Supplementary Materials and Methods

**Plasmid construction**

A series of truncated LMP1, which were generated by PCR using pT7E (Chen et al., 1992) as a template, were cloned into the prey vectors pGAD10 at the EcoRI site or into the bait vector pGBT9 (BD Biosciences, San Jose, CA, USA) at the EcoRI/BamHI sites. The N-terminally FLAG- and GFP-tagged LMP1 as well as its truncated derivatives were generated by ligation of PCR-amplified DNA fragments to HindIII/BamHI-treated pCMV2-FLAG (Kodak) and pEGFP-C3 (BD Biosciences), respectively. The LMP1 export mutants, LMP1\textsubscript{213A215A} and LMP1\textsubscript{225A226A}, were generated by PCR-based site-directed mutagenesis, in which both the residues 213/215 and 225/226 were mutated to Alanine, respectively. The C-terminally FLAG-tagged LMP1, LMP1\textsubscript{187-381}, and the following chimeric LMP1 were generated by insertion of PCR products fused to FLAG epitope into HindIII/XhoI-treated pcDNA3 (BD Biosciences). \textit{B1LMP1\textsubscript{CT}}, \textit{B19LMP1\textsubscript{CT}}, \textit{PALMP1\textsubscript{CT}}, \textit{PAB1LMP1\textsubscript{CT}}, and \textit{PAB19LMP1\textsubscript{CT}} were generated by replacing the N-terminus and membrane-spanning domain of LMP1 (aa 1-186) with the inserts encoding aa 1-161 (B1) or aa 19-161 (B19) of BHRF1 derived from EBV infected B95.8 cells, the sequence for palmitoylation (PA) from GAP-43 (MLCCMRRTKQV), and a combination of the PA sequence with BHRF1 aa 1-161 (PAB1) or aa 19-161 (PAB19), respectively.

The full-length and truncated PRA1s were generated by PCR using human PRA1 cDNA (a kind gift from Dr. C Bruni, University of Napoli, Italy) as a template and sequentially inserted into the prey vectors pGAD10 at the EcoR1 site or into the bait vector pGBT9 (BD Biosciences) at the EcoRI/BamHI sites. To construct GST- and HA-tagged PRA1 and its truncated derivatives, PCR-amplified DNA fragments were
ligated to BamHI/EcoRI-treated pGEX 4T.1 (BD Biosciences) and BamHI/XhoI-treated pcDNA3-HA (provided by Dr. JY Chen, National Taiwan University, Taiwan), respectively. The PRA1 export mutant, PRA1AA, was generated by PCR-based site-directed mutagenesis, in which both the residues 178 and 179 were mutated to Alanine.

For BRET2 assay, the N-terminally Rluc- and GFP2-tagged LMP1 or PRA1 were generated by ligation of PCR-amplified DNA fragments to KpnI/BamHI-treated pRluc(h)-C2 and pGFP2-C1 vectors (PerkinElmer Life and Analytical Sciences, MA, USA), respectively. For FRET experiments, the N-terminally CFP-tagged PRA1 and YFP-tagged LMP1 were generated by insertion of PCR products fused to CFP and YFP, respectively, into HindIII/XbaI-treated pcDNA3 (BD Biosciences).

**siRNA construction**

The RNAi procedure was done as described (Brummelkamp et al., 2002). In brief, a double-strand oligonucleotide was designed to contain a sequence derived from the 5’ end of human PRA1 ORF in forward and reverse orientation separated by a 9-base-pair spacer region (ttcaagaga) to allow formation of the hairpin structure in the expressed oligo-RNA. PRA1 siRNA 1 (nucleotides 319-337): the sense strand, 5’-gatccctGGTTACATTCTCTATCTGCGCttcaagagaGCGCAGATAGAGAATGTAACAtttttggaaa; the antisense strand, 5’-agcttttccaaaaaTGTTACATTCTCTATCTGCGCtctcttgaaGCGCAGATAGAGAATGTAACAggg. PRA1 siRNA 2 (nucleotides 343-361): the sense strand, 5’- gatccctTTGGAGTCCAAGCTTGTGCTCttcaagagaGAGCACAAGCTTGGAGCTCAtttttggaa; the antisense strand, 5’- agcttttccaaaaaTTGGAGTCCAAGCTTGTGCTCtctcttgaaGAGCACAAGCTTGGAGCTCAtttttggaaAagg.

The resulting double-stranded oligonucleotide was cloned into the BglII and HindIII sites of the pSUPER vector for expression under the control of the H1 RNA promoter.
A scrambled sequence was used as a control siRNA. Unless specified, PRA1 siRNA 1 was used in most knockdown experiments.

Yeast two-hybrid assay

Yeast two-hybrid screening was carried out as previously described (Vojtek & Hollenberg, 1995). Briefly, the bait plasmid, pBTM/LMP1, and a cDNA library of human nasopharyngeal carcinoma cells, in which the cDNA was fused to the GAL4-AD, were used sequentially to transform *Saccharomyces cerevisiae* strain Y190. The resulting transformants were then plated on synthetic complete (SC) medium lacking tryptophan (Trp), uracial (Ura), leucine (Leu), lysine (Lys), and histidine (His) (SC-Trp, Ura, Leu, Lys, His) and SC-Trp, Ura, Leu, Lys as a control. His\(^+\) colonies were screened for \(\beta\)-galactosidase activity 3 days later by colony-lift filter assays. His\(^+\)/LacZ\(^+\) colonies were candidate clones of cDNAs encoding LMP1-interacting proteins. After subsequent purification and curing processes, the cDNAs were isolated and sequenced. The resultant DNA sequences were then subjected to a BLAST search in the Gen-Bank\textsuperscript{TM} data base.

To verify the specificity of the LMP1-PRA1 interaction, Y190 cells were co-transfected with the prey vectors pGAD10-LMP1 and the bait pGBT9-PRA1 or in converse according to the manufacturer’s instructions (PT1113-1, BD Biosciences). Protein expression of individual clones was confirmed by Western blotting with specific antibodies. The Ura\(^+\) Trp\(^+\) Leu\(^+\) His\(^+\) transformants were further selected and confirmed via colony-lift filter \(\beta\)-galactosidase assays.

Antibodies and reagents

Polyclonal antibodies against human PRA1 were generated though the immunization of two rabbits injected four times over three months with 1 mg of synthesized peptides
corresponding to either N-terminal residues 1-16 (N16) or C-terminal residues 169-185 (C16; Kelowna International Scientific Inc., Taiwan) that had been mixed with Freund’s adjuvant (Sigma-Aldrich, MO, USA). The antiserum was purified with CNBr-activated Sepharose 4B (Amersham Biosciences, UK) conjugated with corresponding peptides as previously described (Yu et al., 1998). The anti-LMP1 monoclonal antibody (S12) was affinity purified from hybridoma and polyclonal antibody (1B) was generated and purified from antiserum as described above.

Monoclonal antibodies specific to FLAG (M2) and HA (12CA5) were purchased from Sigma, and mAbs to GM130, Vtila, GS15, GS27 and Annexin-II were purchased from BD Transduction Laboratories (BD Biosciences). Polyclonal antibodies against TRAF1, TRAF3, TRADD, calregulin and caveolin-1 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The anti-mannosidase II serum was kindly gifted by Dr. Marilyn G. Farquhar (University of California, San Diego, USA). Goat anti-mouse antibodies conjugated with FITC or TRITC, and goat anti-rabbit antibodies conjugated with rhodamine were purchased from BD Transduction Laboratories. Cycloheximide (CHX), nocodazole (Noco), brefeldin A (BFA), cytochalasin B (Cyto B), monensin sodium (Mon), and N-ethylmaleimide (NEM) were purchased from Sigma-Aldrich and prepared in accordance with the manufacturer’s recommendation.

**In vitro transcription/translation**

The full-length cDNA of LMP1 were constructed and cloned into pcDNA3.1 for in vitro transcription/translation system. The [35S]methionine-labeled proteins were generated with the TNT coupled reticulocyte lysate system (Promega, Madison, WI, USA) by following the manufacturer’s recommendation. Briefly, the reaction was performed in a total of 50 µl reaction mixture containing 40 µl of TNT Quick Master
Mix, 1 µg of pcDNA3.1-LMP1, 2 µl of \(^{[35]}\)S)methionine (1000 Ci/mmol, Amersham Biosciences) at 10 mCi/ml, and 6 µl of nuclease-free water, followed by incubated the reaction at 30°C for 90 min. The \(^{[35]}\)S)methionine-labeled protein was subjected to the GST-pull down assay.

**GST-pull down and precipitation assays**

Cells exhibiting FLAG-LMP1 or/and HA-PRA1 were extracted in a NP40 lysis buffer (1% NP40, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na\(_3\)VO\(_4\), 5 mM EDTA pH 8.0, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) and fractionated by centrifugation (12000 rpm) to obtain cell lysate. For GST pulldown, glutathione-Sepharose beads (Amersham Biosciences) complexed with GST or GST-PRA1 were incubated with a portion of lysate for 2 h at 4°C and washed 5 times with the binding buffer (0.05% NP40, 20 mM Tris-HCl pH 7.5, 125 mM NaCl, 0.1% β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride). For precipitations, 500-1000 µg of cell lysate were incubated with the affinity matrix specific to FLAG (anti-FLAG® M2 affinity gel, Sigma-Aldrich) or HA (anti-HA affinity matrix, Roche, Germany) at 4°C overnight, and washed extensively with the NP40 lysis buffers. The resulting products were eluted with 2X SDS sample buffer and analyzed by Western blotting.

**BRET\(^2\) assay**

HEK293 cells grown in a 10 cm-dish were transfected with the plasmids encoding donor Renilla Luciferase (Rluc)- and acceptor GFP\(^2\)-tagged fusion proteins (1 µg and 3 µg, respectively). For competitive inhibition, Rluc-tagged LMP1 and PRA1 were co-expressed with 2 µg of the plasmids for FLAG-tagged LMP1 and HA-tagged
PRA1, respectively. Cells were detached 24 h after transfection and washed with Dulbecco’s Phosphate Buffered Saline (D-PBS; Invitrogen, CA, USA) and then resuspended in D-PBS to a final concentration of approximately $2 \times 10^6$ cells/ml. Approximately $1 \times 10^5$ cells per well were distributed in a 96-well white polystyrene microplate (Conig, NY, USA). The DeepBlueC coelenterazine substrate (PerkinElmer Life and Analytical Sciences) was added to a final concentration of 5 µM and bioluminescence emissions were monitored immediately by using the Fluoroskan Ascent FL (Thermo Electron Corporation, MA, USA) that allows the sequential integration of the signals detected in the 410-nm and 515-nm windows. The BRET$^2$ ratio is calculated as (emission at 515 nm of transfected cells-emission at 515 nm of non-transfected cells)/(emission at 410 nm of transfected cells-emission at 410 nm of non-transfected cells). Expression level of each fusion protein was analysed by Western blotting with appropriate antibodies.

**Immunofluorescence and FRET experiments**

COS7 or NPC117 cells grown on polylysine-coated coverslides were fixed with 4% formaldehyde, permeabilized and blocked with 0.1% saponin containing 1% BSA for 20 min. The coverslides were incubated with the indicated primary antibodies for 2 h, followed by incubation with the appropriate fluorescence-conjugated secondary antibodies for 45 min at room temperature, and mounting with the VECTASHEILD reagent (Vector Laboratories Inc., CA, USA). Confocal microscopy was performed with a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems Inc., Germany) with a 63×1.32 NA oil immersion objective. For each assay, 80-100 cells were scored according to whether the expressed proteins were highly restricted in the locations of interest. Results (%) were expressed as ratios of number of cells exhibiting these localizations versus the total number of LMP1-expressing cells. For
FRET, COS7 cells expressing CFP- and YFP-tagged proteins were maintained at room temperature throughout the experiments. The images were acquired on an inverted Zeiss Axiovert 200 microscope (Carl Zeiss Light Microscopy, Germany) equipped with CFP-YFP FRET filter sets (Chroma) and CoolSNAP HQ Microscope CCD camera, using a FLUAR 100×/1.3 NA oil-immersion immersion objective lens. Samples were excited by LAMBDA DG-4 laser (Sutter Instrument Company, CA, USA; 470±12.5 nm for CFP and 500±10 nm for YFP). Exposure time was 150 ms and always identical for YFP, CFP an FRET channels within a given sample. Image processing was performed with Stallion (Intelligent Imaging Innovations, Inc.). The pixel alignment of the CFP and FRET images was verified and adjusted, the background taken outside the cells was subtracted and FRET/CFP ratio was computed. Composite figures were prepared using Canvas 9.0 software (Deneba).

**Subcellular fractionation**

Approximately 80% confluent LMP1-Tet-on 293 cells (15-cm dishes) were first transfected with pSUPER-based vectors encoding control or PRA1 siRNA. At 48 h post-transfection, LMP1 expression was induced by incubation with 15 µg of doxycycline for 12 h. The cells were then washed twice in phosphate-buffered saline and scraped into Bud buffer (38 mM potassium aspartate, 38 mM potassium glutamate, 38 mM potassium gluconate, 20 mM potassium MOPS, pH 7.2, 5 mM sodium carbonate, 2.5 mM magnesium sulfate, 2 mM EGTA, 5 mM reduced glutathione, adjusted to pH 7.2 with KOH) with freshly added protease inhibitors. A cytoplasmic extract was prepared by homogenization with 10 strokes in a cell cracker (EMBL, Heidelberg, Germany) and then centrifuged at 800 g for 10 min. The resultant supernatant was layered onto a continuous sucrose gradient (10-45% sucrose in MOPS buffer containing 20 mM EGTA) and centrifuged at 55000 rpm in a SW55
rotor (Beckman, Fullerton, CA, USA) for 1 h. The fractions were collected manually from the bottom of the gradient using a Brandell Tube Piercer (Brandell, Gaithersburg, MD, USA), and a portion of each fraction was subjected to Western blot analysis.

**Protein transport from ER to Golgi**

The cells were first transfected with pSUPER-PRA1 siRNA or the control vector, incubated for 48 h, and then transfected with the plasmid for VSVG<sup>ts045</sup>-GFP, which was kindly donated by Dr. J Lippincott-Schwartz (National Institutes of Health, USA). The cells were incubated at 42°C for 18 h and then shifted to 37°C to allow VSVG transport. The cells were fixed and processed for immunofluorescence analysis. In the case of the biochemical transport assay, the cells were harvested after the temperature shift, lysed in denaturing buffer (0.15 ml/35mm dish) containing 0.5% SDS and 1% β-mercaptoethanol and heated at 100°C for 10 min. A portion of the lysate was digested with Endo H (NEW ENGLAND BioLabs, UK) and then subjected to SDS–PAGE on an 8% gel followed by Western blotting.

**References**


kinase family 1. *Biochem J* **334** (Pt 1): 121-131