

# Minimal protein domain requirements for the intracellular localization and self-aggregation of Epstein-Barr Virus Latent Membrane Protein 2

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Received: 17 March 2007 / Accepted: 14 May 2007 / Published online: 13 June 2007  
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**Abstract** The EBV Latent Membrane Protein 2 (LMP2) may have a role in the establishment and maintenance of in vivo latency. The gene is transcribed into two mRNAs that produce two LMP2 protein isoforms. The LMP2a protein isoform has 12 transmembrane segments (TMs) and an amino terminal cytoplasmic signaling domain (CSD) while the LMP2b isoform is identical but lacks the CSD. There has not been a consensus on the cellular membrane localization being sometimes ascribed to either a plasma membrane or an intracellular location [M. Rovedo, R. Longnecker, J. Virol. 81:89–94, 2007; D. Lynch, J. Zimmerman, D.T. Rowe, J. Gen. Virol. 83:1025–1035, 2002; C. Dawson, J. George, S. Blake, R. Longnecker, L.S. Young, Virology 289:192–207, 2001]. Fluorescent marker and epitope tagged LMP2b truncation mutants progressively removing TMs from the N and C termini were used to assess the localization and aggregation properties of LMP2b. wtLMP2b had an exclusively intracellular perinuclear localization, while all truncations of the protein resulted in localization to the cell surface. By epitope loop-tagging, all the truncated LMP2b proteins were verified to be in the predicted membrane orientation. In co-transfection experiments, the C-terminal region was implicated in the self-aggregation properties of LMP2b. Thus, an intact 12 TM domain was required for intracellular localization and protein–protein interaction while a C-terminal region was responsible for auto-aggregative properties.

**Keywords** Epstein-Barr Virus · LMP2 · Truncation mutants · Membrane protein

## Introduction

Epstein-Barr Virus (EBV) is a human gamma-herpesvirus, which is implicated in infectious mononucleosis, lymphoproliferative disease in the immunocompromised and several epithelial and lymphatic cancers [1]. In the normal host, EBV establishes a latent infection in B-lymphocytes [2]. In vitro, EBV infection of B cells induces proliferation of infected cells that can be subsequently grown as a lymphoblastoid cell line (LCL). LCLs express a limited number of viral gene products with functions associated with episome maintenance and cell survival. Of this restricted protein set, LMP2 is of particular interest due to its ability to maintain viral latency by preventing productive signaling from the B-cell receptor [3].

The LMP2 gene encodes two separate membrane protein isoforms via alternate promoter usage. These promoters, separated by approximately 3 kb, both produce transcripts that cross the fused terminal repeats [4]. The mRNAs have unique 5' first exons and eight shared 3' exons. The messages encode LMP2a, a 497 aa protein with a 119 aa N-terminal cytoplasmic signaling domain (CSD), 12 transmembrane (TM) segments and a short 27 aa C-terminal tail and LMP2b, a 378 aa protein lacking the CSD.

LMP2a has been extensively studied with respect to protein interaction and intracellular signaling. The N-terminal CSD of LMP2a is critical to the production of survival signals and self-degradation [5, 6]. The CSD contains an immunotyrosine activation motif (ITAM), which is constitutively phosphorylated on tyrosines and interacts with protein tyrosine kinases Syk and Lyn [7–10].

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These interactions cause signaling that mimics B-cell receptor complex survival signals in early stages of murine B-cell development [11]. Previous reports also indicated that LMP2a excludes the BCR from detergent-insoluble lipid-enriched membranes [5]. Additionally, two proline rich motifs in the CSD also interact with a family of E3 ubiquitin ligases, mediating degradation of LMP2a and its associated proteins [12, 13].

The nature of the LMP2b contribution to viral pathogenesis has not been determined. A role is suggested by the evolutionary conservation amongst primate lymphocryptoviruses of the ability to produce an LMP2b isoform [14]. Mutational analyses of the LMP2 gene in recombinant viruses have involved replacing regions of the LMP2 gene with antibiotic resistance cassettes, most of which simultaneously affect both LMP2a and LMP2b. From these studies, LMP2b does not appear to contribute to immortalization [15]. Virus mutants that interrupt LMP2a exon1 and leave LMP2b intact, and presumably expressed, suggest that LMP2b has no role in the BCR signaling blockade phenotype of the LMP2 gene [16, 17]. One recent study stated that LMP2b modulates LMP2a activity, however, was unable to detect LMP2b in cells [18]. Studies in epithelial cells demonstrate that LMP2b is able to promote cell spreading and motility, thus indicating that LMP2b does have the ability to mediate some sort of cellular signaling event independent of LMP2a [19]. We previously described the ability of LMP2b to specifically interact with endogenously produced CD19 [20]. Taken together, these studies show that LMP2b is likely to have unique effects on cells, and suggest that LMP2b is probably more than a CSD-less negative regulator of LMP2a. In this study, LMP2b truncations were made and analyzed for localization and multimerization phenotypes. Identification of where LMP2b localizes and how the protein interacts with itself and other cellular proteins will provide important information on how the CSD-less protein could affect other cell processes.

## Materials and methods

### Cell lines and cell culture

BJAB is an EBV-negative Burkitt lymphoma cell line; 293T is a human embryonic kidney cell line containing the SV40 large T antigen. BJAB were maintained in complete RPMI-1640 medium containing 10% inactivated Foetal Bovine serum, 2 mM glutamine, 60  $\mu\text{g ml}^{-1}$  penicillin, 200  $\mu\text{g ml}^{-1}$  streptomycin at 37°C in 5% CO<sub>2</sub>. 293T were grown in complete Dulbecco's modified Eagle's medium (DMEM) under identical growth conditions.

### Construction of recombinant LMP2 plasmids

DNA fragments encoding full-length LMP2a or LMP2b incorporated with a 3× FLAG were constructed as previously described [20]. Truncation mutants of LMP2 were amplified by PCR from full-length constructs. All 5' primers contain an *XhoI* restriction site. All 3' primers contain a *KpnI* restriction site. PCR products were digested with *XhoI/KpnI* and ligated into pC1EGFP or pC1DsRed2 (Clontech) for N-terminal truncations and pN1EGFP or pN1DsRed2 for C-terminal mutations. Full-length PCR products were ligated into pDSRed2 or pN1EGFP (Clontech). All constructs were sequenced for verification.

### Transfections

All DNAs used for transfections were purified on CsCl<sub>2</sub> gradients. For B cell lines, 5 × 10<sup>6</sup> cells were washed once in 4°C PBS and resuspended at room temperature in 0.4 ml of serum- and phenol red-free RPMI 1640 containing 20  $\mu\text{g}$  of the required plasmid. Cells were then transferred to a sterile electroporation cuvette (0.4 cm electrode gap) (BioRad) and pulsed twice. Multiporator (Eppendorf) settings were 400 V for 100  $\mu\text{s}$ . Cells were allowed to settle for 10 min in a 37°C water bath. Cells were removed from the cuvette and added to 10 ml of complete phenol red-free RPMI 1640. For 293T cells, confluent T75 flasks were treated with 10× trypsin (Gibco) and washed. Cells were seeded to achieve 30% confluency within 24 h in a chamber slide and grown in complete DMEM. Once the cells had become 50% confluent, the media were replaced with FBS-free DMEM containing 3  $\mu\text{l}$  of Genejuice (Novagen) and 1  $\mu\text{g}$  of DNA.

### Antibodies and immunofluorescence

Anti-Human CD8, Syntaxin-6, GS15, GM130, and GS27 were purchased from BD Pharmingen. Anti-FLAG M2 was purchased from Sigma. Alexa-fluor secondary antibodies (488 and 594) were purchased from Molecular Probes. Mounting media containing DAPI was purchased from Vector Laboratories. Immunofluorescence was performed by fixing cells to polylysine-coated glass slides (Fisher) using a cytospin (Shanddon) set at 500 rpm for 5 min. Slides were dried for 1 h at room temperature in the dark. Cells were then fixed using freshly prepared 3.7% paraformaldehyde for 1 min at room temperature. Samples were blocked for 30 min using SuperBlock blocking buffer in PBS (Pierce). Primary antibodies were incubated at predetermined dilutions in blocking buffer for 30 min at room temperature. Secondary antibodies were diluted in blocking buffer (1:2,000 dilution) for 30 min at room temperature. Slides were washed three times in cold PBS. Slides were

allowed to air dry in the dark for 30 min. Slides were mounted with Vectashield-mounting media containing DAPI (Vector Laboratories). For cell surface immunostaining, live cells were chilled on ice for 10 min and incubated with anti-FLAG antibody M2 (Sigma) for 30 min at 4°C. Cells were washed in cold blocking buffer, fixed in 3.7% paraformaldehyde and incubated with Alexa-fluor secondary antibodies (Molecular Probes).

#### *Immunoprecipitation and Western Blot*

293T cells were transfected with EGFP and FLAG tagged full-length LMP2b or one of the truncation mutants, and cell lines were established by G418 selection. Immunoprecipitation was performed using  $\mu$ MACS Protein G microbeads (Miltenyi Biotec, Inc.) as per the manufacturer's protocol. Samples were analyzed by SDS-PAGE and transferred to nitrocellulose membranes. The proteins were detected with anti-FLAG M2 antibody (Sigma) via the Pro-Q Western Blot Stain Kit (Molecular Probes) and visualized via the FLA-2000 fluorescent image analyzer (FujiFilm, Inc.).

#### *Microscopy*

Slides were examined with a Nikon E600 microscope. Digital images were captured via a SPOTII CCD digital camera and assembled using METAMORPH software (Universal Imaging). Final image montages were constructed using Adobe Photoshop.

#### *Pixel quantitation and statistical analysis*

Digital images of EGFP and DsRed 2 fluorescence were acquired using a cooled charge-coupled-device camera. Quantification was performed using METAMORPH software. Greyscale images were thresholded by eye to obtain pixel counts for an enriched marker on a single cell. When images were combined, the pixel counts were taken for overlap of the two single color images. The total number of pixels in each of these three single channel pictures were used to obtain a percentage of marker overlap: percentage of red overlapping with green (%R = G), and percentage of green overlapping with red (%G = R). These percentages were used in the Kruskal–Wallis rank sum test [21] via Minitab Release 14 Statistical Software (Minitab, Inc.).

Linescan histograms were generated by creating a 1 by cell width pixel region in each micrograph encompassing a cross section of each cell. Histograms were generated via the METAMORPH software (MDS, Inc.). Each histogram measures the pixel intensity versus pixel number. Standard deviations of mean fluorescent intensities of 10 linescans

for each mutant were compared by *T*-test using SPSS software (SPSS, Inc.).

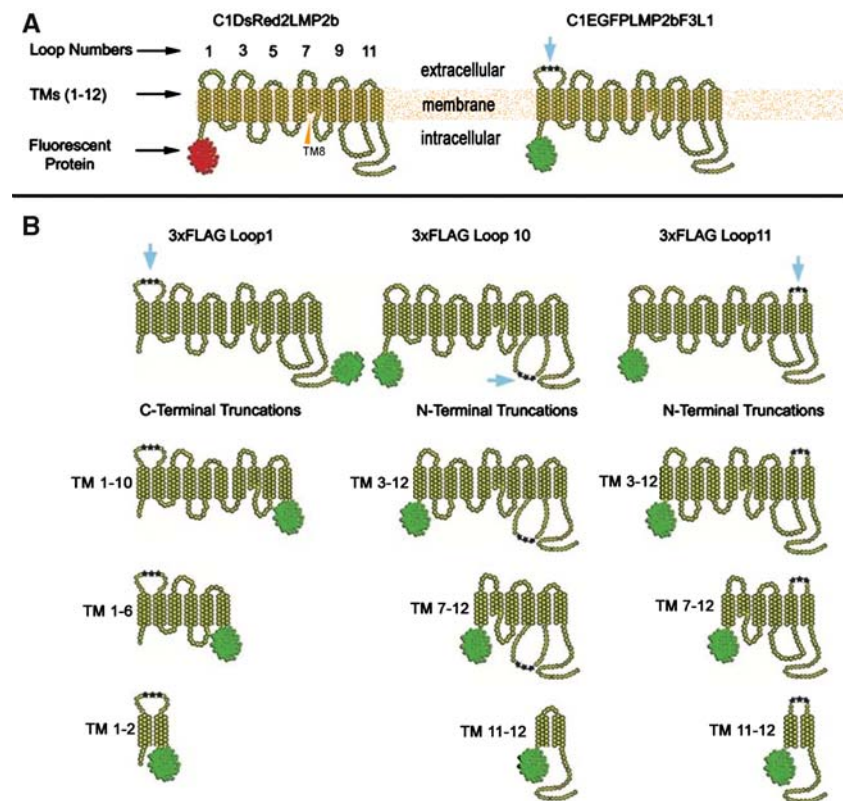
## **Results**

### *Insertion of FLAG epitopes does not affect protein localization*

Due to the lack of antibodies that can independently recognize LMP2b sequences, we inserted artificial peptide (FLAG) epitopes into the loop sequences of the protein. In previous studies, we reported development of a 3 $\times$  FLAG epitope tag inserted into the first extracellular loop of the protein. This epitope was shown to not interfere with localization when compared to wtLMP2b [20]. In this study, we used the same cloning strategy to insert 3 $\times$  FLAG epitope tags into loop 10 connecting TM10 to TM11 (a putative intracellular loop) and into loop 11 connecting TM11 to TM12 (a putative extracellular loop). These constructs were also fused to either DsRed2 or EGFP. (Fig. 1B, top row) Constructs with a fluorescent molecule fused to the N terminus of the full-length LMP2b protein were transiently transfected into BJAB cells (Fig. 2). Protein expression was monitored over the next 3 days. In all experiments, transiently expressed LMP2b proteins localized to a perinuclear region in live cells. This was in agreement with our previous studies that showed that LMP2 proteins with N- or C-terminal fluorescent tags co-localized with wtLMP2. In order to determine if the inserted FLAG epitopes had any affect on localization, co-transfections were performed between all three FLAG containing constructs (Fig. 2). In each co-transfection, one construct was fused to DsRed2 while the other was fused to EGFP. As shown previously for the FLAG epitope insertion into loop 1, FLAG epitopes inserted into loops 10 or 11 did not effect the perinuclear intracellular localization of LMP2b or the ability of differently tagged proteins to co-localize (Fig. 2). The levels of LMP2b fluorescence remained similar in all cells transfected, regardless of time post-transfection, cell type or FLAG modification.

Previously, we reported that full-length LMP2b is able to co-localize with  $\gamma$ -adaptin, a marker for the trans-Golgi network. Immunofluorescent microscopy with antibodies detecting proteins that identify specific subregions of the Golgi were used to further refine the location of perinuclear LMP2b. Cells were probed for gm130 and GS15 (medial Golgi stack), GS27 (trans Golgi stack), and Syntaxin 6 (trans Golgi network). The largest amount of co-localization was seen between LMP2b and Syntaxin 6. Partial overlap was also observed between LMP2b and GS27

**Fig. 1** Modifications and mutations of LMP2b used in this study. (A) LMP2b is a 12 TM protein with short loops connecting the TMs. TM portions are referenced by number, 1 through 12, counting from the left. TM8 is indicated by an orange arrow. Extracellular loop are numbered as indicated. Full-length proteins were fused to a fluorescence marker (either EGFP [*green*] or DsRed2 [*red*]) and have a 3× FLAG epitope (represented by stars) in a loop of the protein (*blue arrow*). (B) C-terminal truncations were derived from N1EGFPLMP2bf3L1 and have deletions of the last 2, 6, or 10 TMs. N-terminal truncations were derived from C1EGFPLMP2bf3L10 or C1EGFPLMP2bf3L11. N-terminal truncations remove the first 2, 6, or 10 TMs



while minor overlap was detected between LMP2b and gm130 or GS15 (Fig. 3, right panels).

#### Removal of TMs changes the localization of LMP2b

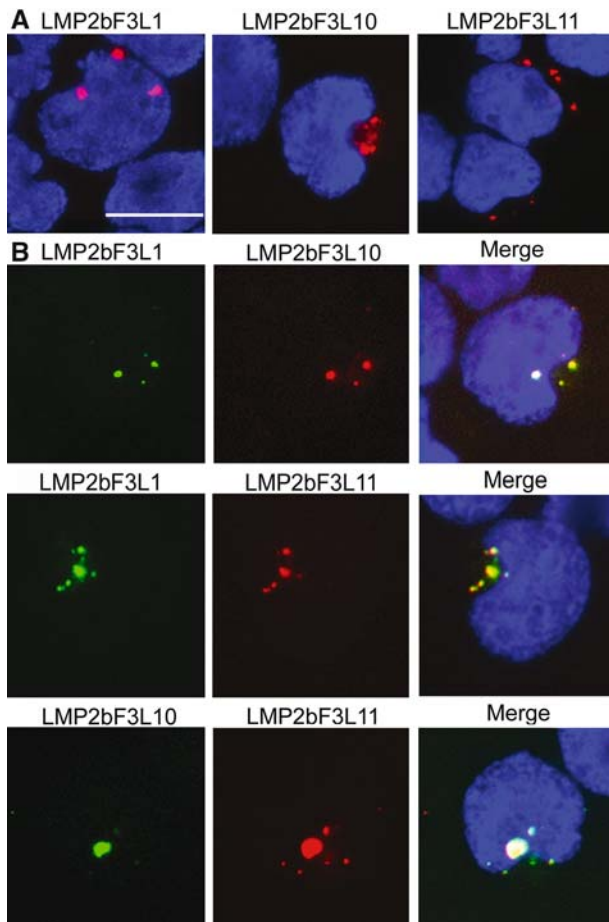
Truncations within the ORFs of the epitope-tagged LMP2b constructs were used to investigate the minimal number of TMs needed for intracellular localization and protein–protein interaction. Truncated proteins removing 2, 6, or 10 TMs from either the N- or C-terminus were made. C-terminal truncations contained the 3× FLAG in the sequence connecting the first and second transmembrane region (loop 1; F3L1). N-terminal truncations contained the 3× FLAG in either the loop connecting transmembrane region 10 to 11 (loop 10; F3L10) or transmembrane region 11 to 12 (loop 11; F3L11) (Fig. 1). We, and others [22], have identified the principal location of LMP2 proteins in cells as a membranous compartment in a perinuclear (Golgi-like) region.

Live impermeabilized BJABs transfected with wtLMP2b containing a loop 11 3× FLAG tag were probed for surface FLAG expression. The epitope tag was not detected regardless of the time post-transfection that the cells were probed or the apparent level of EGFP fluorescence. This indicated that none of the LMP2b was trafficking to the plasma membrane (Fig. 4A). In contrast, the epitope tags were readily detected on the surface of every

cell transfected with a C-terminal truncation mutant with a tag in loop 1 (TM1-2, TM1-6, and TM1-10) or an N-terminal truncation mutant with a tag in loop 11 (TM3-12, TM7-12, and TM11-12) (Fig. 4B and data not shown). Surface expression was detected even at the earliest times post-transfection and was independent of the apparent level of EGFP fluorescence. When mutants with identical truncations carried tags in loop 10 or loop 11 (TM3-12 and TM7-12) were probed for surface expression of the FLAG epitope, proteins with loop 10 tags (a putative intracellular loop) were not detected (Fig. 4C). Loop 10 tags were only detected when the transfected cells were permeabilized (Fig. 4D). In contrast truncated proteins with loop 11 tags (a putative extracellular loop) were detected without permeabilization (Fig. 4B). These data showed that the truncated proteins were expressed at the cell surface and retained the conformation and orientation predicted for LMP2b. Thus, an intact 12 TM protein is necessary for intracellular localization.

The C-terminal region of LMP2b is responsible for self-interaction but not localization

Previous work [23] had shown that a portion of the C-terminus of LMP2 was responsible for ‘clustering’ of the protein. N-terminal truncation mutants (TM3-12, TM7-12, and TM11-12) had a distinct patchiness in the fluorescent

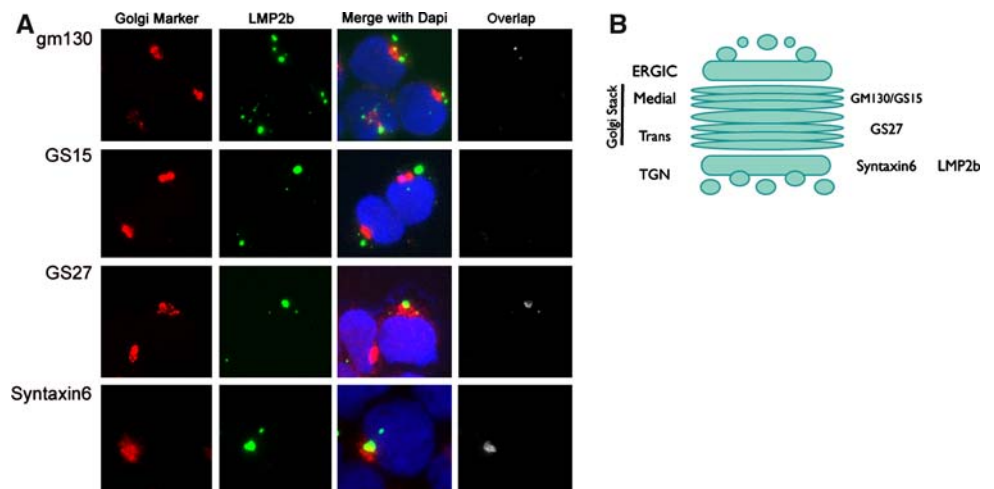


**Fig. 2** FLAG insertion into loops of LMP2b has no effect on protein localization. (A) Plasmids encoding full-length LMP2b fused to DsRed2 were transfected into BJAB cells. Micrographs of BJAB cells expressing single LMP2b proteins. (B) Plasmids encoding full-length LMP2b fused to either EGFP or DsRed2 were transfected into BJAB cells. Micrographs are of cells co-expressing differently FLAG-tagged LMP2

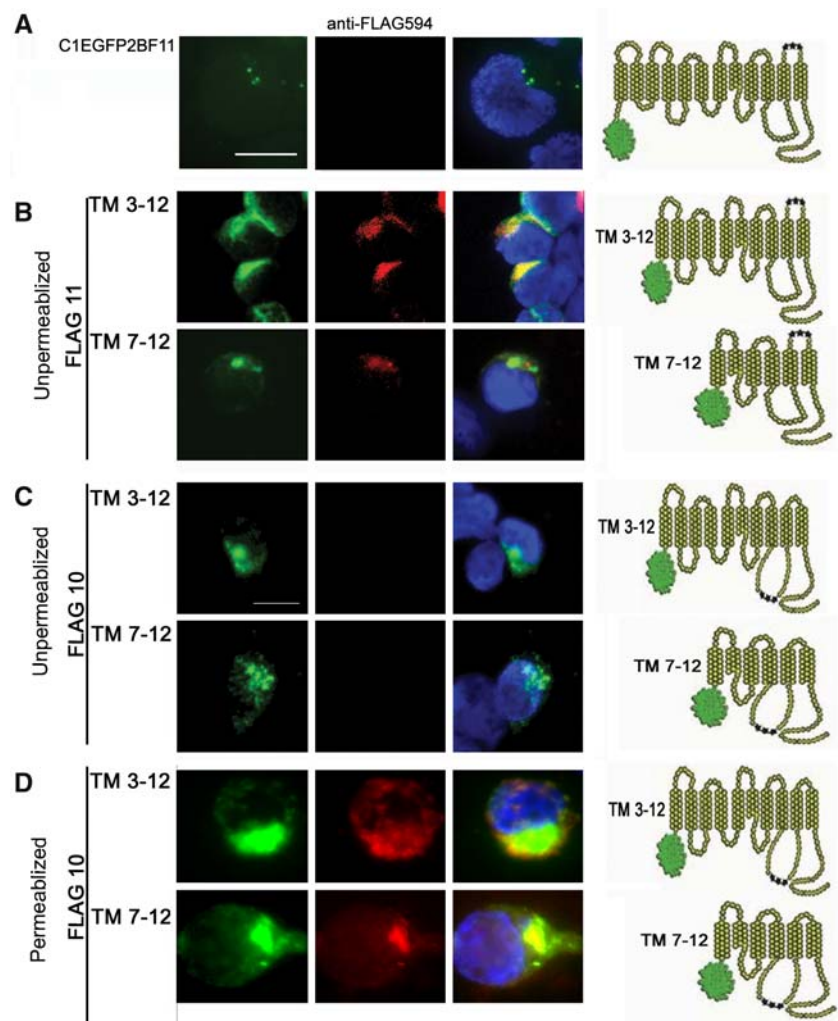
pattern often appearing to cluster into a single patch or cap on the cell surface (Fig. 5A, white arrows indicate clustered areas of protein). The effect was particularly pronounced when compared directly to the more diffuse global distribution of fluorescence observed with C-terminal truncation mutants (TM1-2, TM1-6, and TM1-10). A Western Blot of anti-FLAG immunoprecipitates from transiently transfected cells revealed that LMP2b and all the truncation mutants except TM1-2 migrated as monomers (Fig. 5B). This confirmed that the proteins were intact and not degraded and showed that the clustering detected by immunofluorescence was easily disrupted. Linescan graphs comparing the individual pixel intensity for a cross section of the Fig. 5A micrographs are shown in Fig. 5C. Peaks correspond to close groupings of intensely green pixels and are an indication of clustering. As can be seen in the left column, the N-terminal truncations have more peaks than C-terminal truncations showing less fluctuation, indicating that the protein has a more uniform distribution. We also compared the means of the standard deviations of the individual mutants (Fig. 5D) as well as N- versus C-terminal mutants. We found that the mean standard deviations of the C- and N-terminal mutants were different using a *T*-test with a *P* = <.001 level of significance. The differences in cell surface localization are consistent with the biochemical data that indicates the C-terminus causes LMP2 proteins to interact to form multimers [23].

Proteins containing multiple TMs are often localized to the Golgi via a phenomenon called kin-recognition that involves preferential sorting of proteins with minimal length hydrophobic stretches remaining in membranes of similar thicknesses [24]. Therefore, the shortest LMP2 TM (TM8; Fig. 1) might be a candidate for involvement in the perinuclear localization of LMP2 proteins. However, all the truncations that contained the eighth TM (TM1-10, TM3-12, and TM7-12) localized to the cell surface to the

**Fig. 3** wtLMP2b localizes to the trans-Golgi Network. (A) BJAB cells expressing wt LMP2bEGFP(*green*) were permeabilized and probed for Golgi markers (*red*): GM130, GS15, GS27, and Syntaxin 6. Overlapping pixels (*yellow* in 'Merge with Dapi') are also shown by exclusion, right. (B) Diagram of the Golgi apparatus with regions denoted on left, and markers for regions denoted on right. ERGIC: endoplasmic reticulum-Golgi intermediate compartment



**Fig. 4** LMP2b truncation mutants are expressed on the cell surface. BJAB cells transiently expressing (A) C1EGFP2BL11, (B) unpermeabilized cells expressing TM3-12, or TM7-12 with FLAG epitopes in loop 11, (C) unpermeabilized cells expressing TM3-12, or TM7-12 with FLAG epitopes in loop 10 and (D) permeabilized cells expressing TM3-12, or TM7-12 with FLAG epitopes in loop 10 were grown in coated chamber slides. Cells were chilled and probed for FLAG epitopes on the cell surface. Cells were then fixed and incubated with secondary antibodies (*center*, *red*). Bar, 5  $\mu$ m

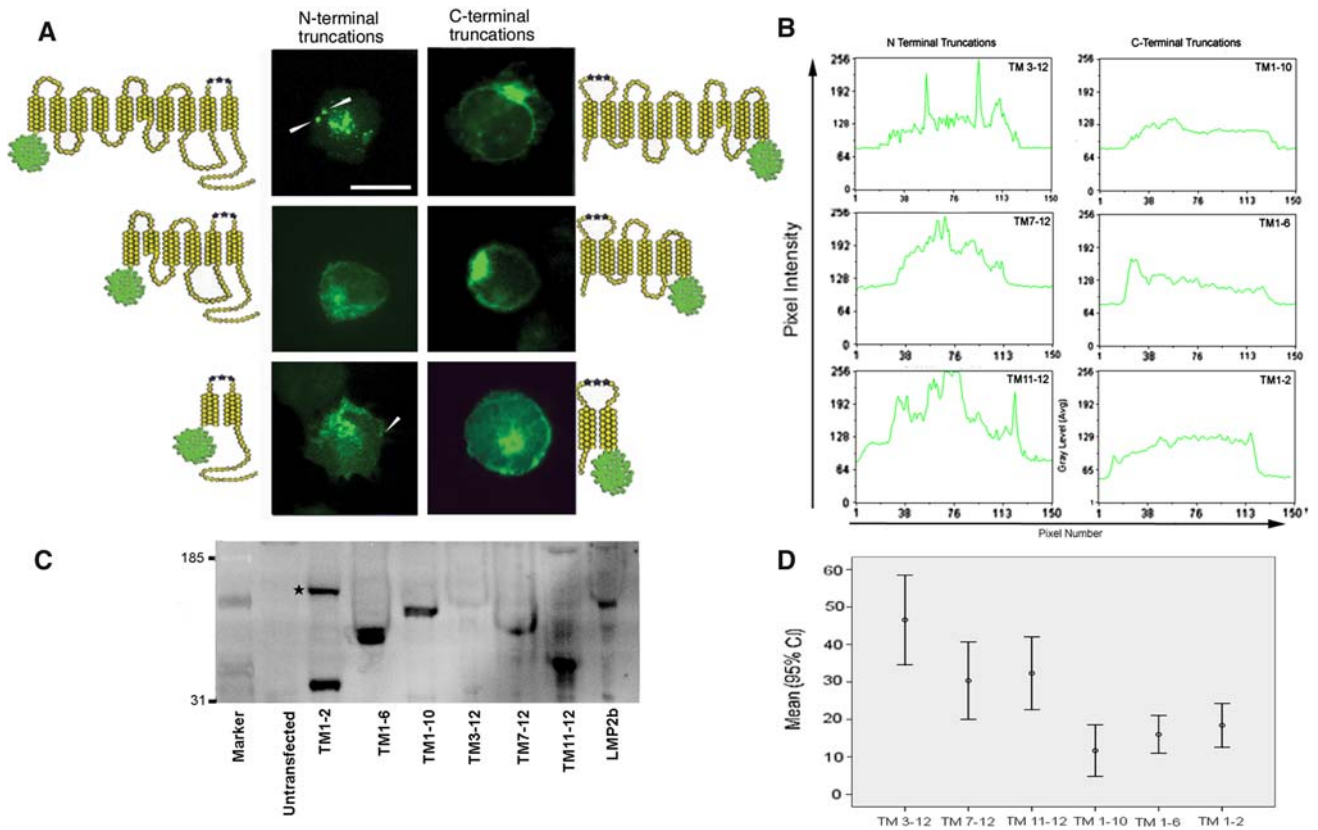


same degree as truncations (TM1-2, TM1-6, and TM11-12) that lacked this TM (Figs. 4 and 5).

The patchy surface expression of the N-terminal mutants suggested that if a C-terminal interaction were to occur between truncated molecules and perinuclear wtLMP2b, that the wtLMP2b might alter the localization of the truncated proteins. In cells co-transfected with DsRed2-tagged wtLMP2b and EGFP-tagged truncation mutants there was always some overlapping fluorescence in the perinuclear region (Fig. 6). We quantitated red, green, and overlapped pixel counts for each of the fluorescent channels. These data, generated from multiple micrographs to control for individual cell expression levels (Table 1), were then used to determine the percentage of green–red overlap (% co-localization). From this image analysis, the C-terminal truncations had a significantly higher percentage green–red overlap compared to N-terminal truncations (Kruskal–Wallis rank sum test,  $P = 0.035$ ). This shows that while an LMP2b–LMP2b C-terminal interaction was capable of clustering LMP2b proteins, a different interaction

appears to be required for trafficking and retention of LMP2b molecules in the intracellular perinuclear compartment. The critical region for this phenotype maps to the N-terminal TMs. Since some truncated molecules appear on the surface, retention seems likely to require multiple protein–protein interactions involving different regions of the intact LMP2b molecule.

The location of the truncation mutant proteins was compared to endogenous Golgi marker proteins (Syntaxin 6) and to an exogenous control cell surface protein (CD8zeta) co-transfected into BJAB cells (Fig. 7). In contrast to the TM mutants, wtLMP2b was not observed on the surface and co-localized with Syntaxin 6 (compare Fig. 3 with Fig. 7). wt2b does not interfere with CD8zeta surface expression [20]. All of the truncation mutants showed some co-localization with Syntaxin 6 indicating that the proteins had some localization in the area where LMP2b is typically found. The remainder of the fluorescence was targeted to the cell surface as indicated by co-localization with CD8zeta.



**Fig. 5** Truncations of LMP2b traffic to the cell surface; N-terminal truncations aggregate in the plasma membrane. (A) BJAB cells transiently expressing individual truncation mutants were fixed 24 h post-transfection and assessed for differences in localization patterns. Cells expressing the N-terminal truncations (green; TM3-12, TM7-12, and TM11-12, respectively) as indicated by the illustrations on the left. C-terminal truncations (TM1-10, TM1-6, and TM1-2) as indicated by the illustrations are on the right. N-terminal truncations exhibit a patch of green when compared to the more uniform green C-terminal truncations as indicated by white arrows. Bar, 5  $\mu$ . (B)

Line scan measurements of pixel intensity of micrographs in (A). Increased fluctuation indicates groupings of pixels with increased intensity. X-axis: Pixel Number, Y-axis: Pixel intensity. (C) 293T cells expressing LMP2b proteins were immunoprecipitated with anti-FLAG and analysed via Western Blot. The TM1-2 protein routinely showed a higher weight band corresponding to a multimer (starred). Sizes of the proteins are consistent with predicted size. (D) Statistical analysis of line scans, showing the mean plus 95% confidence intervals for each truncation mutant

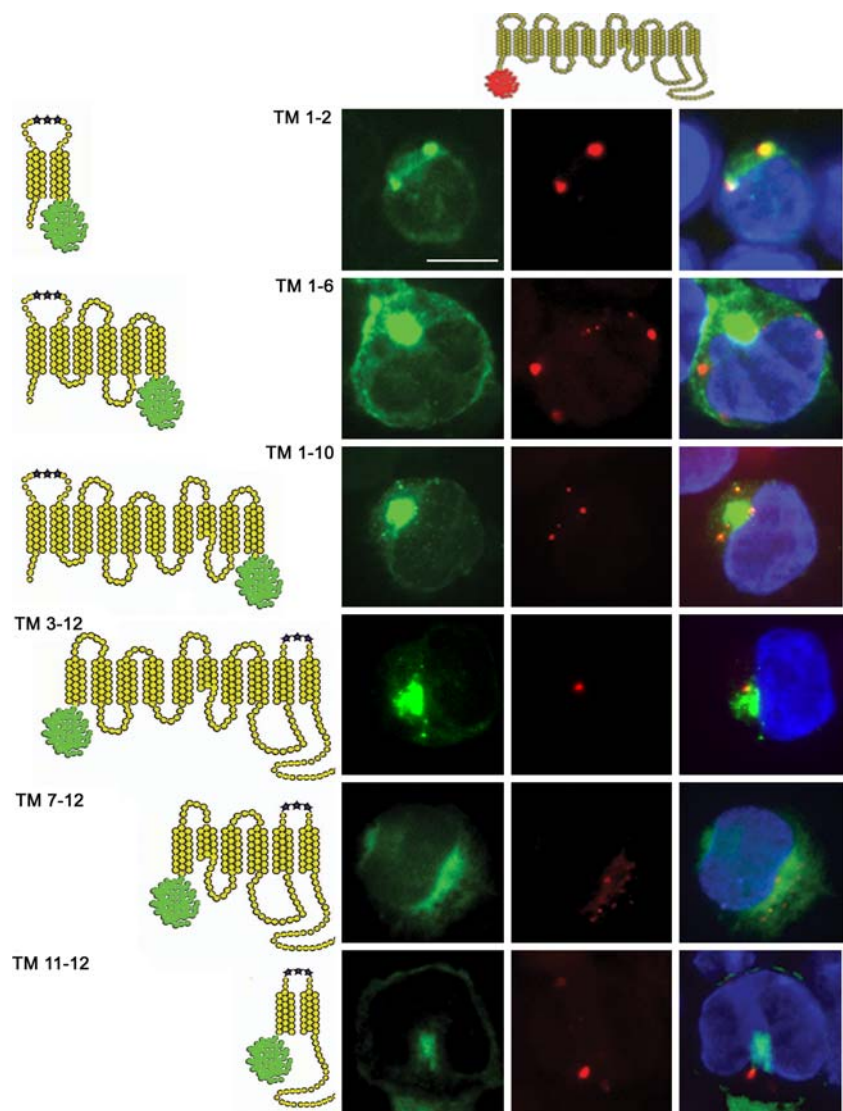
**Discussion**

Most studies of the LMP2 gene have focused on LMP2a isoform. Since LMP2b is essentially LMP2a without the CSD, it has been speculated that LMP2b may act as a negative regulator of LMP2a through interference with complex formation [25]. Also, due to the molecules' similar structures, there has been no means of tracking the two LMP2 protein isoforms independently. In recent studies, we introduced fluorescent tags and peptide epitopes into the LMP2 proteins to address the issues of protein detection and interaction. Fluorescently labeled and epitope-tagged LMP2a truncation mutants were used to determine that LMP2a requires an intact 12 TM region for intracellular localization [20]. The perinuclear localization of LMP2a has been independently confirmed in both B-lymphocytes and epithelial cells [22].

We have extended these studies with epitope tags in loop 10 and 11 and with N and C terminal truncations. The loop epitope tags were used to track and orient LMP2 proteins. The predicted membrane insertion structure of the LMP2 isoforms (proteins composed of 12 TM segments with alternating short extracellular and intracellular loops) has never been experimentally verified. In the studies described here, loop FLAG epitope-tagged truncation mutants all localized to the plasma membrane of live cells. All the predicted external loop tags were detected on the surface by anti-FLAG immunofluorescence while none of the internal loop tagged proteins were. All the results were in agreement with the predicted membrane orientation for the LMP2 loops and TMs.

The localization of LMP2a to a trans-Golgi network-like area might involve a retention signal. Several specific sequence motifs have been implicated in post-Golgi

**Fig. 6** Co-transfection of wtLMP2b with LMP2 truncation mutants. BJAB cells were transiently transfected with full-length DsRed2LMP2bF3 and one of the truncation mutants fused to EGFP (*green*). Diagrammatic representations of the mutant constructs are to the left of the panels. Red and Green images were merged with DAPI-stained nuclei (*blue*) in the panel on the right. Overlap between the truncated LMP2b (*green*) and full-length LMP2b (*red*) is indicated by yellow fluorescence. Bar, 5  $\mu$ m



**Table 1** LMP2b is able to sequester C-terminal truncation mutants more efficiently

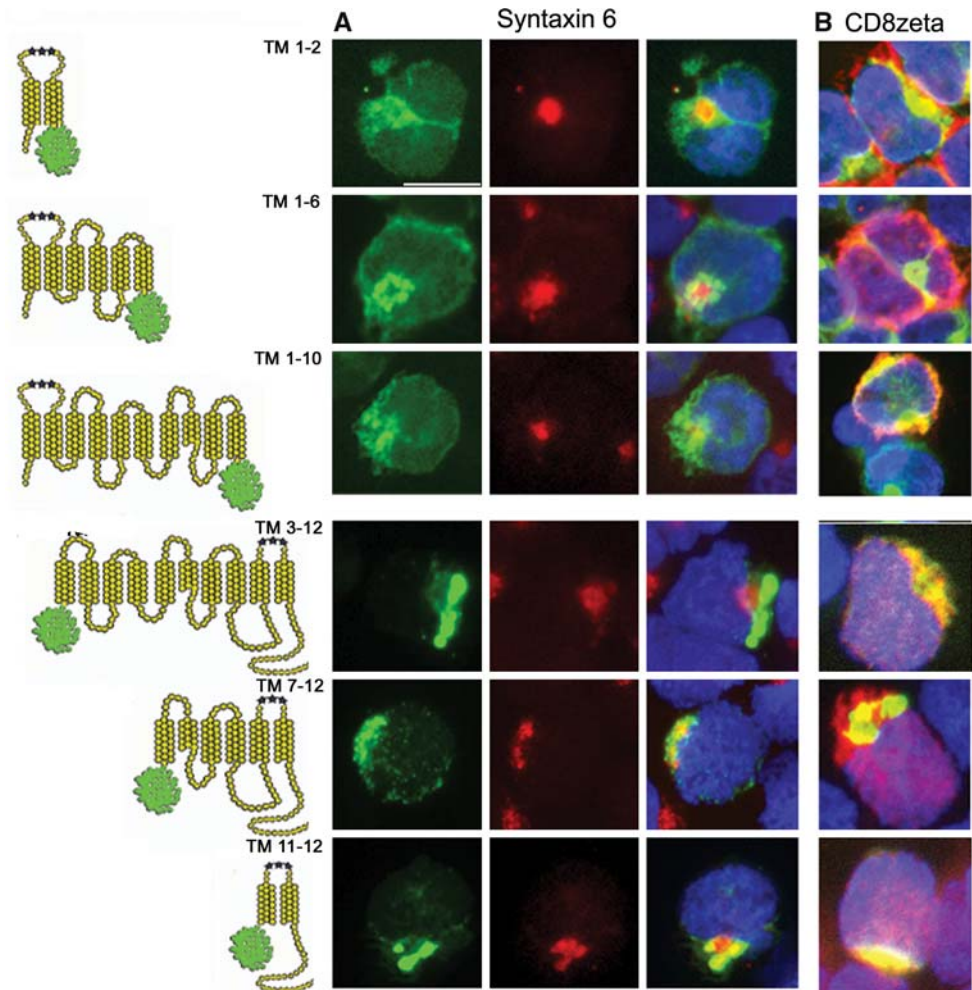
| Construct | Median (% overlap) | Ave. rank            | Z           |
|-----------|--------------------|----------------------|-------------|
| TM1-2     | 54.27              | 19.7*                | 2.34        |
| TM1-6     | 13.3               | 13.8*                | 0.77        |
| TM1-10    | 26.8               | 16.7*                | 1.48        |
| TM3-12    | 2.75               | 8.0                  | -1.66       |
| TM7-12    | 1.4475             | 6.6                  | -1.00       |
| TM11-12   | 0.51               | 7.7                  | -1.49       |
|           | $H=11.96$          | Total = 11.5<br>DF=5 | $P = 0.035$ |

Percentage of green pixels that equal red pixels based on mutant co-transfected. For each construct the number of overlapping pixels (yellow, see Fig. 6) was taken as a percentage of the total number of green pixels. These numbers were used in the Kruskal–Wallis mean rank sum test. Numbers above  $H$  denote a difference in mean

\*Constructs containing the N-terminal regions of LMP2b are being sequestered more efficiently by full-length LMP2



**Fig. 7** Co-localization of LMP2 of truncation mutants with cellular compartment marker proteins. **(A)** Transiently transfected BJAB cells were permeabilized and probed for Syntaxin 6 (a Golgi marker) to demonstrate that truncated LMP2b proteins (*green, left*) trafficked through the Golgi apparatus. Anti-Syntaxin 6 was detected with Alexa-fluor 594 labeled anti-mouse secondary antibody (*red, center*). Red and green images were merged with DAPI-stained nuclei (*blue*) in the panel on the right. Co-localization was indicated by yellow fluorescence. **(B)** BJAB cells were co-transfected with an LMP2b mutant protein (*green*) and CD8zeta (an exogenous cell surface marker). CD8zeta was detected with anti-CD8 and anti-mouse Alexa-fluor 594 (*red*). Diagrammatic representation of the mutants used in each row are to the left of the panels. Bar, 5  $\mu$ m



transport of membrane proteins [26, 27]. The best-recognized TGN retention motifs, YXX $\phi$  and DE, are not present in the LMP2 coding sequence. TGN retention may also be mediated by non-sequence related structural features such as coiled-coil motifs, which are used by resident Golgi proteins to remain in this organelle [28]. Sequence analysis and protein folding predictions for LMP2 proteins do not predict these motifs. Another feature, kin recognition, directs proteins to certain cisternae of the Golgi by the virtue of the length of the shortest TM segment [23]. Both isoforms of LMP2 contain the atypically short TM8 that could be implicated in retention via kin recognition. Our analysis of the localization of LMP2b truncations suggests that the presence of TM8 did not correlate with retention to a perinuclear location. A more specialized mutational analysis involving just alterations to TM8 will be required to determine if this TM (or any of the TMs) has a unique and critical role.

Previously, LMP2a was shown to have a ‘clustering’ domain in the 27 aa C-terminal tail [21]. This feature was analyzed by biochemical assays detecting multimerized

proteins in cell lysates. Our truncation mutant study indicated that the self-interaction domains previously mapped to the C-terminus of the protein were not sufficient for intracellular localization. Truncated proteins that had intact C termini were not sequestered in an intracellular compartment. However, consistent with the presence of a C terminal aggregation motif, only truncated proteins with intact C termini showed aggregates on the cell surface.

When the truncated proteins were expressed alone in cells, a portion of the protein was always detected in an intracellular perinuclear location suggesting that the mutants might be transiting slowly through the compartment in which wtLMP2b was localized. We performed co-transfection experiments to determine if a resident wtLMP2b could influence the localization of the truncation mutants. There was a significantly greater intracellular co-localization with LMP2b of C-terminal truncation mutants (TM1-2, TM1-6, and TM1-10) compared to N-terminal truncation mutants (TM3-12, TM7-12, and TM11-12). Thus, a previously unrecognized N-terminal region has been detected that is involved in either trafficking or

clustering (or both). The appearance of readily detectable N-terminal truncation mutant proteins on the surface indicates that the C-terminal tail is not alone sufficient to mediate internal sequestration.

The aggregative properties of the LMP2 proteins can be seen as having functional consequences. Due to the structural similarities between LMP2a and LMP2b, it has been suggested that LMP2b expression would have a functional effect on LMP2a. Speculation focuses on a model of LMP2b regulation of LMP2a through stochastic molecular interactions [18, 21]. In this model, the C-terminal clustering domain of LMP2b competes for clustering with LMP2a tails, decreasing LMP2a self-aggregation, phosphorylation, and downstream signaling. This clustering model of regulation is supported by our data showing that in addition to the C-terminal clustering domain, there is an N-terminal domain that is also involved in aggregation. An addition of a N-terminal clustering domain would add a block in protein multimerization closer to the CSD of LMP2a, which could add an additional impediment to signaling by this isoform.

Full-length LMP2 proteins are detected as a discrete patch of fluorescence in the perinuclear region of cells, a location normally ascribed to the Golgi network. Previously, we reported co-localization with gamma-adaptin, a marker of the late TGN and early endosomes, here we show full-length LMP2b co-localizing with another Golgi marker (Syntaxin 6), but excluding others. Truncated LMP2 molecules show partial overlap with Syntaxin 6, indicating that some, possibly transient, localization to a compartment specific for wtLMP2 molecules occurs. Truncation mutants trafficked to the cell surface where co-localization with a transiently expressed exogenous control (CD8zeta) was detected. This demonstrated that neither LMP2b nor the truncation mutants interfere with the ability of other proteins to traffic to the cell surface. Since the distribution of CD8zeta on the surface was not affected by the mutants of LMP2b, we found no evidence for a global reorganization of surface proteins. Thus, LMP2b appears to have a specific intracellular compartmental localization that can be disrupted by N or C terminal truncations affecting the transmembrane regions.

**Acknowledgement** The authors would like to thank Dr. L. Kingsley for expert statistical advice.

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