PRA1 promotes the intracellular trafficking and NF- κ B signaling of EBV latent membrane protein 1

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Latent membrane protein 1 (LMP1), which is an Epstein–Barr virus (EBV)-encoded oncoprotein, induces nuclear factor-kappa B (NF- κ B) signaling by mimicking the tumor necrosis factor receptor (TNFR). LMP1 signals primarily from intracellular compartments in a ligand-independent manner. Here, we identify a new LMP1-interacting molecule, prenylated Rab acceptor 1 (PRA1), which interacts with LMP1 for the first time through LMP1's transmembrane domain, and show that PRA1 is involved in intracellular LMP1 trafficking and LMP1-induced NF- κ B activity. Immunofluorescence and biochemical analyses revealed that LMP1 physically interacted with PRA1 at the Golgi apparatus, and the colocalization of LMP1 and PRA1 to the Golgi was sensitive to nocodazole and brefeldin A. Coexpression of a PRA1 export mutant or knockdown of PRA1 led to redistribution of LMP1 and its associated signaling molecules to the endoplasmic reticulum and subsequent impairment of LMP1-induced NF- κ B activation, but had no effect on CD40- and TNFR1-mediated signaling or the functional integrity of the Golgi apparatus. These novel findings provide important new insights into LMP1, and identify an unexpected new role for PRA1 in cellular signaling.

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Introduction

The ubiquitous human γ -herpesvirus, Epstein–Barr virus (EBV), is closely associated with many human malignancies, including nasopharyngeal carcinoma (NPC), Burkitt's lymphoma, T-cell lymphoma, gastric carcinoma, and invasive breast cancer. One of the EBV gene products, a 62-kDa integral membrane protein called latent membrane protein

1 (LMP1), is expressed in 70% of NPC. LMP1 can transform rodent cells and gives a higher invasive capacity to cells grown in soft agar, primarily by mimicking tumor necrosis factor receptor (TNFR) family members, such as TNFR1 and CD40 (Kilger *et al*, 1998; Uchida *et al*, 1999). Through its C-terminal cytoplasmic tail, LMP1 binds TNFR-associated factors (TRAFs) and TNFR1-associated death domain protein (TRADD) (Sandberg *et al*, 1997) to activate several signaling pathways, including the well-known nuclear factor-kappa B (NF- κ B)-mediated transcription pathway (Gires *et al*, 1997; Eliopoulos *et al*, 2002). Unlike TNFR-based signaling, however, LMP1 signaling appears to occur in a ligand-independent fashion (Gires *et al*, 1997; Eliopoulos and Rickinson, 1998). LMP1 acts as a constitutively activated receptor-like molecule; a portion of LMP1 associates with lipid rafts at a steady state, where it interacts readily with TRAFs and recruits them to the lipid rafts (Ardila-Osorio *et al*, 1999; Higuchi *et al*, 2001; Kaykas *et al*, 2001). The N-terminus and transmembrane domain of LMP1 mediate this association with lipid rafts (Higuchi *et al*, 2001; Coffin *et al*, 2003), and further mediate the aggregation of LMP1 molecules and the assembly of a signaling complex (Gires *et al*, 1997), all of which are required for efficient LMP1 signaling (Kaykas *et al*, 2001). Notably, a major portion of LMP1 in B lymphoid cells colocalizes with Golgi protein GS15 at steady state (Flanagan *et al*, 2003); more strikingly, LMP1 in various types of cells precisely colocalizes with cholera toxin (a lipid raft marker) at the perinuclear region and actively recruits TRAFs to the site, demonstrating that LMP1 is able to signal from intracellular sites containing lipid rafts (Lam and Sugden, 2003).

Here, we sought to further elucidate the mechanism underlying ligand-independent LMP1 signaling by exploring the cellular factors mediating the intracellular trafficking of LMP1. Through a yeast two-hybrid screen, we identified the human prenylated Rab acceptor 1 (PRA1) as a new interacting partner of LMP1. PRA1, a 21-kDa protein conserved from yeast to human (Bucci *et al*, 1999), contains two extensive hydrophobic domains and is predicted to be an integral membrane protein (Lin *et al*, 2001). PRA1 predominantly localizes at the Golgi and late endosomes (Abdul-Ghani *et al*, 2001; Figueroa *et al*, 2001; Sivars *et al*, 2003). Previous studies have shown that PRA1 interacts with multiple prenylated Rabs in the yeast two-hybrid system (Martincic *et al*, 1997; Bucci *et al*, 1999), associates with VAMP2 and GDP dissociation inhibitor 1 (GDI1) (Martincic *et al*, 1997; Hutt *et al*, 2000), and acts catalytically to dissociate endosomal Rabs from GDI-bound complexes (Sivars *et al*, 2003). These findings suggest that PRA1 may functionally link various vesicle trafficking proteins and participate in protein transport through the Golgi apparatus.

Here, we not only elucidate a new role for intracellular trafficking of LMP1 via NF- κ B signaling, but also reveal a novel mechanism underlying this process, namely the interaction of LMP1 with PRA1.

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Results

LMP1 is predominantly found at the Golgi apparatus

To explore the basis for the intracellular localization of LMP1, we first examined the spatial and temporal distribution of LMP1 transiently expressed as a GFP-fusion protein in COS7 and NPC cells. Protein expression was first detected 12 h after transfection. The intracellular distributions of GFP-LMP1 were classified with regard to four patterns on the basis of predominant colocalization with calregulin (endoplasmic reticulum, ER), GM130 (Golgi), both (ER/Golgi), or unclassified vesicular structures (vesicle). During transient expression, GFP-LMP1 was predominantly observed at the Golgi apparatus in NPC117 cells (Supplementary Figure 1A) and COS7 cells (data not shown). Furthermore, treatment of this experimental system with cycloheximide (CHX) apparently reduced the Golgi localization of GFP-LMP1, suggesting that the appearance of LMP1 at the Golgi apparatus requires protein biosynthesis (Supplementary Figure 1A).

Golgi localization of LMP1 is critical for LMP1-mediated NF- κ B signaling

As a major fraction of LMP1 is localized to the Golgi, we sought to elucidate a role for this intracellular localization in the function of LMP1. Previous studies have shown that NF- κ B signaling is essential for LMP1-associated cell transformation and tumorigenicity (Horikawa *et al*, 2000; Lo *et al*, 2004). We thus examined whether interruption of Golgi transport of LMP1 affected LMP1-induced nuclear translocation of active p65, a NF- κ B molecule. Without treatments, nuclear translocation of p65 was seen in most GFP-LMP1-expressing COS7 cells (Supplementary Figure 1B). Treatment of LMP1-expressing cells with brefeldin A (BFA), monensin, both of which are known to interrupt the Golgi transport (Machamer and Cresswell, 1984; Klausner *et al*, 1992), or a microtubule-depolymerizer nocodazole (Storrie *et al*, 1998) significantly perturbed the Golgi localization of LMP1 and inhibited LMP1-induced nuclear translocation of p65 (Supplementary Figure 1B and C). In contrast, treatment with an actin-depolymerizer cytochalasin B (Hirschberg *et al*, 1998) did not affect the Golgi localization of LMP1 and subsequent nuclear translocation of p65 in LMP1-expressing cells. Similar results were obtained from NF- κ B reporter assays in NPC cells (Supplementary Figure 1D). Collectively, these data suggest that the proper localization of LMP1 is critical for its efficient activation of NF- κ B signaling.

LMP1 interacts with its novel partner, PRA1, at the Golgi apparatus in living cells

To further elucidate the mechanism underlying the linkage between LMP1 trafficking and signaling, we sought to explore the cellular factors mediating the intracellular trafficking of LMP1. To identify intracellular proteins that interact with LMP1, we screened a complementary DNA library derived from human nasopharyngeal carcinoma using LMP1 as bait. One distinct clone encoding the main part of human PRA1 was isolated. The possible interaction between LMP1 and PRA1 was confirmed with a yeast two-hybrid assay (Supplementary Table 1). The intracellular localization of PRA1 was determined by coimmunostaining with two Golgi resident proteins, GM130 and GS27, which reveals

that PRA1 predominantly localizes at the Golgi apparatus (data not shown).

To assess the LMP1-PRA1 interaction inside cells, we then performed the fluorescence resonance energy transfer (FRET) microscopy, which is a protein-protein interaction assay based on energy transfer from a CFP donor to an YFP acceptor protein upon excitation. As shown in Figure 1A, FRET was significantly detected at the perinuclear region in COS7 cells coexpressing CFP-fused PRA1 and YFP-fused LMP1, but not observed in the case of coexpression of CFP-fused galactosyltransferase (CFP-GT, a Golgi-resident transmembrane protein) and YFP-LMP1. As CFP-PRA1 and CFP-GT

Figure 1 Analysis of LMP1-PRA1 interaction in live cells by FRET and BRET² assays. (A) FRET was measured in COS7 cells coexpressing the indicated CFP and YFP constructs as described in Materials and methods. The pseudocolor images depict FRET efficiency, calculated as FRET/CFP ratio, with the color indicating the relative value at each pixel. Bar, 10 μ m. (B) BRET² ratios were measured in HEK293 cells expressing the indicated constructs. Cells were detached at 24 h post-transfection and applied to the BRET² assay as described in Materials and methods. Expression of the GFP²-fused *Renilla* Luciferase (GFP²-Rluc) serves as a BRET²-positive control. The data represent the mean $\pm$ s.d. of three independent readings. To analyze the expression level of fusion proteins as indicated, a portion of cells was lysed and subjected to Western blot analysis (30 μ g each) with Ab specific to LMP1 (S12; upper panel) or PRA1 (C16; lower panel).

both colocalized with YFP-LMP1 (data not shown), the data reveal that the observed FRET should be achieved by the interaction between LMP1 and PRA1 rather than their coincidental colocalization to the Golgi or random association owing to hydrophobicity. To further evidence this, we then performed an advanced bioluminescence resonance energy transfer (BRET²) assay using live HEK293 cells. This technology uses *Renilla* luciferase (Rluc) as the donor and a GFP² as the acceptor molecule in an assay analogous to FRET, but without the need for the use of an excitation light source. As shown in Figure 1B, BRET was observed only in the cells coexpressing either Rluc-LMP1/GFP²-PRA1 or Rluc-PRA1/GFP²-LMP1, with the comparable extent to that of Rluc-PRA1/GFP²-PRA1 dimerization or the GFP²-Rluc positive control. Moreover, the expression of FLAG-LMP1 and HA-PRA1, in combination with Rluc-LMP1/GFP²-PRA1 and Rluc-PRA1/GFP²-LMP1, respectively, was able to decrease the BRET ratio resulted from LMP1-PRA1 interaction (owing to competition), providing the evidence for specificity. Expression level of each fusion protein was comparable as analyzed by Western blot. Collectively, the data strongly

suggest that LMP1 interacts with PRA1 at the Golgi under physical conditions.

Structural domains responsible for the interaction between LMP1 and PRA1

To verify the direct interaction between LMP1 and PRA1, we performed *in vitro* affinity chromatography assays using recombinant GST-PRA1 fusion proteins and *in vitro*-translated LMP1. Our results revealed that [³⁵S]-labeled LMP1 was precipitated by immobilized GST-PRA1 but not by GST alone, suggesting that LMP1 directly binds to PRA1 (Figure 2A). To determine the region within LMP1 responsible for its interaction with PRA1, we performed co-precipitation assays with various LMP1 deletion mutants (Figure 2B; Supplementary Figure 2A). The short N-terminus and the entire C-terminal tail of LMP1 were dispensable for its interaction with PRA1. However, deletion of the transmembrane segments 3–4 (ΔTM3/4) or through 3–6 (ΔTM3–6) of LMP1, or replacement of the entire transmembrane domain of LMP1 with that derived from CD40 (CD40-CT) or from GT (GT-CT), largely impaired the interaction of LMP1 with PRA1, revealing that this trans-

Figure 2 Domains responsible for LMP1–PRA1 interaction. **(A)** Purified GST and GST-PRA1 fusion proteins immobilized on glutathione-agarose were incubated with [³⁵S]methionine-labeled LMP1 separately. After washing, labeled proteins were eluted from the beads and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The amounts of GST fusion proteins were demonstrated by Coomassie blue staining. **(B)** Schematic representations of LMP1 and its mutants are shown with the short N-terminus (aa 1–20), the transmembrane-spanning domain (TM; aa 21–186), and the C-terminal region comprising two function domains termed CTAR1 and CTAR2. **(C)** Mapping of the PRA1-interacting domain of LMP1 using coprecipitation analysis. Cell extracts were harvested from HEK293 cells expressing FLAG-tagged LMP1 or its derived mutants. FLAG-tagged wide-type or mutant LMP1 proteins were precipitated with anti-FLAG affinity matrix and the immunocomplexes were analyzed by Western blotting with anti-PRA1 and anti-FLAG Abs. **(D)** Schematic representations of PRA1 and its mutants are shown with the N-terminal region (aa 1–78), the transmembrane domain (TM; aa 79–164), and the short C-terminus (aa 165–185). **(E)** Mapping of the LMP1-interacting domain of PRA1 using co-precipitation analysis. Cell extracts were harvested from HEK293 cells expressing FLAG-LMP1 in conjunction with HA-PRA1 or its mutants. FLAG-LMP1 proteins were precipitated as previously described and the immunocomplexes were analyzed by Western blotting with Abs specific to FLAG and HA. Molecular weight markers are indicated in kDa.

membrane region of LMP1 is responsible for its specific interaction with PRA1 (Figure 2C; Supplementary Figure 2B).

We next performed a deletion analysis on PRA1 to define the domain required for its interaction with LMP1 (Figure 2D). We found that deletion of the first 30–54 amino acids within the N-terminus of PRA1 largely impaired its interaction with LMP1 (Figure 2E), although the PRA1 transmembrane domain (amino acid (aa) 70–164) could render a minimal interaction. The interaction was hardly detected once the proximal transmembrane region (aa 70–120) of PRA1 was deleted. These data indicate that the transmembrane domain of PRA1 is required for the PRA1–LMP1 interaction, in which the PRA1 N-terminus may play an accessory role.

Characterization of the LMP1–PRA1 interaction at the Golgi apparatus

To examine whether LMP1 colocalized with endogenous PRA1, COS7 cells expressing GFP-LMP1 were immunostained with an anti-PRA1 antibody (Ab). Precise colocalization of GFP-LMP1 with endogenous PRA1 was observed at the Golgi apparatus (Figure 3A). Consistent results were obtained from the experiments using NPC cells (Figure 4A) and the EBV-positive B lymphoid cells, B95.8, which naturally express LMP1 (Supplementary Figure 3A), indicating that the colocalization of LMP1 with PRA1 is not restricted to cell types. To further characterize the phenomena, the Golgi-located GFP-LMP1 and PRA1 fractions were tested for sensitivity to a variety of reagents that have been reported to interfere with secretory protein trafficking. GFP-LMP1 and endogenous PRA1 present at the Golgi apparatus were both dispersed by

treatment with BFA (Figure 3A), which is known to cause disassembly and redistribution of the Golgi apparatus into the ER. Treatment with Monensin, an ionophore that inhibits protein transport within the Golgi, led to the accumulation of GFP-LMP1 and PRA1 at the early cisternae of the Golgi apparatus (Figure 3A). To test whether the localization of these proteins to the Golgi apparatus resulted from direct targeting, we examined the effect of *N*-ethyl-maleimide (NEM), which blocks a wide range of vesicular fusion events in post-Golgi transport and endocytosis (Bivona *et al*, 2004; Kasahara *et al*, 2004). Treatment with NEM did not affect the coappearance of GFP-LMP1 and PRA1 at the Golgi (Figure 3A), suggesting that their localization to the Golgi apparatus may not require vesicular transport from other cellular compartments. Furthermore, we found that treatment with the microtubule-depolymerizer nocodazole induced fragmentation of both perinuclear GFP-LMP1 and PRA1 into multiple ministack structures (Figure 3A). In contrast, treatment of cells with cytochalasin B, which disrupts the actin cytoskeleton, did not affect the colocalization of LMP1 and PRA1 at the Golgi apparatus, despite the fact that this treatment appeared to block vesicular GFP-LMP1 formation (Figure 3B). These findings collectively indicate that co-trafficking of LMP1 and PRA1 to the Golgi apparatus acts in a BFA-sensitive and microtubule-dependent manner.

PRA1 is involved in LMP1 trafficking and NF- κ B signaling

To investigate the role of the LMP1–PRA1 interaction, we tested whether this interaction affected the intracellular loca-

Figure 3 Characterization of the LMP1–PRA1 colocalization at the Golgi. (A) COS7 cells transfected with the plasmid for GFP-LMP1 (G-LMP1) were cultured for 18 h and then treated without or with 5 μ M/ml nocodazole (Noco), 10 μ M/ml BFA, 25 μ M monensin (Mon), 1 μ M/ml cytochalasin B (Cyto B), or 300 μ M NEM for 2–6 h. The cells were fixed and stained with Ab specific to PRA1 and the localization of GFP-LMP1 was observed with the confocal fluorescence microscopy. Bar, 16 μ m. (B) Quantitation of LMP1 localization. The numbers of cells in which GFP-LMP1 was located at ER (ER), both ER and Golgi (ER/Golgi), Golgi apparatus (Golgi), and vesicular and/or patch-like structures (Vesicle) were individually counted. In all, about 100 cells were analyzed.

Figure 4 Correlation of LMP1–PRA1 interaction to the localization and NF- κ B activation of LMP1. **(A)** Intracellular localization of LMP1 and LMP1 Δ TM3–6. NPC117 cells transfected with the plasmids for FLAG-LMP1 or -LMP1 Δ TM3–6 were fixed at 24 h post-transfection and stained with Abs specific to FLAG and PRA1. Bar, 16 μ m. **(B)** Activation of NF- κ B-mediated transcription by LMP1 and LMP1 Δ TM3–6. Cell extracts from NPC117 cells expressing FLAG-LMP1 or -LMP1 Δ TM3–6 were harvested and subjected to the assay for NF- κ B activation as described in Materials and methods. The basal activities in the absence of LMP1 were set as 1. **(C)** Interaction of PRA1 export mutant, PRA1 Δ AA, with LMP1. HEK293 cells were transfected with the plasmid for HA-PRA1 or -PRA1 Δ AA in conjunction with the plasmid for FLAG-LMP1. Cell extracts were harvested at 24 h post-transfection and applied to coprecipitation with LMP1 and anti-HA affinity matrix. The immunocomplexes were analyzed by Western blotting with Abs specific to FLAG and HA. **(D)** LMP1 localization in the presence of exogenous wide-type or mutant PRA1. COS7 cells expressing GFP-LMP1 in conjunction with HA-PRA1 or -PRA1 Δ AA were fixed at 24 h post-transfection and stained with anti-HA Ab. Bar, 20 μ m. **(E)** Quantitation of LMP1 localization. The numbers of cells in which GFP-LMP1 localization was classified into four patterns (ER, ER/Golgi, Golgi, vesicle) were individually counted. In all, about 100 cells were analyzed. **(F)** NF- κ B activation by LMP1 in the presence of exogenous wide-type or mutant PRA1. Cell extracts from NPC117 cells coexpressing FLAG-LMP1 with HA-PRA1 or -PRA1 Δ AA were harvested and subjected to the assay for NF- κ B activation as described in Materials and methods.

lization of LMP1 and its ability to activate NF- κ B. The localization of Δ TM derivatives of LMP1, which are defective to interact with PRA1 (Figure 2C; Supplementary Figure 2B), was examined in NPC117. The majority of FLAG-LMP1 Δ TM3–6 or -LMP1 Δ TM3/4 was unexpectedly seen in ER-like structures rather than the Golgi (Figure 4A; Supplementary Figure 2C). This altered intracellular distribution coincided with impaired NF- κ B activation as analyzed by reporter assays in NPC117 cells (Figure 4B; Supplementary Figure 2D), although the binding of LMP1 Δ TM with TRAF was unaffected (Supplementary Figure 2B). These findings support the possibility that PRA1 may play a role in LMP1 localization and signaling.

To further examine whether PRA1 affected the localization and signaling of LMP1, we generated a PRA1 export mutant (PRA1 Δ AA). The C-terminus of PRA1 contains the DXE motif essential for Golgi localization (Abdul-Ghani *et al*, 2001); thus, mutations in this motif resulted in retention of PRA1 Δ AA in the ER (Figure 4D). Regardless of the retention, the mutant PRA1 was able to interact with LMP1 in co-precipitation assays (Figure 4C). Consistently, GFP-LMP1 was largely entrapped in the pre-Golgi compartments in cells coexpressing PRA1 Δ AA, whereas no such interference was observed in the presence of wild-type PRA1 (Figure 4D and E). Finally, LMP1-mediated NF- κ B activation was markedly enhanced by coexpression of wild-type PRA1, but was repressed by expression of the export mutant (Figure 4F). These data collectively indicate that PRA1 functions in LMP1-induced signaling, at least in part by mediating the trafficking of LMP1.

Depletion of PRA1 leads to redistribution of LMP1 and TRAFs

To investigate the requirement of PRA1 for the trafficking and activity of LMP1, we used pSUPER-based small interfering RNA (siRNA) knockdown (Brummelkamp *et al*, 2002) to deplete PRA1 from LMP1-Tet-on 293 cells, in which LMP1 expression is induced by doxycycline. Following PRA1 knockdown and LMP1 induction, we performed the subcellular fractionation using postnuclear extracts to corroborate the localization of LMP1 (Figure 5A). In control siRNA-treated cells, LMP1 and PRA1 were markedly observed in the GS27- and Vti1a-containing fractions (i.e. the Golgi fractions), concomitant with notable levels of TRAF1, TRAF3, and TRADD. In contrast, the fractions from PRA1-depleted cells showed significant redistribution of LMP1 and TRAFs to the ER fractions (identified by the ER-resident protein, calregulin). To examine this result in greater detail, we observed the localization of GFP-LMP1 in NPC117 cells expressing two different PRA1 siRNAs. Consistent with the above results, siRNA-based PRA1 knockdown led to redistribution of GFP-LMP1, as well as TRAF1 and TRAF3, from the Golgi to the ER (Figure 5B and D; data not shown), although the binding affinity between LMP1 and TRAFs was not significantly affected by knockdown of PRA1 (data not shown). Taken together, these results suggest that PRA1 is required for the proper localization of LMP1 and the sequential targeting of its associated molecules. These events appear to be crucial for efficient signaling mediated by LMP1.

The properties of the Golgi apparatus are unaffected by PRA1 depletion

To explore whether depletion of PRA1 universally affected the trafficking of membrane proteins to the Golgi, we first examined the localization of Golgi proteins that have been implicated in distinct but overlapping aspects of its function (Supplementary Figure 4A). In PRA1-depleted NPC cells, no visible change was observed in the distribution of the Golgi matrix protein, GM130, or the *cis*/medial-Golgi protein, mannosidase II (Man II). There was also no obvious change in the localizations of GS15 and Vti1a, which are mainly involved in trafficking within the early cisternae of Golgi (Xu *et al*, 2002) and the *trans*-Golgi network (Xu *et al*, 1998), respectively. The distribution of GS27, which participates in transport from the *cis*/medial- to *trans*-Golgi network (Lowe *et al*, 1997),

Figure 5 Effect of PRA1 knockdown on LMP1 localization and TRAFs recruitment. (A) Cell extracts from LMP1-Tet-on 293 cells pretreated with control or PRA1 siRNA were prepared and subjected to subcellular fractionation as described in Materials and methods. Every other fraction was analyzed by Western blotting with the indicated Abs. The following proteins were used as organelle markers: calregulin (ER), GS27 and Vti1a (Golgi), and Annexin-II (plasma membrane, PM). (B) NPC117 cells were transfected with pSUPER-based vector encoding control or PRA1 siRNA (1 or 2). After 48 h, cells were further transfected with the plasmid for GFP-LMP1, followed by fixation and staining with anti-PRA1 Ab. Bar, 16 μm. (C) Quantitation of LMP1 localization. The numbers of cells in which GFP-LMP1 localization was classified into four patterns (ER, ER/Golgi, Golgi, vesicle) were individually counted. About 50 cells were analyzed in each experiment. (D) NPC117 cells were pretreated with control or PRA1 siRNA. After 48 h, cells were further transfected with the plasmid for GFP-LMP1, followed by fixation and staining with Ab specific to GM130 or TRAF3. Bar, 16 μm. In most knockdown experiments, PRA1 siRNA 1 was used unless specified elsewhere.

was also unaffected by PRA1 knockdown, as analyzed by subcellular fractionation (Figure 5A).

To further assess the effect of PRA1 depletion on vesicular transport from the ER through the Golgi, we conducted a morphological VSVG-GFP transport assay (Presley *et al*, 1997) using NPC117 cells. In both control and PRA1-depleted cells, VSVG-GFP was transported to the ER exit sites and/or the Golgi apparatus at 25 min and reached the plasma membrane by 75 min after the temperature shift (Supplementary Figure 4B and C). To confirm this, we investigated the endoglycosidase H (Endo H) sensitivity of VSVG-GFP, which acquires Endo H resistance when it is transported from the ER to the Golgi. At 75 min after temperature shift, a substantial amount of VSVG-GFP acquired Endo H resistance in both PRA-depleted and control cells (Supplementary

Figure 4D). Consistently, no detectable interaction between VSVG-GFP and PRA1 was seen in co-precipitation assays (Supplementary Figure 4E). These findings strongly suggest that the PRA1 depletion-induced redistribution of LMP1 is not due to a global defect in the Golgi localization and vesicular transport, but is instead a consequence of reduced interactions between LMP1 and PRA1.

PRA1 depletion selectively represses LMP1-induced NF-κB activity

We next performed reporter assays to corroborate the effect of PRA1 knockdown on LMP1-mediated NF-κB signaling. HEK293 cells with reduced PRA1 expression showed significant repression of FLAG-LMP1-induced NF-κB activity (Figure 6A). This repression could be largely recovered by

reconstitution of HA-PRA1, indicating that this effect was specific to PRA1 depletion. Similar results were obtained in NPC117 cells. As shown in Figure 6B, the PRA1 depletion-associated impairment of FLAG-LMP1-induced NF- κ B activity could be restored by wild-type PRA1 but not the export mutant PRA1_{AA} or PRA1₁₋₇₈, the latter of which is defective to interact with LMP1. These data confirm that LMP1-induced NF- κ B signaling requires the LMP1-PRA1 interaction, which mediates export.

To test whether PRA1 selectively affected LMP1-mediated signaling versus that mediated by other TNFR-related proteins, we investigated the effect of PRA1 deletion on NF- κ B activation stimulated by overexpression of CD40 in HEK293 cells (Figure 6C) or treatment with TNF α in LMP1-Tet-on 293 cells (Figure 6D). Neither CD40- nor TNFR1-induced NF- κ B activity was markedly affected by PRA1 depletion. In addition, coprecipitation analyses showed no evidence of an interaction between FLAG-CD40 and PRA1 (Supplementary Figure 2B). These results suggest that PRA1 specifically interacts with LMP1, perhaps providing a mechanistic basis

for the differences between LMP1 signaling and that mediated by CD40 or TNFR1.

The LMP1-PRA1 interaction is critical for LMP1-activated NF- κ B signaling

To investigate whether PRA1 mediates LMP1 function purely by assuring its localization to the Golgi, we generated a set of LMP1 derivatives designed to further elucidate the role of the LMP1-PRA1 interaction (Figure 7A). We first fused the palmitoylation (PA) sequence derived from GAP-43 (McLaughlin and Denny, 1999) to the non-PRA1-interacting C-terminus of LMP1 (LMP1_{CT}), causing the latter to be translocated from the cytoplasm to the Golgi membrane (Figure 7B). The results from reporter assays revealed that targeting of PALMP1_{CT} to the Golgi apparatus was not sufficient to trigger NF- κ B activation (Figure 7D), suggesting that PRA1 may not merely serve as an interacting transporter for LMP1. To verify this hypothesis, we introduced a PRA1-interacting motif (B₁) derived from BHRF1 (Li *et al*, 2001), or a noninteracting truncated version of this motif (B₁₉), into

Figure 6 Selective repression of LMP1-mediated NF- κ B activation by PRA1 depletion. (A) Recovery of LMP1-induced NF- κ B activation by reconstitution of PRA1. HEK293 cells pretreated with control or PRA1 siRNA for 48 h were further transfected with the plasmid for FLAG-LMP1 (+) or control vector (–), in conjunction with the plasmid for HA-PRA1 (WT) or pcDNA3-HA (V). Cell extracts were harvested and applied to the assay for NF- κ B activity and Western blotting as described in Materials and methods. *Exogenous PRA1. (B) Recovery of LMP1-induced NF- κ B activation by reconstitution of wide-type but not mutant PRA1. NPC117 cells pretreated with control or PRA1 siRNA for 48 h were further transfected with the plasmid for FLAG-LMP1 (+), in conjunction with the plasmid for HA-PRA1 (WT), -PRA1_{AA} (AA), -PRA1₁₋₇₈ (1-78), or vector control (V). Cell extracts were harvested and applied to the NF- κ B activity assay and Western blotting as previously described. *Exogenous PRA1, in which HA-PRA1₁₋₇₈ cannot be detected by PRA1 specific Ab (C16), which recognizes the C-terminus of PRA1. (C) No effect of PRA1 knockdown on CD40-induced NF- κ B activation. HEK293 cells pretreated with control or PRA1 siRNA were further transfected with the plasmid for FLAG-LMP1, -CD40, or vector alone. Cell extracts were harvested and applied to the assay for NF- κ B activity as previously described. (D) No effect of PRA1 knockdown on TNF α -mediated NF- κ B activation. LMP1-Tet-on 293 cells pretreated with control or PRA1 siRNA for 48 h were further incubated without or with doxycycline (Dox, 10 μ g/ml) or recombinant TNF α (25 μ g/ml) for 24 h. The NF- κ B activity assay was performed as previously described. In all, a portion of each cell lysate was analyzed by Western blotting with indicated Abs. The basal activities in the vector alone or the blank control were set as 1.

Figure 7 Requirement of PRA1 interaction for LMP1-induced NF-κB activation. (A) Schematic representations of LMP1 derivatives. B₁LMP1_{CT} is a fusion of the N-terminus of BHRF1 (aa 1–161) fused to the C-terminus of LMP1 (LMP1_{CT}; aa 187–381). B₁₉LMP1_{CT} is a fusion of the N-terminal aa 19–161 of BHRF1 fused to LMP1_{CT}. PALMP1_{CT}, PAB₁LMP1_{CT}, and PAB₁₉LMP1_{CT} consist of the 11-amino acid N-terminal palmitoylation (PA) sequence derived from GAP-43 fused to LMP1_{CT}, B₁LMP1_{CT}, and B₁₉LMP1_{CT}, respectively. All derivatives contain C-terminal FLAG tags. (B) Intracellular localizations of LMP1 derivatives. COS7 cells expressing LMP1_{CT} or the derivatives were fixed at 24 h post-transfection and stained with Abs specific to FLAG and GM130 (red). Bar, 20 μm. (C) Interaction between LMP1 derivatives and PRA1. Cell extracts from HEK293 cells expressing LMP1 or the derivatives were harvested at 24 h post-transfection. FLAG-tagged proteins were precipitated and the immunocomplexes were analyzed as previously described. (D) NF-κB activation by LMP1 derivatives. NPC117 cells were transfected with 250 or 500 ng of the plasmid encoding LMP1_{CT} or the derivatives. Cell extracts were harvested at 24 h after transfection and applied to the NF-κB activity assay as previously described.

PALMP1_{CT} to substitute LMP1's transmembrane domain, which is responsible for LMP1 interaction with PRA1. Both PAB₁LMP1_{CT} and PAB₁₉LMP1_{CT} were found to localize to the Golgi apparatus (Figure 7B), whereas coprecipitation assays revealed that PAB₁LMP1_{CT} but not PAB₁₉LMP1_{CT} interacted with endogenous PRA1, although to a markedly lesser extent than wild-type LMP1 (Figure 7C). To further investigate the effect of this weak interaction, we examined the NF-κB activity induced by increasing amounts of PAB₁LMP1_{CT} in NPC cells (Figure 7D). Consistent with the coprecipitation data, only PAB₁LMP1_{CT} was able to activate NF-κB signaling, and this activation was enhanced by expression of increased levels of PAB₁LMP1_{CT}.

In conclusion, we herein newly identified an interaction between the transmembrane domain of LMP1 and PRA1, and showed that this interaction is not only involved in the intracellular localization of LMP1, but is also crucial for LMP1-mediated NF-κB signaling.

Discussion

The ligand-independent receptor, LMP1, has been shown to signal primarily from the intracellular perinuclear compartments in various cell types, including epithelial and B lymphoid cells (Lam and Sugden, 2003). In the current study, we demonstrate that localization of LMP1 to the Golgi apparatus

is critical for the efficient function of LMP1 in NF- κ B signaling; more importantly, this process requires the interaction of LMP1 through its transmembrane domain with a newly identified interacting partner, PRA1.

The results from FRET (Figure 1A) and fluorescence recovery after photobleaching (FRAP; data not shown) indicate that newly synthesized LMP1, accompanied by PRA1, localizes to the Golgi and primarily interacts with PRA1 at the site. Consistently, CHX-induced inhibition of protein synthesis markedly reduces the Golgi localization of LMP1 (Supplementary Figure 1A). Disruption of the actin cytoskeleton with cytochalasin B results in the accumulation of LMP1 only at the Golgi apparatus (Figure 3A). As the actin cytoskeleton has been shown to be involved in endocytosis, the data further suggest that LMP1 and PRA1 are directly targeted to the Golgi, rather than returning from the plasma membrane. Moreover, treatment with BFA disperses the Golgi localization of both LMP1 and PRA1 (Figure 3), reminiscent of the previous finding that BFA causes profuse microtubule-dependent tubulation of Golgi membranes and relocation of the Golgi contents in the ER (Lippincott-Schwartz *et al*, 1995). Interestingly, the microtubule-depolymerizing reagent, nocodazole, triggers fragmentation of both Golgi-localized LMP1 and PRA1 into multiple ministacks (Figure 3A) near transitional ER sites (Hammond and Glick, 2000). Together, these results strongly suggest that LMP1 likely interacts with PRA1 after protein synthesis, and sequentially colocalize with PRA1 to the Golgi via microtubule-dependent, potentially COPII-mediated transport (Murshid and Presley, 2004). This idea is supported by the evidence that the sorting signals recognized by COPII coat (Barlowe, 2003; Bonifacino and Glick, 2004) are found in both the C-terminus of LMP1 and PRA1, such as di-acidic motifs fitting the consensus [DE]X[DE] (where D is aspartate, X is any amino acid, and E is glutamate) and a short hydrophobic motif LL (double leucines), all of which are necessary for LMP1 and PRA1 export (Figure 4D; Supplementary Figure 5A and B).

The ER-to-Golgi transport of LMP1 appears to be critical for its efficient signaling, as LMP1-mediated NF- κ B activation is significantly impaired when this localization is disturbed by interfering reagents (Supplementary Figure 1B–D) or more specifically, by genetic alterations (Supplementary Figure 5B and D). In addition to their presence in the plasma membrane, lipid rafts have been shown to occur in intracellular compartments, particularly the Golgi (Heino *et al*, 2000; Ikonen, 2001). A previous report speculated that LMP1 probably associates with lipid rafts in the Golgi apparatus (Lam and Sugden, 2003); this theory is supported by our observation that both LMP1 and PRA1 colocalize with the Golgi pool of caveolin, which is an indicator of lipid raft (unpublished data). Despite the possibility that a portion of LMP1 may proceed from the Golgi apparatus to the plasma membrane, we herein demonstrate that the process of Golgi-directed LMP1 trafficking is essential for the full function of LMP1, and further show that this process involves the interaction of LMP1 with PRA1.

The LMP1–PRA1 interaction requires transmembrane segments 3–6 of LMP1 and the transmembrane domain of PRA1 with assistance from the PRA1 N-terminus (Figure 2), indicating that the membrane localization of both proteins may enhance or allow their interaction. The transmembrane segments 3–6 of LMP1 are involved in its intermolecular

interactions (Yasui *et al*, 2004). The short N-terminus and transmembrane segments 1–2 of LMP1 have been shown to be required for targeting of LMP1 to the lipid raft (Rothenberger *et al*, 2002; Yasui *et al*, 2004); however, these regions are not responsible for the LMP1–PRA1 interaction as demonstrated by experiments using Δ TM derivatives of LMP1, in particular LMP1 $_{\Delta$ TM3–6 (Figure 2C; Supplementary Figure 2B). These LMP1 variants substantially retain in the pre-Golgi structures and have impaired ability to signal (Figure 4A and B; Supplementary Figure 2C and D), suggesting a linkage between PRA1 interaction and oligomerization of LMP1, two events that likely also mediate LMP1 trafficking. In support of this possibility, we found that coexpression of the minimal LMP1-interacting region of PRA1 (PRA1 $_{70-164}$) leads to concomitant impairment of the oligomerization, the ER-to-Golgi translocation, and NF- κ B signaling of LMP1 (Supplementary Figure 6A–C). More importantly, PRA1 depletion results in redistribution of LMP1 from the Golgi to the ER and impairment of LMP1-induced signaling (Figures 5 and 6B), in agreement with the observation that PRA1 depletion appears to reduce the level of LMP1 oligomerization (Supplementary Figure 6D). Although the N-terminus of LMP1 mediates targeting to the lipid raft, our results strongly suggest that PRA1 interaction may trigger or modulate the oligomerization/stabilization of LMP1, leading to subsequent trafficking of LMP1 to the sites where it can function efficiently (Figure 8). Oligomerization of LMP1 triggered by PRA1 association may support LMP1 packaging into the COPII-mediated vesicle and transport (see above), similar to the movement of sterol regulatory element binding protein to the Golgi (Dobrosotskaya *et al*, 2003), and this process is required for efficient signaling of LMP1. In support of the model, we found that targeting of the LMP1 C-terminus to the Golgi by fusion with a palmitoylation sequence derived from GAP-43 is insufficient to trigger LMP1-mediated signaling

Figure 8 Model for the role of PRA1 in LMP1's trafficking and NF- κ B signaling. Through protein–protein interaction, PRA1 may mediate or enhance the oligomerization/stabilization of LMP1 into the membrane, leading to subsequent trafficking of LMP1 and its associated TRAFs along microtubules to the Golgi apparatus where LMP1 efficiently activates the NF- κ B signaling. This process can be interfered by nocodazole or BFA. Knockdown of PRA1 results in impaired oligomerization/stabilization of LMP1 and retention of LMP1 and its associated TRAFs in the ER, leading to impairment of NF- κ B activation. PRA1, which is free from LMP1-associated complexes, is omitted in this model.

unless the LMP1 derivative is competent to interact with PPA1 (Figure 7). Thus, our identification of PRA1 as a new interacting partner of LMP1 may suggest a mechanistic explanation for the differences between LMP1-mediated signaling and that triggered by CD40 or TNFR (Figure 6C and D; Supplementary Figure 2B and D).

Although PRA1 has been reported to interact with numerous molecules implicated in vesicular trafficking, its precise trafficking functions still remain unknown. Indeed, PRA1 is better known to function in endocytic pathways, where it acts as a GDI-displacement factor that catalytically dissociates endosomal Rab complexes and delivers them to membranes (Sivars *et al*, 2003). Despite our observation that a portion of PRA1 localizes in pericellular endosome-like structures, LMP1 does not appear to localize in these compartments, suggesting that PRA1 may function differentially based on its localization to distinct subcellular compartments in association with different molecules. The function of PRA1 in protein trafficking between the ER and Golgi may be distinct from syntaxin-mediated vesicular transport, since localization of PRA1 to the Golgi is resistant to NEM treatment (Figure 3; Hirose *et al*, 2004). In support of this idea, we noted that depletion of PRA1 does not affect the cellular distribution of NEM-sensitive factor attachment protein receptors such as GS27, GS15, and Vt1a (Figure 5A; Supplementary Figure 4A). However, in contrast to the results of our knockdown experiments, a previous report demonstrated that stable overexpression of mutant PRA1 resulted in disruption of Golgi morphology, as represented by perturbed Man II localization and impaired VSVG trafficking (Gougeon *et al*, 2002). This discrepancy may result from gain of functions owing to expression of mutant PRA1, or unexpected interference of other molecules. Alternatively, residual levels of PRA1 in our PRA1-depleted cells may have been sufficient to enable the efficient transport of Man II and VSVG, but not LMP1, to which PRA1 with respect to its function and/or interaction is intimately related.

In sum, the present results provide new insights into the properties of LMP1, and show for the first time that PRA1 plays an unexpected role in linking protein trafficking to signaling. It will be of great interest to further identify the molecular basis of the LMP1–PRA1 interaction, as this could shed additional light on the biological properties of PRA1 and the trafficking-based function of LMP1.

Materials and methods

Plasmid and siRNA construction

Details are provided as Supplementary data.

Yeast two-hybrid assay, Abs, and reagents

Details are provided as Supplementary data.

In vitro transcription/translation, GST-pull down, and precipitation assays

Details are provided as Supplementary data.

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BRET² assay

BRET² assay was performed under the manufacturer's instructions (PerkinElmer Life and Analytical Sciences, MA, USA). In brief, HEK293 cells expressing donor *Renilla* Luciferase (Rluc)- and acceptor GFP²-tagged fusion proteins were detached 24 h after transfection and applied for BRET² assay. The BRET² ratio is calculated as (emission at 515 nm of transfected cells–emission at 515 nm of nontransfected cells)/(emission at 410 nm of transfected cells–emission at 410 nm of nontransfected cells). Details are provided as Supplementary data.

Immunofluorescence and FRET experiments

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. For FRET, the images were acquired on an inverted Zeiss Axiovert 200M microscope (Carl Zeiss Light Microscopy, Germany) with a FLUAR 100 × 1.3 NA oil-immersion immersion objective lens. Details are provided as Supplementary data.

Assay for NF-κB activity

NPC076 and NPC117, which were derived from NPC biopsies, or HEK293 cells were grown to 70% confluence in six-well culture plates, and each well was transfected with 250 ng of pFLAG-LMP1 or the empty vector, in conjunction with 100 ng of NF-κB-luciferase reporter plasmid and 100 ng of expression plasmid for *Renilla* luciferase. Cells were harvested 24 h post-transfection, lysed in a luciferase lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 10% glycerol and 1% Triton X-100), and assayed for luciferase activity using a luminometer (LB593; Berhold, Germany). For double transfections, cells were initially transfected with pSUPER-based vectors expressing either control or PRA1-specific siRNA (2 μg per well) for 48 h, followed by a second transfection as described above. Transfection efficiencies were normalized with regard to *Renilla* luciferase activities. Half of each cell lysate was applied for Western blot analysis.

Subcellular fractionation

In brief, LMP1-Tet-on 293 cells pretreated with control or PRA1 siRNA for 48 h were incubated with doxycycline for LMP1 induction. Cells were homogenized and centrifuged, and the resultant supernatant was layered onto a continuous sucrose gradient (10–45% sucrose) and centrifuged at 55 000 r.p.m. in a SW55 rotor (Beckman, Fullerton, CA, USA) for 1 h. The fractions were collected manually from the bottom of the gradient and a portion of each fraction was subjected to Western blotting. Details are provided as Supplementary data.

Protein transport from ER to Golgi

Details are provided as Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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