylation (Ub) of LMP2A and perhaps LMP2A-associated tyrosine kinases, thereby regulating the stability of LMP2A-kinase complexes. This effectively limits the degree of signaling through LMP2A and may be important for providing a weak, tonic signal for the survival of EBV-positive T cell tumors.
LMP2A in T Cells

The recruitment of T cell tyrosine kinases to LMP2A suggests that LMP2A itself has a signaling function in T cells. However, we were unable to see LMP2A-mediated activation of downstream signaling events in Jurkat T cells transiently expressing LMP2A (Fig. 4 and data not shown) or stably expressing LMP2A (Fig. 6). In fact, tyrosine phosphorylation of cellular proteins and activation of ZAP-70 and Erk were reduced in the LMP2A-expressing stable cell lines compared with the parental cell line. It remains possible that LMP2A-induced signals in Jurkat T cells are weak and thus hard to observe or that LMP2A has more pronounced effects in other T cell types. Regardless, our data clearly show that LMP2A recruits key signaling proteins in T cells.

The in vivo role that LMP2A and, for that matter, EBV plays in T cells is still unclear. It is unlikely that T cells represent an additional reservoir to look at the function of LMP2A in T cells in the context of EBV-positive T cell lymphomas and their precursors.

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