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High Molecular Weight Complex Analysis of Epstein-Barr Virus Latent Membrane Protein 1 (LMP-1): Structural Insights into LMP-1's Homo-Oligomerization and Lipid Raft Association

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Abstract

LMP-1 is a constitutively active Tumor Necrosis Factor Receptor analog encoded by *Epstein-Barr virus*. LMP-1 activation correlates with oligomerization and raft localization, but direct evidence of LMP-1 oligomers is limited. We report that LMP-1 forms multiple high molecular weight native LMP-1 complexes when analyzed by BN-PAGE, the largest of which are enriched in detergent resistant membranes. The largest of these high molecular weight complexes are not formed by purified LMP-1 or by loss of function LMP-1 mutants. Consistent with these results we find a dimeric form of LMP-1 that can be stabilized by disulfide crosslinking. We identify cysteine 238 in the C-terminus of LMP-1 as the crosslinked cysteine. Disulfide crosslinking occurs post-lysis but the dimer can be crosslinked in intact cells with membrane permeable crosslinkers. LMP-1/C238A retains wild type LMP-1 NF-κB activity. LMP-1's TRAF binding, raft association and oligomerization are associated with the dimeric form of LMP-1. Our results suggest the possibility that the observed dimeric species results from inter-oligomeric crosslinking of LMP-1 molecules in adjacent core LMP-1 oligomers.

Keywords

EBV; LMP-1; oligomerization; complex formation; BN-PAGE; Lipid Raft

1. Introduction

The human herpesvirus Epstein-Barr virus (EBV), is the causative agent of infectious mononucleosis and is causally associated with a number of human malignancies (Pagano et

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al., 2009). EBV infects B lymphocytes and profoundly alters their signaling environment via manipulation of key B cell signaling pathways (Raab-Traub et al., 2009).

The viral Latent Membrane Protein-1 (LMP-1) plays an essential role in EBV's regulation of B cell signaling. LMP-1 is a polytopic membrane protein with six predicted membrane-spanning domains (Dambaugh et al., 1984; Hudson et al., 1985). Originally thought to be localized exclusively in the plasma membrane (Dambaugh et al., 1984), LMP-1 can also be found in discrete patches, visible by fluorescence microscopy, in intracellular membrane compartments (Lam and Sugden, 2003; Liebowitz et al., 1986). Known as the EBV transforming protein, LMP-1 transforms rodent cells to anchorage independent growth and is required for EBV immortalization of primary human B cells (Kaye et al., 1993; Wang et al., 1985). LMP-1 functions in part as a constitutive CD40 mimic (Gires et al., 1997; Kilger et al., 1998; Mosialos et al., 1995). CD40 is a B cell surface receptor of the Tumor Necrosis Factor Receptor (TNFR) superfamily that plays an essential role in B cell proliferation, survival, and differentiation to memory (Xie et al., 2008). LMP-1 and CD40 bind to many of the same Tumor Necrosis Factor Receptor Associated Factors (TRAFs) via carboxy-terminal activating regions (CTARs) in their cytoplasmic C-termini and activate many of the same pathways (i.e. NF-κB and JNK) to elicit similar, but not identical, phenotypic changes in cells (Bishop et al., 2007; CareagaBrodeur et al., 1997; Careaga and Falke, 1992; Devergne et al., 1996; Kulwichit et al., 1998; Raab-Traub et al., 2009; Sandberg et al., 1997; Uchida et al., 1999; Xie et al., 2008). Unlike CD40, LMP-1 signaling is ligand-independent and constitutive.

Activated TNFRs, such as CD40, signal as TRAF-binding trimers from lipid raft microdomains (Hueber et al., 2002; Hueber, 2003; Muppidi et al., 2004; Vidalain et al., 2000). TNFR activation is the result of a combination of factors including ligand-induced trimerization, ligand-induced conformational changes, and formation of receptor aggregates (Siegel et al., 2000a). The extent of CD40 multimerization can determine the nature of the biological response (Baccam and Bishop, 1999; Fanslow et al., 1994; Pound et al., 1999).

Studies of LMP-1 activation and signaling have proven more difficult because of the constitutively active nature of LMP-1 signaling. A number of studies show a correlation between LMP-1's oligomerization and/or raft association and activation of NF-κB signaling (Ardila-Osorio et al., 1999; Ardila-Osorio et al., 2005; Coffin et al., 2003; Floettmann et al., 1998; Floettmann and Rowe, 1997; Hatzivassiliou et al., 1998; Higuchi et al., 2001; Lee and Sugden, 2007; Yasui et al., 2004). Little is known about the stoichiometry of LMP-1 homo-oligomers and the mechanism of their formation. The existence of LMP-1 homo-oligomers in the cell, prior to cell lysis, has yet to be demonstrated. Insight into the mechanisms of LMP-1's constitutive activation is essential for designing strategies to interfere specifically with LMP-1 signaling. Understanding LMP-1's activation mechanism will require in-depth analyses of the mechanism of LMP-1 oligomerization and raft association. Here we report that LMP-1 forms multiple native high molecular weight complexes in both tranfected B cells and EBV immortalized lymphoblastoid cells. We identify a cysteine disulfide-linked dimeric form of LMP-1. The relationship between the native LMP-1 complexes and the dimeric form of LMP-1 and their relevance to LMP-1 function, are explored, as are the implications for the stoichiometry of the LMP-1 homo-oligomer in LMP-1 signaling complexes.

2. Materials and Methods

2.1 Cell culture and cell lines

721 is an *in vitro* transformed EBV-positive lymphoblastoid cell line (Kavathas et al., 1980). DG75 is an EBV-negative Burkitt-like lymphoma cell line (Ben-Bassat et al., 1977). Both

cell lines were grown in RPMI supplemented with 10% bovine calf serum (R10C). HEK293T is a T antigen-positive human embryonic kidney cell line and was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (D10C). All cell lines were maintained at 37°C, in high humidity, with 5% CO_2.

2.2 Antibodies and reagents

Anti-LMP-1 antiserum is an affinity-purified polyclonal rabbit serum raised against LMP-1's C-terminus (residues 188–352) fused to glutathione-S-transferase. CS1-4 is a pool of monoclonal antibodies recognizing epitopes in LMP-1's C-terminus (Dako). The rabbit LMP-1 antibody was used for Western blotting unless specifically noted. Mouse monoclonal myc antibody (9E10) was from Santa Cruz Biotechnology, Inc. Anti-HA (HA-11) is a mouse monoclonal antibody from Berkeley Antibody Company (BabCo). Anti-TRAF3 (H20) is a goat polyclonal antibody (sc-948-G) from Santa Cruz Biotechnology, Inc. Anti-LMP-1 antibodies and horseradish peroxidase-conjugated secondary antibodies (Promega) were used at a dilution of 1:2500 for Western blotting. Antibodies (anti-TRAF3, anti-myc and anti-HA) were used at a concentration of 1 μg/ml for immunoprecipitation studies. Horseradish peroxidase conjugated secondary antibodies were from Promega. BMH (Bismaleimidohexane), EGS (ethylene glycol bis[succinimidyl]succinate), and TMEA (Tris-[2-maleimidoethyl]amine) were from Pierce. 1,10-Phenanthroline was from Sigma-Aldrich.

2.3 Plasmids

pCMV-LMP-1 and pCMV-ΔLMP-1/TMD5,6 are pCDNA3-based expression vectors encoding LMP-1 and the N-terminally truncated form of LMP-1 expressed during EBV's lytic cycle (referred to as lyLMP-1); ΔLMP-1/TMD5,6 encodes LMP-1 residues 129–386 which include the 5th and 6th transmembrane domains and cytoplasmic C-terminus (Erickson and Martin, 2000)). pCMV-LMP-1*myc* and pCMV-ΔLMP-1/TMD5,6*myc* encode C-terminal myc epitope tags. The following cysteine substitution mutants are all constructed in the pCMV-LMP-1 background: pCMV-LMP-1/C*sub*A1 has an alanine codon in place of cysteine 78 (C78A); pCMV-LMP-1/C*sub*A1,2 has alanine codons in place of cysteines 78 and 84 (C78A; C84A); pCMV-LMP-1/C*sub*A3 has an alanine in place of cysteine 116 (C116A); pCMV-LMP-1/C*sub*A1-3 has alanine codons in place of cysteines 78, 84, and 116 (C78A; C84A; C116A); pCMV-LMP-1/C*sub*A4 has an alanine codon in place of cysteine 238 (C238A); pCMV-LMP-1/C*sub*A1-4 has alanine codons in place of cysteines 78, 84, 116, and 238 (C78CA; C84A; C116A; C238A). Cysteine substitution mutants were generated by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis XL-II, Stratagene) and confirmed by sequencing. pRSV-LMP-1 (LMP-1), pRSV-LMP-1/TMD1,2 (LMP-1 deletion lacking TMDs 3–6, and pRSV-LacZ are pRC-RSV (Empty Vector) based expression vectors (Invitrogen) and have been described previously (Coffin et al., 2003; Geiger and Martin, 2006). pRSV-LMP-1/TMD1,6 (LMP-1 deletion lacking TMDs 2–5, with LMP-1 residue 51 (alanine) fused in frame to residue 166 (threonine); The cysteine substitution mutant C*sub*A4 was subcloned into pRC-RSV to generate pRSV-LMP-1/C*sub*4. p1242 is a luciferase reporter in which the luciferase gene is driven by the minimal *fos* reporter with 3 upstream kB binding sites from the MHC class I gene (Mitchell and Sugden, 1995). pRSV-LacZ encodes the lacZ gene in the pRC-RSV vector.

2.4 Transient Transfections

DG75 cells were electroporated in 0.4 cm gapped cuvettes using a Bio-Rad gene pulser (0.25 kV, 960 μF, 5×10^6 cells/0.35 ml R10C). HEK 293T cells were transfected using Mirus TransIT-293 transfection reagent according to manufacturer's instructions. Transfected cells were assayed two days post-transfection.

2.5 Membrane isolation

Cells were resuspended in hypotonic lysis buffer (10 mM HEPES-KOH, pH 7.9, 0.5 mM KCL, 0.5 mM $MgCl_2$, 0.1 mM ethylene glycol tetraacetic acid (EGTA), 0.5 mM DTT), incubated on ice for 30 minutes and triturated 10 times through a 26.5 gauge needle. The lysate was centrifuged at 13,000xg for 10 minutes (low speed spin) and the resulting supernatant was centrifuged at 105,000xg for 60 minutes (high speed spin) and the pellet was triturated and centrifuged at 10,000xg for 10 minutes. The pellet from the high-speed spin was combined with the low speed pellet in low salt buffer (LSB)(50 mM HEPES-KOH, pH 7.4, 100 mM B-glycerolphosphate, 25 mM NaF, 1 mM $MgCl_2$, 1 mM EGTA, 5% glycerol, 1 mM PMSF). This membrane preparation was used as the source of material for nonreducing SDS-PAGE (Fig. 1 and 2). For preparation of solubilized membranes, an equal volume of LSB/10% Triton X-100 was added to the membrane pellet and the solution was incubated on ice for 30 minutes before centrifugation at 13,000xg for 15 minutes. The supernatant from this spin was centrifuged at 100,000xg for 60 minutes and the resulting supernatant was used for experiments shown in Fig. 4B and 5).

2.6 BMH Crosslinking

721 cells (2×10^7 cells/μl) were resuspended in R10C containing 1 mM BMH and incubated at 37°C at 5% CO_2 for 1 hour. The reaction was quenched in 180 mM DTT for 10 minutes 37°C at 5% CO_2. Cells were then washed 2 times with 1xPBS, diluted 1:1 in 4x SDS sample buffer (100mM TrisHCL pH6.8, 5%SDS, 50mM DTT, 10% Glycerol, 0.02g Bromophenol Blue) with 50mM DTT, and heated to 85°C for 15 minutes.

2.7 Tris-[2-maleimidoethyl]amine (TMEA) and Ethylene glycol bis-(succinimidylsuccinate) (EGS) Crosslinking

721 cells (2×10^4 cells/μl) were lysed in Syn7 lysis buffer (50 mM $NaPO_4$, pH 7.4, 150 mM NaCl, 25 mM sucrose, 5% glycerol, 1% Triton X-100). Insoluble material was removed by centrifugation and the supernatant was used for crosslinking reactions. *EGS crosslinking:* EGS was added to the soluble material at final concentrations of 500 μM, 1mM and 1.5 mM (in DMSO; volume of EGS/DMSO was 5% of total volume of lysate) and incubated for 30 minutes at room temperature with rocking. The crosslinking reaction was quenched by the addition of 1M Tris, pH 7.5 to a final concentration of 20 mM followed by 15 minutes of incubation. *TMEA crosslinking:* TMEA was added to the soluble material after adjusting the pH to 7.0 (final TMEA concentrations of 125 μM, 250 μM and 375 μM (in DMSO; volume of TMEA/DMSO was 5% of total volume of lysate) and incubated at room temperature for 30 minutes. 4X SDS-sample buffer containing 50 mM DTT was added to crosslinked lysates (1:1 dilution) and samples were heated to 85°C for 15 minutes and sonicated.

2.8 NEM treatment

721 cells were pretreated with or without the membrane permeable, irreversible alkylating agent N-ethylmaleimide (NEM; 20 mM)(Pierce) at 10^6 cells/ml in R10C for 60 minutes at 37°C. Cells were washed in ice cold phosphate buffered saline (PBS) and homogenized with Dounce homogenizer (20 strokes) on ice in NEM lysis buffer (NLB; 50 mM NaH_2PO_4, pH 7.0, 300 mM NaCl, 20 mM NEM, 1% Triton X-100, protease inhibitors). This extract was diluted 1:1 with 4X nonreducing SDS-sample buffer, heated to 85°C for 15 minutes in the presence or absence of 50 mM DTT. For the experiment shown in Fig. 3B, 721 cells pretreated with and without NEM were lysed in NLB lacking NEM, incubated at 4°C for the indicated times and diluted 1:1 with 4X nonreducing SDS-sample buffer containing NEM. Samples were heated to 85°C for 15 minutes and analyzed by SDS-PAGE and Western blot for LMP-1. For the experiment shown in Fig. 6, 721 cells were treated with NEM as described above and fractionated through sucrose step gradients as described below.

2.9 SDS-PAGE and Western blotting

Samples were solubilized in 4X nonreducing SDS-sample buffer, sonicated and heated to 85°C for 15 minutes in the presence or absence (for nonreducing SDS-PAGE) of 50 mM DTT. Samples were then resolved on either 10% or 7.5% gels Tris/Glycine SDS page gels and transferred to Immobilon-P membranes (Millipore) as described previously (Coffin et al, 2001). Proteins were visualized on blots using the ECL Plus Western Blotting Detection System (GE Healthcare) following incubation with primary and secondary antibodies. Protein standards used were BenchMark Pre-Stained, SeeBlue Plus2 Pre-Stained Standard (Invitrogen) or Dual Color Precision Plus Recombinant Protein Standards (Bio-Rad).

2.10 NF-κB reporter assay

HEK293T cells were cotransfected with LMP-1 expression vectors, an NF-κB luciferase reporter, and the β-galactosidase reporter pRSV-lacZ. Each transfection was performed in duplicate. Cells were harvested two days posttransfection and cell lysates were assayed in triplicate by luciferase and β-galactosidase activity using the Tropix Dual Light assay system (Applied Biosystems). Luciferase relative light units are corrected for transfection efficiency by normalizing to β-galactosidase relative light units.

2.11 Immunoprecipitation

Solubilized membranes from 721 cells were used as starting material for immunoprecipitation analysis. Antibody was added to the precleared starting material, incubated for 30 minutes on ice, and immune complexes were recovered with Protein-G agarose (Roche Diagnostics). Beads were washed five times in LSB/0.035% Triton X-100 and heated to 85°C for 15 minutes in 4X nonreducing SDS-sample buffer.

2.12 Lipid raft assay

721 cells were pretreated with or without 20 mM NEM as described above and homogenized on ice with a Dounce homogenizer (10 strokes) in 1 ml of MNE (25 mM MES [morpholineethanesulfonic acid, pH 6.5], 150 mM NaCl, 5 mM EDTA) containing 0.2% Triton X-100. Extracts were diluted 1:1 in 80% sucrose/MNE, overlaid with 2 ml of 30% sucrose/MNE and 1 ml of 5% sucrose, and centrifuged at 4°C for 18 hours in an SW50.1 rotor at 200,000xg. Gradients were harvested from the top of the gradient in 400 μl fractions and mixed with 200 μl of 4X nonreducing SDS-sample buffer and heated to 85°C for 15 minutes in the presence or absence of 50 mM DTT.

2.13 Blue Native PAGE

1×10^7 cells were harvested with or without NEM treatment (see NEM treatement section). Cells were lysed on ice for 30 minutes in 500 μl of native blue lysis buffer (20 mM Bis-Tris, 500 mM ε-aminocaproic acid, 20 mM NaCl, 2mM EDTA, 10% glycerol, 0.2% Triton-X100 pH 7.0). Insoluble material was removed by centrifugation at 13,000 RPM for 10 minutes in a microfuge. For antibody gel shift experiments, antibody was added to lysates and rocked for 30 minutes at 4°C. 25 μl of 5% Coomassie G-250 was added to the lysis solution and allowed to rock for 30 minutes. Samples were loaded onto a 3%–18% Native PAGE precast gradient gel (Invitrogen). Gels were run with anode buffer (500 mM Bistris pH 7.0) and cathode buffer (150 mM BisTris, 500 mM Tricine, 0.2% Coomassie G-250 pH7.0). Halfway through the run the cathode buffer was replaced with cathode buffer lacking Coomassie G-250. Gels were run until the dye front reached the bottom of the gel (~3 hours). After electrophoresis, gels were soaked in Gel Soak buffer (48 mM Tris base, 39 mM Glycine, 0.25% SDS) for 30 minutes at 65°C, and transferred using Bio-Rad Semi Dry Blotter at 20V for 1 hour with Semi-Dry Transfer Buffer (48 mM Tris, 39 mM Glycine, 20% MeOH, 0.1% SDS). Cells were blocked and western blotted as previously described (Coffin et al., 2001).

2.14 Two Dimensional Gel Electrophoresis

After resolution in the first dimension (by BN-PAGE), lanes were cut out and soaked in 4xSDS sample buffer, allowed to equilibrate at room temperature for 20 minutes, heated in the microwave for 20 seconds, and then allowed to incubate for another 10 minutes at room temperature. Each gel lane was then placed horizontally into the well of an SDS-PAGE gel and run as described in the SDS-PAGE section.

2.15 Baculovirus generation and Flag-LMP-1-6xHis purification

Baculovirus vectors: Flag-LMP-1-6xHis/pFastBac1 was generated by the addition of a 6xHis tag to LMP-1's C-terminus in FLAG-LMP-1 by PCR and then the FLAG-LMP-1-6xHis cassette was cloned into pFastBac1 (Invitrogen). Flag-LMP-1-6xHis/pFastBac1 was transformed into DH10 Bac E. coli (Invitrogen) for recombination into its resident bacmid. FLAG-LMP-1-6xHis-bacmid was purified from DH10 Bac cells and transfected into Sf9 cells to generate Flag-LMP-1-6xHis expressing baculovirus. FLAG-LMP-1-6xhis baculovirus stocks were used to infect Sf9 cells and recombinant LMP-1 was purified as described below.

2.16 Recombinant Flag-LMP-1-6xHis expression and purification

120 ml of Sf9 cells at 2×10^6 cells/ml were infected with FLAG-LMP-1-6xHis baculovirus and incubated for 72 hours at 27°C with shaking at 130 revolutions/min. Harvested cells were washed twice with 1xPBS and lysed in 4 ml Syn7 lysis buffer (50 mM NaPO4, pH 7.4, 150 mM NaCl, 25 mM sucrose, 5% glycerol, 1% Triton X-100) with 1mM DTT and P8849 Protease Inhibitor Cocktail (Sigma) at 4°C by vortex for 45 min. Lysate was centrifuged in a refrigerated microfuge at 20,000xG for 15 min. Supernatant was filtered through a 0.45 μm filter and filtered lysate was purified over a NiNTA column according to manufacturers directions (Qiagen). NiNTA column-purified eluate was then purified over a FLAG M2 affinity column according to manufacturer's directions except the column buffers contained 0.65% Triton X-100 (Sigma). Column eluate (purified Flag-LMP-1-6xHis) was snap frozen in liquid nitrogen and stored at −80°C until use. Purity of Flag-LMP-1-6xHis was verified by Silver stain and Western blot for LMP-1.

3. RESULTS

3.1 Latent Membrane Protein-1 forms multiple high molecular weight complexes

LMP-1's activation of downstream signaling pathways is proposed to require homo-oligomerization, but the nature of the LMP-1 oligomer is unknown. The size and heterogeneity of native LMP-1 oligomeric complexes was examined to test the hypothesis that LMP-1 forms high order complexes to signal to its downstream effectors. 721 cells, an EBV positive lymphoblastoid cell line, were lysed and analyzed by Blue Native PAGE (BN-PAGE). BN-PAGE is well suited for resolving large native multi-protein complexes on the basis of shape and size. This method has been used successfully to characterize native mitochondrial complexes from cellular lysates and purified mitochondria membranes (Nijtmans et al., 2002; Schägger, 2001). Cells were lysed in hypotonic lysis buffer (containing Triton X-100) and resolved by BN-PAGE. At least two major LMP-1 immunoreactive bands migrating at ~440 kDa and ~669 kDa were resolved. Very high molecular weight immunoreactive material > 669kDa was also observed (Fig. 1). To verify that the high molecular weight complexes were not the result of post-lysis oxidation and crosslinking of LMP-1 monomers, 721 cells were pretreated with N-ethylmaleimide (NEM) prior to lysis. NEM is a membrane permeable alkylating agent that irreversibly and specifically alkylates free sulfhydryl groups at neutral pH and does not disrupt pre-existing disulfide bonds (Gorin et al., 1966). NEM treatment increased the resolution of high molecular weight LMP-1 immunoreative bands, suggesting that post-lysis oxidation of

sulfhydryls contributed to the relatively poor resolution of complexes in lysates from non-NEM treated cells. Importantly, the ladder of very high molecular weight bands (>669 kDa) observed in lysates from NEM treated cells does not result from post-lysis disulfide crosslinking.

3.2 High molecular weight LMP-1 immunoreactive complexes contain monomeric LMP-1 building blocks

To confirm that the high molecular weight LMP-1 immunoreactive species resolved by BN-PAGE correspond to LMP-1 containing native complexes and are not proteins that cross react with primary or secondary antibody during staining, the migration of complexes from lysates pre-incubated with anti-LMP-1 antibody was assessed (Fig. 2A). The addition of LMP-1 antibody to lysates prior to electrophoresis shifted complexes to a higher molecular weight. Lower molecular weight species (≤ 669 kDa) disappeared and new higher molecular weight species appeared following incubation of lysates with anti-LMP-1 antibody prior to BN-PAGE. Incubation with either an affinity purified polyclonal serum specific for LMP-1's C-terminus or a pool of monoclonal antibodies specific for epitopes in LMP-1's C-terminus generated the same gel shift pattern (data not shown). Importantly, pre-incubation with antibodies to GFP, a non-specific control antibody, had no effect on the migration of LMP-1 complexes (Fig. 2A). LMP-1 immunoreactivity could be depleted from lysates by incubation with anti-LMP-1 antibody followed by clearing with Protein-G agarose (Fig. 2B). To further characterize the LMP-1 content of each high molecular weight species observed in BN-PAGE gels, samples were resolved by BN-PAGE in one dimension and then resolved in a second dimension by SDS-PAGE. If the high molecular weight complexes contain LMP-1, immunoreactive dots migrating at the molecular weight of monomeric LMP-1 could be detected in the second dimension Western blot. High molecular weight complexes were resolved by BN-PAGE and then subsequently resolved by SDS-PAGE (Fig. 2C). Detection of monomeric LMP-1 in the second dimension from native complexes resolved in the first dimension shows that the higher molecular weight bands observed by BN-PAGE are composed of monomeric LMP-1 building blocks and are not the result of antibody cross-reaction with a cellular protein.

3.3 Native LMP-1 complexes are unequally distributed between detergent resistant membranes and detergent soluble fractions

LMP-1's constitutive activation of signaling is linked to its ability to localize in lipid raft microdomains (Ardila-Osorio et al., 1999; Ardila-Osorio et al., 2005; Coffin et al., 2003; Higuchi et al., 2001; Lee and Sugden, 2007; Yasui et al., 2004). Specifically, LMP-1's ability to activate NF-κB correlates tightly with its localization in detergent resistant membranes (DRMs). To ask if the association with DRMs reflects the presence of higher order LMP-1 megameric complexes, and to begin to determine if any of the native LMP-1 complexes resolved by BN-PAGE have active signaling potential, 721 cells lysates were fractionated into detergent resistant and detergent soluble fractions by sucrose gradient fractionation. Native LMP-1 complexes in gradient fractions were then resolved independently by BN-PAGE (Fig. 3A) and SDS-PAGE (Fig. 3B). SDS-PAGE analysis of gradient fractions showed LMP-1 was enriched in DRMs (fractions 2 and 3 of the gradient) and was also found in the bottom of the gradient in the detergent soluble fractions. Analysis of the same gradient fractions by BN-PAGE revealed that the highest molecular weight complexes (>669kDa) were found in DRMs, whereas the smaller complexes (<669kDa) were enriched in detergent soluble fractions. Although LMP-1 is found in both the detergent resistant and detergent soluble membrane fractions, the complement of native complexes within these fractions differs. These results appear to be consistent with a model that active LMP-1 signals as an oligomer from raft microdomains and active LMP-1 can form large multi-protein complexes.

3.4 LMP-1 is not sufficient for formation of very high molecular weight native complexes

The high molecular weight native LMP-1 complexes observed by BN-PAGE are likely to represent LMP-1 oligomers and associated cellular proteins. To ask whether any or all of the observed complexes are composed of "pure" LMP-1 homo-oligomers, baculovirus expressed Flag/LMP-1/6xHis was purified from insect cells and its migration in BN-PAGE and reducing SDS-PAGE gels was compared to the migration of LMP-1 expressed in transfected 293T cells (Fig. 4A). Both very high molecular weight LMP-1 complexes (> 669 kDa) and LMP-1 complexes migrating in the ~440 to 669 kDa range (lower molecular weight) were observed in lysates from 721 cells and 293T cells (Fig. 4A; compare to Fig. 1). In contrast, purified recombinant LMP-1 migrated exclusively with the lower molecular weight complexes (≤ 669 kDa). This suggests that formation of the larger of the high molecular weight complexes (>669 kDa) requires interaction with cellular factors or processes (post-translational modification, for example), and the complexes migrating at or below 669 kDa represent LMP-1 homo-oligomers. Analysis of LMP-1 from 293T cell extracts and purified recombinant LMP-1 by SDS-PAGE and Western blot demonstrated that LMP-1 degradation was not the cause of the lack of very high molecular weight complexes observed with recombinant LMP-1 (Fig. 4A). We observed however, a high molecular weight band of ~120 kDA in the recombinant LMP-1 sample (Fig. 4A, right), most likely due to a combination of the high concentration of purified protein and resulting incomplete reduction. Together, these results suggest that the smaller complexes (≤ 669 kDa) represent LMP-1 homo-oligomers and the high molecular weight complexes (> 669 kDa) found in DRMs are hetero-oligomers and/or require cellular processing to form the higher order homo-oligomers. Purified recombinant LMP-1 alone is sufficient for formation of the smaller native complexes but is not competent to form the highest molecular weight native complexes.

3.5 LMP-1's TMD is required for formation of high molecular weight native complexes

Thus far we have shown that LMP-1 is present in B cells in multiple high molecular weight native complexes. The functional relevance of these complexes was investigated by comparing the migration of LMP-1 to the migration of functional and non-functional transmembrane domain deletion mutants of LMP-1 by BN-PAGE (Fig. 4B). 293T cells were transfected and harvested 48 hours post transfection and extracts resolved by BN-PAGE and analyzed by Western blot (Fig. 4C). As observed for purified LMP-1 (Fig. 4A), transmembrane domain deletion mutants (LMP-1/TMD1,6; LMP-1/TMD1,2; ΔLMP-1/TM5,6) were unable to form the larger subset of higher molecular weight complexes. Uniformly, these mutants exhibit varying degrees of signaling deficiencies relative to full length LMP-1 (Coffin et al., 2003; Erickson and Martin, 2000) (unpublished results). Both LMP-1/TMD1,2 and LMP-1/TMD1,6 are compromised in their ability to activate NF-κB and inhibit IFNα signaling (Coffin et al., 2003) (not shown). ΔLMP-1/TM5,6 is unable to activate NF-κB signaling (Erickson and Martin, 2000; Mitchell and Sugden, 1995). These results suggest that the very high molecular weight complexes represent "active" LMP-1 signaling complexes. The fact that these very high molecular weight complexes are enriched in DRMs (Figs. 3 and 10), from where LMP-1 is believed to signal, is consistent with this conclusion. The smaller LMP-1 complexes (<669 kDa) may represent homo-oligomers of LMP-1 that are yet to form mature, active, signaling complexes.

3.6 An SDS-resistant, higher molecular weight form of LMP-1 is found in B cell extracts

To ask whether multimeric LMP-1 could be detected in cell extracts from B cells expressing LMP-1, we analyzed the migration of LMP-1 in nonreducing SDS-polyacrylamide gels. 721 cells were grown and harvested for SDS-PAGE gel analysis. LMP-1's apparent molecular weight on reducing SDS-polyacrylamide gels is ~60 kDa. We observed both the ~60 kDa species and an additional higher molecular weight LMP-1 species that migrated between the

85 kDa and 190 kDa markers (Fig. 5A) The observed SDS-resistance and sensitivity to reduction is consistent with a cysteine-disulfide linkage between two LMP-1 monomers or between LMP-1 and an interacting protein.

3.7 The higher molecular weight LMP-1 species is a dimer

Current models of LMP-1 signaling predict that LMP-1 signals as a constitutive trimer, akin to activated CD40. The higher molecular weight LMP-1 species described above could be a homo-dimer, a homo-trimer, or LMP-1 crosslinked to a cellular interactor of similar size. To distinguish these possibilities, the migration of LMP-1 and an LMP-1 deletion mutant lacking the last 55 amino acids from the carboxy-terminus were compared. LMP-1/CΔ55 was chosen because it encodes the entire hydrophobic transmembrane domain of LMP-1 and therefore retains the ability to homo-oligomerize (Coffin et al., 2001; Coffin et al., 2003). The higher molecular weight LMP-1 species in extracts from cells expressing LMP-1 migrated at a molecular weight of ~125 kDa on 10% nonreducing SDS-polyacrylamide gels (Fig. 5B), approximately twice that of the ~60 kDa LMP-1 monomer. Importantly, a higher molecular weight form of the truncated LMP-1 mutant (LMP-1/CΔ55) was also observed, and migrated with a molecular weight of ~85 kDa, approximately twice that of the LMP-1/CΔ55 monomer (~45 kDa). These results suggest that the slower migrating form of LMP-1 represents an LMP-1 homo-dimer.

3.8 Cysteine 238 is required for formation of the LMP-1 homo-dimer

Earlier results suggested that crosslinking of LMP-1 may occur post-lysis as pretreatment with NEM blocked formation of some high molecular weight complexes (Fig. 5). To test the possibility that these high molecular weight bands and the LMP-1 homodimer form because of oxidized cysteine residues LMP-1 mutants were created. LMP-1 possesses four cysteine residues, at positions 78, 84, 116, and 238 (referred to here as cysteines 1, 2, 3, and 4, respectively). Cysteine's 1–3 are located in the hydrophobic transmembrane domain while cysteine 4 is located in the cytoplasmic carboxy-terminus (Fig. 6A). Cysteine to alanine substitutions were introduced into the LMP-1 coding sequence with the goal of identifying which of the cysteine residue(s) participated in the disulfide linkage. Substitution of cysteines 1–3, either individually or in combinations (LMP-1/C*sub*A1, LMP-1/C*sub*A1,2, LMP-1/C*sub*A3 and LMP-1/C*sub*A1-3) had no effect on the formation of the dimeric form of LMP-1 (Fig. 6B). In contrast, LMP-1 mutants with cysteine 4 replaced with alanine (LMP-1/C*sub*A4), or substitutions of all four cysteines with alanines (LMP-1/C*sub*A1-4), were unable to form the crosslinked dimeric species. These results suggest strongly that the cytoplasmic cysteine 238 is responsible for the formation of the dimeric form of LMP-1.

3.9 Cysteine 238 is not required for activation of NF-κB by LMP-1

To test the requirement of cysteine 238 in LMP-1 signaling, NF-κB activation by LMP-1/*Csub*A4 was compared to NF-κB activation by LMP-1 using an NF-κB reporter assay. HEK 293T cells co-transfected with LMP-1 expression vectors and an NF-κB luciferase reporter were assayed for luciferase accumulation (Fig. 6C). LMP-1 stimulated NF-κB activity ~13 fold relative to empty vector. LMP-1/C*sub*A4 activated NF-κB to wildtype LMP-1 levels whereas the negative control, ΔLMP-1/TMD5,6, was inactive. ΔLMP-1/TMD5,6 (often referred to as lyLMP-1) is a nonfunctional N-terminally truncated form of LMP-1 lacking the cytoplasmic N-terminus and first 4 transmembrane domains of LMP-1 (Baichwal and Sugden, 1989; Hudson et al., 1985; Wang et al., 1988). These results demonstrate that cysteine 238 is not essential for LMP-1's CTAR dependent signaling to NF-κB.

3.10 Disulfide crosslinking of cysteine 238 is blocked by N-ethylmaleimide

The lack of requirement of cysteine 238 in activation of NF-κB by LMP-1 suggested that formation of active LMP-1 signaling complexes does not depend on the formation of a disulfide crosslink at cysteine 238. The observed disulfide crosslinking could occur fortuitously if the β carbons of two cysteine 238 residues in adjacent LMP-1 monomers are in close enough proximity (within 3.4 to 4.6 angstroms) to allow disulfide bond formation (Balaji et al., 1989; Careaga and Falke, 1992). To address whether the observed disulfide-linked LMP-1 dimers were formed before or after cell lysis, 721 cells were treated with NEM prior to harvest. Live cells were treated with 20 mM NEM for 60 minutes prior to harvest and analyzed by nonreducing SDS-PAGE and Western analysis. NEM pretreatment blocked the formation of the LMP-1 dimer, indicating that disulfide bond formation occurred post cell lysis (Fig. 7A). To determine the time course of disulfide bond formation after cell lysis, 721 cells were treated with and without 20 mM NEM for 60 minutes and lysates were incubated at 4°C for up to 24 hours (Fig. 7B). Dimeric LMP-1 is undetectable 20 minutes post-lysis and peak levels are detected by 24 hours. As in Fig. 7A, formation of the dimeric form of LMP-1 is completely blocked by pretreatment with NEM. These results demonstrate that LMP-1 monomers are not covalently linked via disulfide bonds within the cell prior to lysis. Rather, cysteine disulfide crosslinking occurs following cell lysis and the extent of crosslinking increases with time following lysis. These results are not inconsistent with the relative red/ox state of the cytoplasm versus the cell lysate or with the formation of LMP-1 oligomers prior to cell lysis. Prior to cell lysis, LMP-1's carboxy-terminus is localized within the reducing environment of the cytosol. The oxidizing environment encountered post-lysis, promotes covalent linkage between sulfhydryl groups in close enough proximity to allow disulfide bond formation (i.e. between interacting LMP-1 monomers within an LMP-1 oligomer).

To test the hypothesis that LMP-1 monomers physically interact in the cell prior to lysis, 721 cells were treated with the cell-permeable, sulfhydryl-reactive, bifunctional crosslinker bismaleimidohexane (BMH). BMH has a 13.0 angstrom spacer arm. The dimeric LMP-1 species was observed in 721 cells pre-treated with BMH before lysis and analyzed by SDS-PAGE and Western blot (Fig. 8A). Thus, the observed LMP-1 dimeric species pre-exists in cells prior to lysis and can be crosslinked by the addition of a chemical crosslinker. These results demonstrate that LMP-1 C-termini are within crosslinkable proximity in intact cells, and that the formation of the crosslinked dimer post-lysis is not due to nonspecific aggregation followed by oxidation of cysteines.

3.11 Formation of higher molecular weight LMP-1 species upon crosslinking sulfhydryl and amine groups in LMP-1

Disulfide bonding between adjacent cysteine 238 residues depends upon their close proximity to one another (within 3–5 angstroms). LMP-1 molecules within oligomeric complexes are likely to make intermolecular contacts mediated by noncovalent interactions that occur over greater distances. We asked whether additional higher molecular weight LMP-1 species could be crosslinked by the addition of bifunctional or trifunctional crosslinkers. We chose to use the amine-specific bifunctional crosslinker ethylene glycol bis-(succinimidylsuccinate) (EGS; 16.1 angstrom spacer arm) and the sulfhydryl-specific crosslinker Tris[2-maleimidoethyl]amine (TMEA; 10.3 angstrom spacer arm). TMEA was chosen because it is trifunctional and thus has the potential to crosslink three adjacent sulfhydryls (i.e. three cysteine 238 residues in a potential trimer).

Solubilized extracts of 721 cells were incubated with EGS or TMEA and analyzed by SDS-PAGE and Western blotting (Fig. 8B). TMEA crosslinking was efficient in that the majority of LMP-1 was shifted to a higher molecular weight species. The predominant species

detected following TMEA treatment was dimeric LMP-1. No evidence of a trimeric form of LMP-1 was detected, although there appeared to be additional, lower abundance, higher molecular weight species. The predominant species observed in EGS crosslinked samples was of very high molecular weight (greater than 250 kDa and beyond the resolution of the gel); low levels of the dimeric LMP-1 species was also observed in EGS crosslinked samples. The size of high molecular weight species varied depending on the type of crosslinker used. If nonspecific aggregation of LMP-1 were occurring upon cell lysis, we predict that we would consistently see a very high MW species that fails to enter the gel, independent of the type of crosslinker used. These results demonstrate that LMP-1 forms oligomeric complexes that can be captured in SDS-soluble dimeric units by sulfhydryl crosslinking and in a high molecular weight multimeric form(s) by crosslinking of amine groups.

3.12 Homo-oligomerization correlates with disulfide-crosslinking

If formation of the dimeric form of LMP-1 is a reflection of LMP-1's oligomeric status in the membrane, then dimeric forms of non-oligomerizing LMP-1 mutants of LMP-1 should be undetectable via nonreducing SDS-PAGE. We tested this prediction by comparing homo-oligomerization and disulfide-crosslinking properties of LMP-1 and the nonfunctional LMP-1 mutant ΔLMP-1/TM5,6. ΔLMP-1/TM5,6 cannot oligomerize with itself (submitted) or with LMP-1 (Yasui et al., 2004). DG75 cells were cotransfected with expression vectors encoding LMP-1 and LMP-1*myc* or ΔLMP-1/TM5,6 and ΔLMP-1/TM5,6*myc*. ΔLMP-1*myc* and LMP-1/TM5,6*myc* were immunoprecipitated from solubilized membranes and analyzed by SDS-PAGE and Western blot for LMP-1 and LMP-1/TMD5,6 (Fig. 9A). As expected, LMP-1 is detected in the myc immunoprecipitate from LMP-1 and LMP-1*myc* coexpressing cells, and ΔLMP-1/TM5,6 is not found in the myc immunoprecipitate from ΔLMP-1/TM5,6 and ΔLMP-1/TM5,6*myc* coexpressing cells. Importantly, although dimeric LMP-1 is detected in extracts from LMP-1 expressing cells, dimeric ΔLMP-1/TMD5,6 was not present in extracts from ΔLMP-1/TMD5,6 expressing cells (Fig. 9B). These results and the finding that LMP-1/CΔ55 (an oligomerizing deletion mutant) can be resolved in a disulfide-linked dimeric form (Fig. 5) suggest that disulfide-linked dimer formation correlates with homo-oligomerization.

3.13 TRAF3 selectively co-purifies with dimeric LMP-1

A large body of evidence suggests TRAF-mediated LMP-1 signaling requires constitutive homo-oligomerization of LMP-1 (Floettmann et al., 1998; Floettmann and Rowe, 1997; Gires et al., 1997; Hatzivassiliou et al., 1998). Detection of disulfide-linked LMP-1 dimers provides strong evidence that individual LMP-1 molecules in the membrane are in close enough proximity to form crosslinks between adjacent cysteine 238 residues. Disulfide-linked LMP-1 dimers could represent two LMP-1 molecules in an individual TRAF-binding LMP-1 oligomer, or, LMP-1 molecules from two adjacent TRAF-binding LMP-1 oligomers. We have shown that LMP-1 forms multiple native LMP-1 complexes we next asked if TRAF3 selectively associates with disulfide-linked dimeric LMP-1 in detergent extracts of EBV-positive 721 cells. TRAF3 was immunoprecipitated from 721 cell lysates and the immunoprecipitates analyzed by nonreducing SDS-PAGE and Western blot for LMP-1 (Fig. 9C). The monomeric and dimeric forms of LMP-1 were present in the starting material in a 1:1 ratio. TRAF3 immunoprecipitates were dramatically enriched for dimeric LMP-1, while LMP-1 was not detected in the negative HA immunoprecipitation control. Dimeric LMP-1 in the starting material and in the TRAF3 immunoprecipitate was reduced to the monomeric form upon treatment with DTT. These results suggest that dimeric LMP-1 is representative of the TRAF-binding oligomeric form of LMP-1 in EBV-immortalized B cells. Taken together with the finding that disulfide-linked dimer formation correlates with homo-

oligomerization (Fig. 9A and 9B), these results support a model in which the dimeric form of LMP-1 represents the active, TRAF binding LMP-1 oligomer.

3.14 The dimeric form of LMP-1 is restricted to lipid rafts

The association between oligomerization, disulfide-linked dimer formation, and the co-immunopreciptiation of TRAF3 with the dimeric form of LMP-1 (Fig. 6 and 9) suggests that the dimeric form of LMP-1 is a "marker" for the active, TRAF-binding LMP-1 oligomer. LMP-1's constitutive activation of signaling is linked to its ability to localize in lipid raft microdomains (Ardila-Osorio et al., 1999; Ardila-Osorio et al., 2005; Coffin et al., 2003; Higuchi et al., 2001; Lee and Sugden, 2007; Yasui et al., 2004). Specifically, LMP-1's ability to activate NF-κB correlates tightly with its localization in detergent resistant membranes (DRMs). We asked whether dimeric LMP-1 selectively partitioned with DRMs in sucrose gradients in an attempt to link the formation of disulfide-linked dimers to LMP-1's signaling activity. 721 cells were treated with or without NEM for 60 minutes and then detergent lysates were analyzed by flotation in sucrose step gradients. Gradient fractions were analyzed by reducing and nonreducing SDS-PAGE and Western blot for LMP-1 (Fig. 10). As expected, LMP-1 is found in the DRM fractions and the detergent soluble fractions. The dimeric form of LMP-1 is found only in extracts from non-NEM treated cells and is restricted to the DRM fractions. In addition, we observe LMP-1 in the DRM fraction is independent of NEM treatment, demonstrating that LMP-1's ability to fractionate with DRMs is not a result of post-lysis crosslinking. These results suggest strongly that raft-associated LMP-1 is in oligomeric complexes that bring the carboxy-termini of adjacent LMP-1 molecules together to allow crosslinking to occur when extracts are oxidized following lysis. These results agree with our earlier results that high molecular weight oligomers can be found in detergent resistant membranes and that the crosslinked dimeric LMP-1 species found in DRMs is likely to arise from very high molecular weight native LMP-1 oligomers.

4. DISCUSSION

LMP-1's constitutive signaling activity depends on its homo-oligomerization and lipid raft association, both of which are mediated by LMP-1's amino-terminus and transmembrane domains. We have identified that LMP-1 participates in forming multiple high molecular weight complexes (Fig. 1). These complexes are specific for LMP-1 (Fig. 2). BN-PAGE analysis demonstrates that the largest subset of the high molecular weight complexes are enriched in the detergent resistant fractions of sucrose gradients (rafts) while the smaller complexes are enriched in the soluble fractions (Fig. 3). Importantly, nonfunctional LMP-1 mutants are enriched in the detergent soluble fractions and form only the smaller native complexes (Coffin et al., 2003) (Fig. 4). These results are consistent with the model that the higher molecular weight complexes contain (>669 kDa) complexes represent 'active' LMP-1 signaling complexes. Unlike LMP-1 from cell extracts, recombinant LMP-1 purified from Sf9 cells is not able to form the higher molecular weight complexes, suggesting that the larger complexes are hetero-oligomers composed of cellular proteins and/or requires processing within the cell (Fig. 4). In addition to the high molecular weight complexes we have identified a homo-dimeric form of LMP-1 that is stabilized via disulfide bond formation between cysteines within LMP-1's cytoplasmic carboxy-terminus upon cell lysis (Fig. 5 and 6). This dimeric species is crosslinkable by treatment of living cells with a cell permeable crosslinker, demonstrating that the C-termini of at least two adjacent LMP-1 monomers are in close enough proximity to crosslink in intact cells (Fig. 8A). The fact that cysteine 238 can be crosslinked via disulfide formation provides direct evidence that a minimum of two LMP-1 monomers interact, either as homo-dimers or as part of a larger homo-oligomeric complex, in the intact membrane environment. This assertion is supported by two findings. First, LMP-1 dimers and larger species are observed following crosslinking

with the trifunctional sulfhydryl-specific crosslinker TMEA (Fig. 8B). Second, LMP-1 variants that have been shown to homo-oligomerize via coimmunoprecipitation from solubilized cell extracts can form disulfide-linked homo-dimers while non-oligomerizing LMP-1 variants cannot (Fig. 5 and 9). Nonreducing SDS-PAGE analysis of TRAF3 immunoprecipitates demonstrates that dimerized LMP-1, but not monomeric LMP-1, copurifies with TRAF3 (Fig. 9). Importantly, the disulfide-linked dimeric form of LMP-1 is restricted to DRMs (lipid rafts) in EBV-immortalized B cells (Fig. 9). Based on these results, we believe that the crosslinked dimeric form of LMP-1 is a "marker" of functional LMP-1 oligomers. Multiple high molecular weight native LMP-1 complexes can be visualized by BN-PAGE analysis of 721 cells (Fig. 10).

Crosslinking studies reveal that actively signaling LMP-1 in DRMs is organized minimally into dimers. Consistent with the crosslinking results, oligomerization in the membrane is a common mechanism by which cell surface receptors initiate signaling in response to ligand binding. Ligand activation of many immune receptor classes triggers their association with raft microdomains where they can interact with, or recruit, critical signaling molecules to initiate signal transduction (Bouillon et al., 2003; Cheng et al., 2001; Eren et al., 2006; Grassme et al., 2002; Hueber et al., 2002; Kovacs et al., 2005; Pierce, 2002; Sadra et al., 2004; Vidalain et al., 2000). LMP-1 is unusual in this regard as it is a ligand-independent "receptor" and signals as a constitutive raft-associated oligomer.

The structure and dynamics of the ligand-activated oligomerization of members of the TNFR superfamily have been extensively studied. It has long been appreciated that trimerized TNFR1 interacts with trimerized TNFα ligand (Banner et al., 1993; Loetscher et al., 1991; Pullen et al., 1999). TNFRs have also been shown to self-assemble into trimers in a ligand-independent fashion via their pre-ligand-binding assembly domains (PLADs) (Chan et al., 2000; Clancy et al., 2005; Papoff et al., 1999; Siegel et al., 2000b). Ligand-activated trimeric TNFRs form higher order multimeric complexes as well. The degree of TNFR multimerization can dictate the strength and specificity of TRAF binding and thereby affect downstream signaling outcome (Baccam and Bishop, 1999; Fanslow et al., 1994; Liu et al., 2002; Pullen et al., 1999) CD40 signaling activity has been linked not only to trimerization but to the formation of CD40 dimers (Girouard et al., 2005; Reyes-Moreno et al., 2004; Reyes-Moreno et al., 2007; Werneburg et al., 2001). Preformed disulfide-linked CD40 homo-dimers are found at low levels in EBV-transformed B cells and their levels dramatically increase following co-culture with CD40L-expressing fibroblasts or treatment with soluble trimeric CD40L. Formation of the disulfide-linked homo-dimer is required for CD40L activation of phosphatidylinositol 3-kinase and IL-8 secretion (Reyes-Moreno et al., 2004; Reyes-Moreno et al., 2007). As we have observed with disulfide-linked LMP-1 dimers (Fig. 3 and 10), CD40L-induced disulfide-linked homo-dimers are restricted to DRMs and the target for disulfide oxidation is a cysteine residue in CD40's cytoplasmic signaling domain, just downstream of the PXQXT TRAF binding motif (Reyes-Moreno et al., 2007).

Less is known about the oligomeric structure of LMP-1. A large body of evidence suggested early on that LMP-1 functions as a constitutively active, oligomeric receptor (Baichwal and Sugden, 1987; Devergne et al., 1996; Hennessy et al., 1984; Huen et al., 1995; Laherty et al., 1992; Liebowitz et al., 1986; Mann et al., 1985; Mann and Thorley-Lawson, 1987; Martin and Sugden, 1991; Mitchell and Sugden, 1995; Mosialos et al., 1995; Sandberg et al., 1997). This hypothesis has since been supported by results from chimeric analyses (Floettmann et al., 1998; Floettmann and Rowe, 1997; Gires et al., 1997; Hatzivassiliou et al., 1998), as well as structure/function and coimmunoprecipitation studies (Gires et al., 1997). Interestingly, studies comparing the effects of forced trimerization and/or lipid raft targeting of LMP-1's cytoplasmic C-terminus on LMP-1 signaling output raised the question that

LMP-1's lipid raft association may play a more significant role in signaling activation than does LMP-1 oligomerization (Kaykas et al., 2001). The stoichiometry of LMP-1 functional units (i.e. dimer, trimer etc.) has not been defined and the possibility that these functional units organize into multimerized oligomers has not been explored.

Together, our results shed light on the nature of the LMP-1 oligomer and are consistent with the hypothesis that active LMP-1 signaling complexes are oligomerized and raft-associated. Cysteine 238 is within ~24 residues of the TRAF binding consensus in CTAR1 (Devergne et al., 1996). LMP-1's core TRAF3 binding peptide (P_{204}QQATDD$_{210}$) binds to the same binding crevice on TRAF3 as does CD40 (Wu et al., 2005), suggesting that LMP-1 and CD40 interact with TRAF trimers similarly. If LMP-1 and CD40 interact with trimeric TRAFs in a similar fashion, why do we see a crosslinked dimeric, and not a trimeric, LMP-1 species? TRAF2 trimers bind to CD40 within the interior of trimerized carboxy-terminal TRAF binding domains (McWhirter et al., 1999). If trimeric TRAF3 binds to the CTAR1 motifs of trimerized LMP-1 in an analogous fashion, it is unlikely that cysteine 238 of two adjacent LMP-1 carboxy-termini of a TRAF3-bound LMP-1 trimer could be within the necessary 3–5 angstrom disulfide-crosslinkable distance of each other. The proximity of cysteine 238 to CTAR1, together with the unstructured nature of its cytoplasmic domain, suggests strongly that cysteine 238 is unlikely to form intra-trimer disulfide links.

Our findings are consistent with a model in which cysteine 238 of adjacent LMP-1 trimers in a TRAF-binding LMP-1 multimer, or "supercluster", are within disulfide-crosslinkable distance (Fig. 11). The formation of multimers of TRAF-binding trimers is consistent with what is known about TNFR multimers and also with the very large size of EGS crosslinked LMP-1 species (Fig. 8B) and of native LMP-1 complexes (Fig. 1). This model suggests the interesting possibility that the "core" LMP-1 trimer is localized in the "bulk" membrane and associates with lipid rafts only upon formation of multimeric complexes. Consistent with this is our finding that dimeric LMP-1, which most likely represents multimeric LMP-1 complexes, is found only in the raft fraction of sucrose flotation gradients and the fact that a considerable amount of LMP-1 is found in the non-raft fraction (Ardila-Osorio et al., 1999; Ardila-Osorio et al., 2005; Clausse et al., 1997; Coffin et al., 2003; Higuchi et al., 2001; Lee and Sugden, 2007; Yasui et al., 2004). This model raises several key questions. Does the putative core LMP-1 trimer have signaling activity? If so, what is the relationship of this signaling (both quantitatively and qualitatively) to LMP-1 signaling from raft-associated superclusters? Are the sequence requirements for formation of the core LMP-1 trimer distinct from those governing the formation of the LMP-1 supercluster? Does LMP-1 actively promote clustering of microdomains to potentiate its own signaling? Experiments are in progress to address these questions with the goal of better understanding native LMP-1 complex formation.

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Abbreviations

EBV	Epstein Barr Virus
LMP-1	Latent Membrane Protein-1

TRAF	Tumor Necrosis Receptor Associated Factor
BN-PAGE	Blue Native Polyacrylamide Gel Electrophoresis
IFNα	Interferon alpha
TMEA	*Tris-[2-maleimidoethyl]amine*
EGS	ethylene glycol bis[succinimidylsuccinate
BMH	Bismaleimidohexane
NEM	N-ethylmaleimide

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Highlights (for review)

LMP-1 forms multiple high molecular weight complexes as resolved by BN-PAGE.

High molecular weight forms of LMP-1 correlate with activation of signaling.

Higher order LMP-1 signaling complexes signal from lipid rafts.

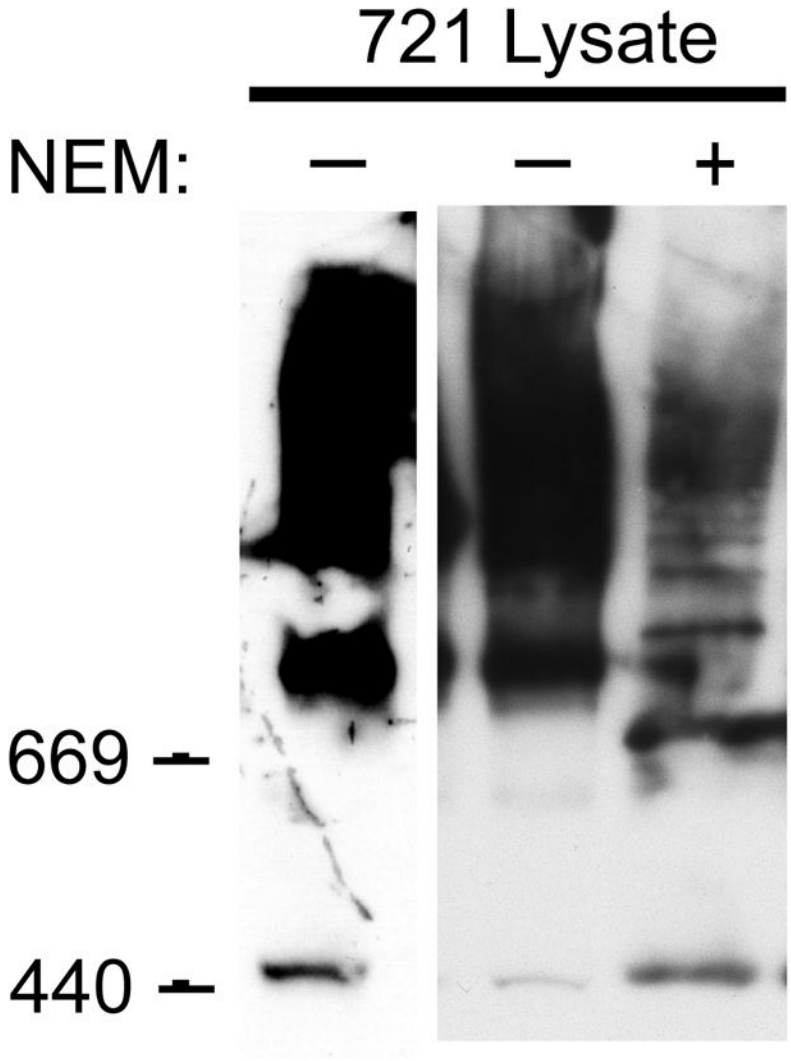

Fig. 1.

LMP-1 forms multiple high molecular weight native complexes. 721 cells (4×10^5 cells) pre-treated with or without NEM for 30 minutes, were lysed and resolved in 3–15% gradient gels by BN-PAGE and analyzed by Western blot for LMP-1 using rabbit polyclonal antibody for LMP-1 C-terminus. The migration of protein standards (in kDa) is noted to the left of the blot, and treatment with NEM is noted above the blot.

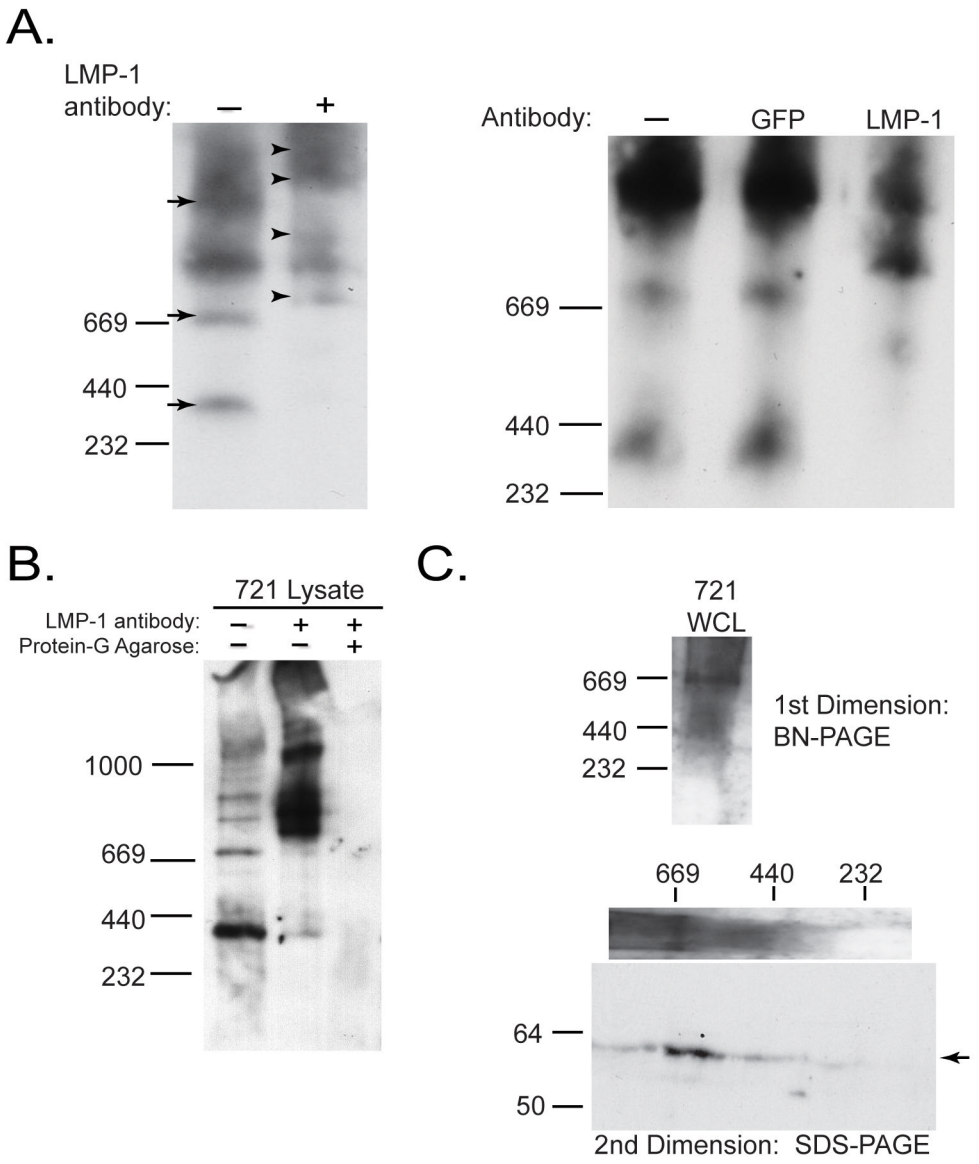

Fig. 2.

Complexes resolved by BN-PAGE contain LMP-1 *A)* 721 lysates (prepared as described in Fig. 1) were incubated with LMP-1 specific antibodies (right and left blots) or with anti-GFP antibody (right blot) for 1 hour prior to BN-PAGE and Western analysis for LMP-1. Arrows mark immunoreactive complexes that are shifted upon addition of antibody, arrowheads indicate the position of representative bands of immunoreactivity shifted from antibody binding. Addition of antibody is noted above each blot. *B)* 721 cell lysates were incubated with LMP-1 specific antibodies for 1 hour followed by addition of Protein-G agarose beads for 30 minutes. Beads were removed by centrifugation, and the resulting supernatant was resolved by BN-PAGE and analyzed by Western blot for LMP-1. The addition of antibody (−/+) and protein G-agarose (−/+) is noted above the blot. *C)* 2D PAGE analysis of high molecular weight native complexes. 721 cell lysates were resolved by BN-PAGE (*top blot;* 1st Dimension, BN-PAGE). An untransferred lane from the BN-PAGE gel was cut out and placed in the well of an SDS-PAGE gel for resolution in the second dimension (as described in Materials and Methods)(*bottom blot,* 2nd Dimension*).* Both gels were transferred and

analyzed by Western blot for LMP-1. The arrow to the right of the bottom blot marks the migration of monomeric LMP-1, and the migration of protein standards (in kDa) are noted to the left of each blot.

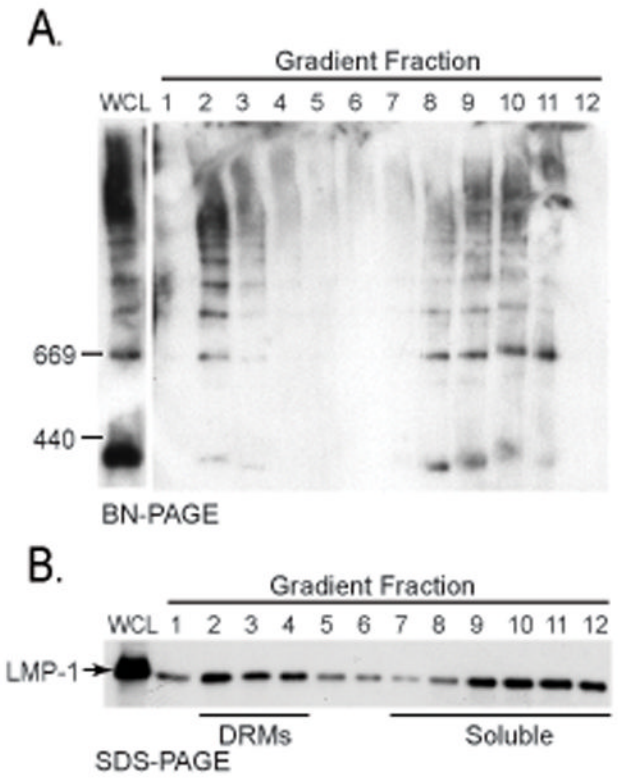

Fig. 3.

High molecular weight native LMP-1 complexes are in enriched in DRMs. 721 cells were homogenized in MNE/0.2% Triton X-100 and fractionated by flotation through sucrose step gradients to obtain DRMs and soluble fractions (fractions were taken from the top of the gradient (fraction 1, top of gradient) Each fraction was analyzed by both BN-PAGE (A)and SDS-PAGE (B). *A)* Samples from gradient fractions were resolved in a 3–15% acrylamide BN-PAGE and analyzed by Western blot for LMP-1. *B)* Samples from gradient fractions were diluted 1:1 with 4 x Sample buffer and resolved by SDS-PAGE and analyzed by Western blot for LMP-1. DRM and detergent soluble fractions are identified below the blot in “B” and specific fractions are numbered above each blot. DRM fractions were identified by staining blots for Lyn kinase, a DRM marker (not shown). The migration of protein standards (in kDa) is noted to the left of the blot and the arrow points to the position of monomeric LMP-1.

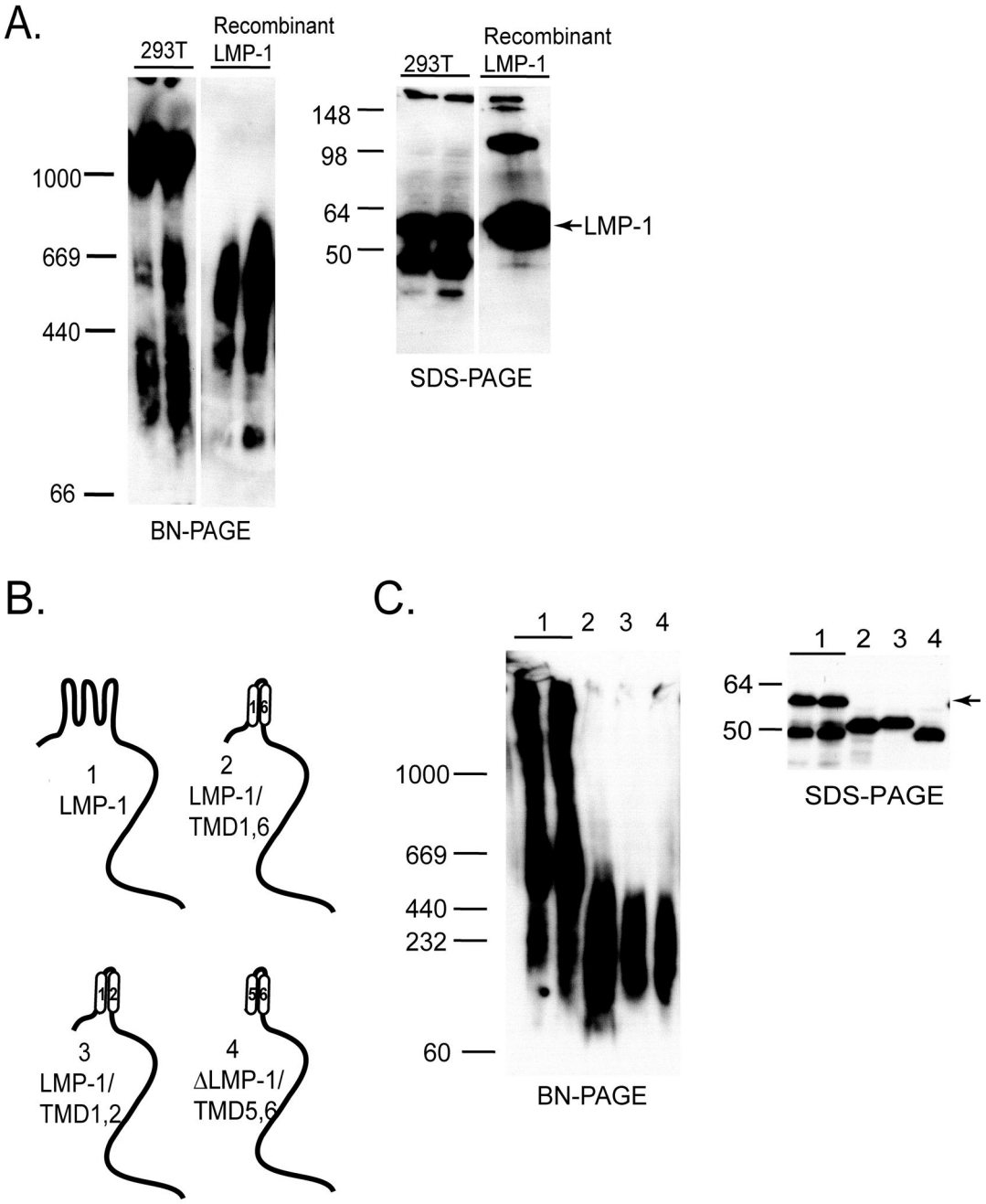

Fig. 4.

Recombinant Flag/LMP-1/6xHis and LMP-1/TMD mutants cannot form the highest molecular weight complexes. *A)* Analysis of purified recombinant LMP-1 by BN-PAGE (*left blot)* and SDS-PAGE (*right blot)*. Purified Flag/LMP-1/6xHis and 293T extracts from cells expressing LMP-1 were lysed and analyzed by BN-PAGE and Western blot for LMP-1 as described in the legend to Fig. 1 (*left blot*) or by SDS-PAGE and Western blot for LMP-1 *(right blot)*. Migration of protein standards (in kDa) are shown on the left of each blot. The arrow on the right marks the migration of monomeric LMP-1 (*right blot). B)* Schematics of LMP-1 TMD mutants (described in Materials and Methods)*C)* 293T cells were transfected with expression vectors encoding the indicated LMP-1 or LMP-1 deletion mutants (marked

1–4) and lysates were analyzed by BN-PAGE (*left blot)* or SDS-PAGE (*right blot)* followed by Western blot analysis for LMP-1. The migration of protein standards (in kDa) is noted to the left of each blot and the arrow points to the position of monomeric LMP-1 (*right blot).*

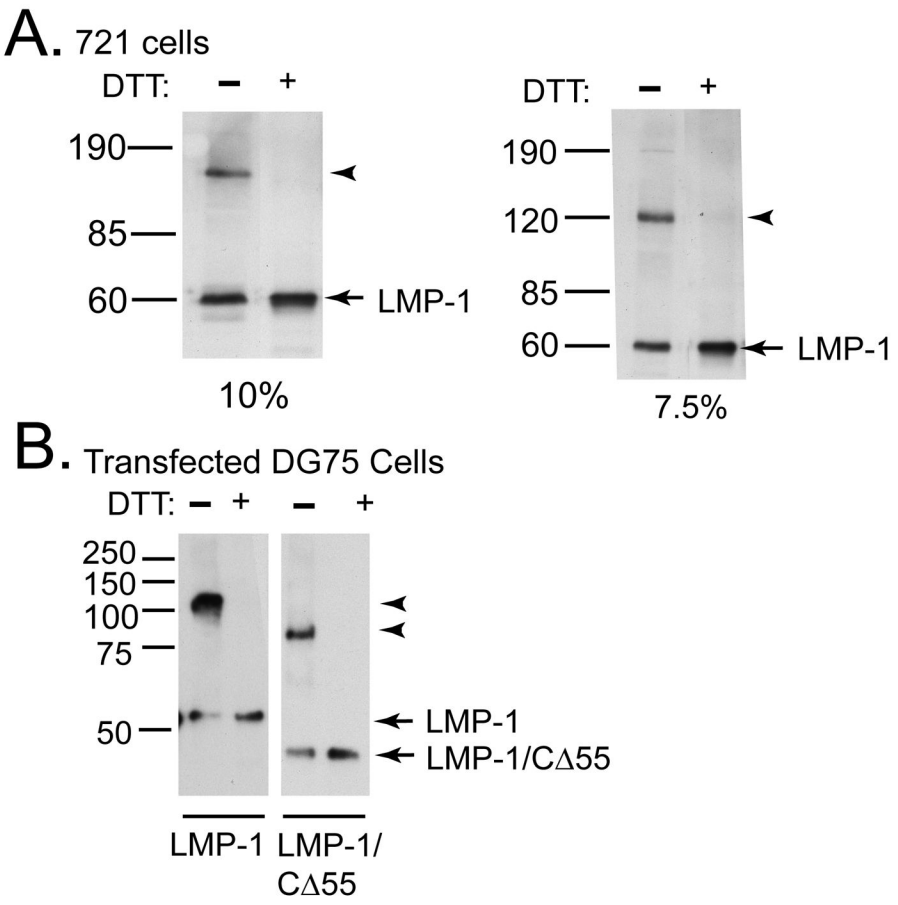

Fig. 5.

B-cells expressing LMP-1 contain an SDS-resistant, homo-dimeric form of LMP-1. *A)* Membranes isolated from EBV-immortalized 721 cells were solubilized in 4X nonreducing SDS-sample buffer and heated to 85°C for 15 minutes in the absence or presence of 50 mM DTT. Samples were resolved in 10% or 7.5% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1 using anti-LMP-1 antibodies. *B)* DG75 cells were transfected with LMP-1 or LMP-1/CΔ55 and harvested 48 hours later. Membranes were solubilized in 4X nonreducing SDS-sample buffer and heated to 85°C, in the presence or absence of 50 mM DTT, for 15 minutes. Samples were resolved on 7.5% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1. Treatment with DTT is noted above each blot, and the expressed protein is noted below the blots (B). The migration of protein standards (in kDa) are indicated to the left of each blot, arrows mark the migration of LMP-1 and LMP-1/CΔ55, and arrowheads mark the migration of the higher molecular weight LMP-1 and LMP-1/CΔ55 species.

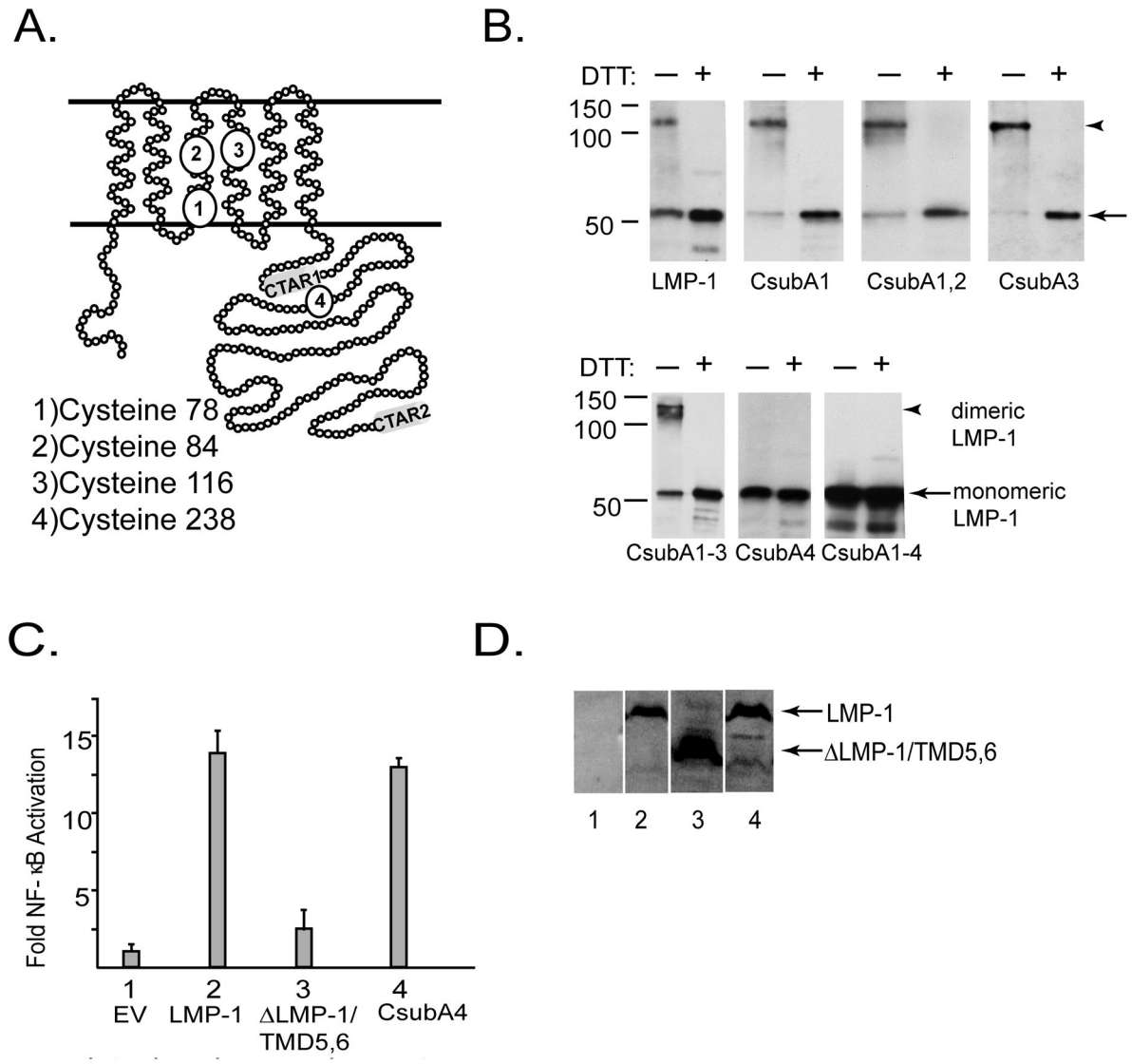

Fig. 6.

Cysteine 238 is required for formation of LMP-1 homo-dimers but is not essential for activation of NF-κB. *A)* Schematic of LMP-1 showing the relative position of its 4 cysteines (LMP-1 residues 78 (1), 84 (2), 116 (3), and 238 (4)). The relative positions of LMP-1's CTAR1 and CTAR2 motifs are also noted. *B)* DG75 cells were transfected with pCMV-LMP-1 or pCMV-LMP-1 substitution mutant expression vectors harboring cysteine to alanine substitutions. Cysteine 78 is substituted with alanine in C*sub*A1; cysteines 78 and 84 are substituted with alanines in C*sub*A1,2; cysteine 116 is substituted with alanine in C*sub*A3; cysteines 78, 84, and 116 are substituted with alanines in C*sub*A1-3; cysteine 238 is substituted with alanine in C*sub*A4; cysteines 78, 84, 116, and 238 are substituted with alanines in C*sub*A1-4. Membranes were solubilized in nonreducing 4X SDS-sample buffer and heated to 85°C, with and without 50 mM DTT, for 15 minutes. Samples were resolved on 10% SDS- polyacrylamide gels and analyzed by Western blot for LMP-1. Data for LMP-1, C*sub*A4 and C*sub*1-4 are from the same blot; data for C*sub*A1, C*sub*A1,2, C*sub*A3 and C*sub*A1-3 are from different blots. Treatment with DTT is noted above the blots, and the expressed LMP-1 protein is noted beneath each blot. The migration of protein standards

(in kDa) are shown on the left of the blots, and the arrows on the right marks the migration of monomeric LMP-1 and the arrowhead marks the migration of dimeric LMP-1. *C)* HEK 293T cells were cotransfected with the indicated LMP-1 expression vectors, an NF-kB luciferase reporter (p1242) and a β-galactosidase reporter (pRSV-lacZ). Cells were harvested 48 hours later and assayed for luciferase and β-galactosidase accumulation and LMP-1 expression. Data are shown as fold NF-kB activation relative to pRC-RSV (empty vector). *D)* Extracts from "C" were resolved on 10% SDS- polyacrylamide gels and analyzed by Western blot for LMP-1; numbers below each lane correspond to the numbers below the introduced expression vector in "C". *Lane 1*, pRC-RSV; *lane 2*, pRSV-LMP-1; *lane 3*, pRSV-ΔLMP-1/TM5,6; *lane 4*, pRSV-LMP-1/C*sub*A4. Data are representative of three experiments.

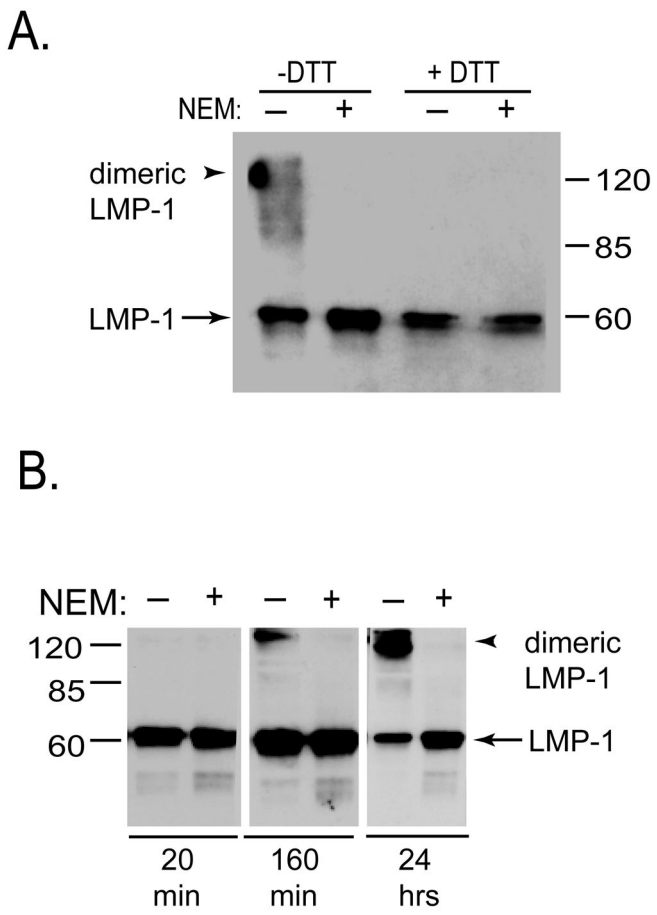

Fig. 7.

Cysteine-disulfide crosslinking occurs post cell lysis. *A)* 721 cells were treated with or without 20 mM NEM for 60 minutes, solubilized in nonreducing SDS-sample buffer, heated to 85°C in the presence or absence of 50 mM DTT for 15 minutes and analyzed by 10% SDS-PAGE and Western blot for LMP-1. Treatment with NEM and DTT is noted above the blot. Migration of protein markers (in kDa) and of monomeric (arrow) and dimeric (arrowhead) forms of LMP-1 are noted to the right and left of the blot, respectively. *B)* 721 cells pretreated with or without 20 mM NEM for 60 minutes were lysed in phosphate buffer containing 1% Triton X-100. Lysates were incubated for up to 24 hours at 4°C, diluted 1:1 with 4X nonreducing sample buffer containing NEM and analyzed by SDS-PAGE and Western blot for LMP-1. Treatment with or without NEM is noted at the top of each gel. Length of incubation is noted below the blot. Migration of protein standards (in kDa), and of monomeric (arrow) and dimeric (arrowhead) LMP-1, are shown on the left and right of each blot, respectively.

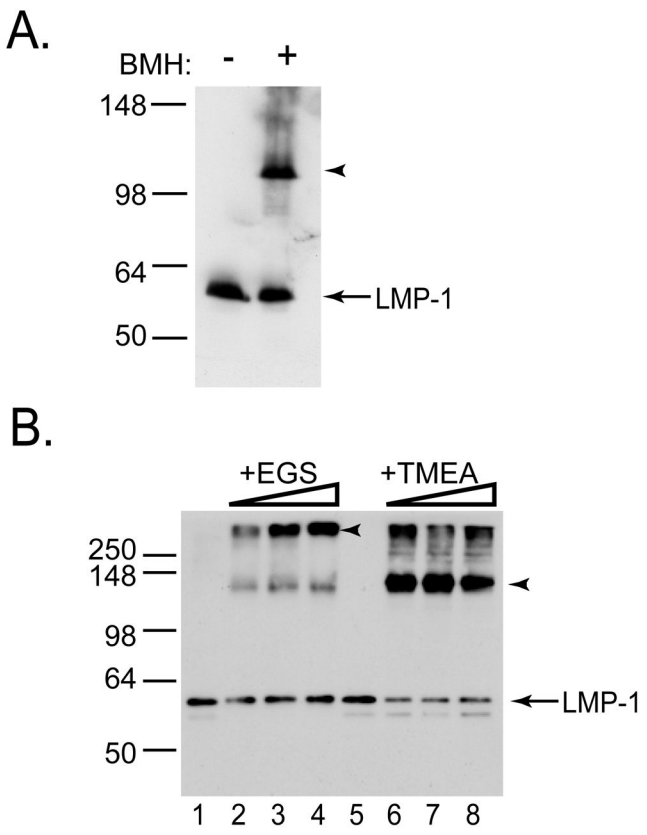

Fig. 8.

Generation of high molecular weight forms of LMP-1 following crosslinking. *A)* 721 cells were treated with 1 mM BMH at 37°C for 1 hour. The reaction was quenched in 180 mM DTT. Cells were resuspended in 4xSample buffer with 100 mM DTT and resolved in a 10% SDS-polyacrylamide gel and analyzed by Western Blot for LMP-1. *B)* 721 cells were lysed in Syn7 buffer and soluble material was incubated with increasing concentrations of EGS or TMEA as described in Materials and Methods. Following crosslinking, extracts were diluted in 4X SDS-sample buffer containing 50 mM DTT, heated to 85°C, resolved in 10% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1 using anti-LMP-1 monoclonal antibodies (CS1-4). *Lanes 1 and 5*, no crosslinker (DMSO control); *Lanes 2–4*, crosslinked with 500 μM, 1.0 mM, and 1.5 mM EGS, respectively; *lanes 6–8*, crosslinked with 125 μM, 250 μM, and 375 μM TMEA, respectively. Migration of protein standards (in kDa), and of monomeric (arrow), dimeric and higher molecular weight LMP-1 species (arrowheads) are shown to the left and right of the blots, respectively.

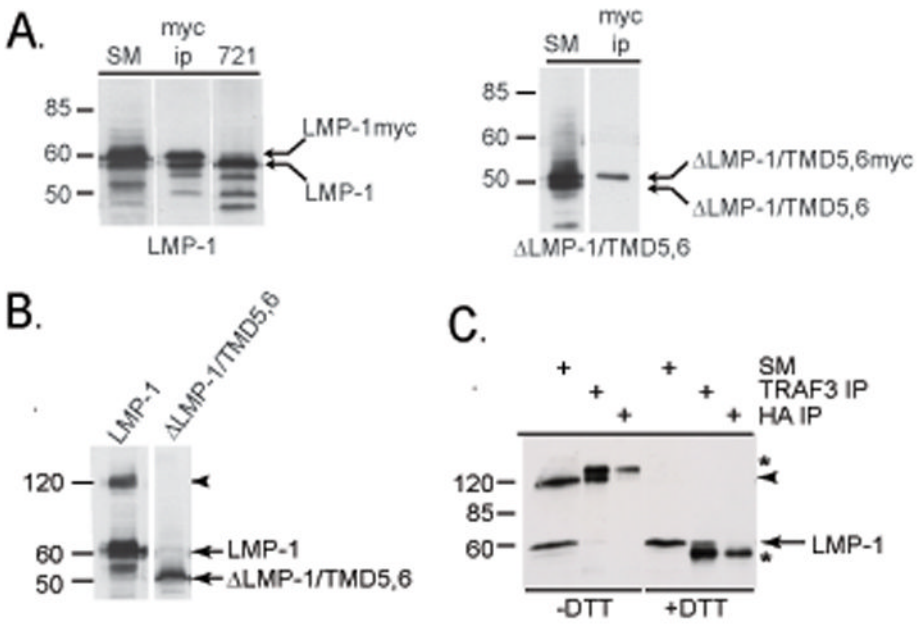

Fig. 9.

Dimeric LMP-1 is not found in cells expressing a non-oligomerizing LMP-1 variant but co-purifies with TRAF3 and is restricted to lipid rafts. *A)* DG75 cells were cotransfected with pCMV-LMP-1 and pCMV-LMP-1*myc* or pCMV-ΔLMP-1/TM5,6 and pCMV-ΔLMP-1/TM5,6*myc* and harvested 48 hours later. Triton-solubilized extracts were immunoprecipitated with myc antibodies (myc IP), immunoprecipitates resolved on 10% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1; *SM*, starting material for immunoprecipitation; *myc IP*, anti-myc immunoprecipitate; *721*, 721 cell extract included as LMP-1 standard. The blots on the left and right show results for extracts of cells expressing LMP-1 and ΔLMP-1/TM5,6, respectively. Arrows point to LMP-1, LMP-1*myc*, ΔLMP-1/TM5,6, ΔLMP-1/TM5,6*myc* proteins (right). *B)* DG75 cells were transfected with pCMV-LMP-1 or pCMV/ΔLMP-1/TM5,6, harvested 48 hours later and solubilized in 4X nonreducing SDS-sample buffer. Extracts were heated to 85°C and resolved on 10% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1 and ΔLMP-1/TM5,6. Migration of monomeric LMP-1 and ΔLMP-1/TM5,6 (arrows) and dimeric LMP-1 (arrowhead) is shown to the right of the blot. *C)* Triton-solubilized 721 cell extracts were immunoprecipitated with TRAF3 (TRAF3 IP) or HA (HA IP) antibodies. Triton-solubilized starting material (SM) and immunoprecipitates were diluted in 4X nonreducing SDS-sample buffer and heated to 85°C in the presence or absence of 50 mM DTT, resolved on 10% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1. The HA antibody was used as an irrelevant antibody control for the TRAF3 immunoprecipitation. Treatment with DTT is noted above the blot. Migration of monomeric (arrow), dimeric and higher molecular weight LMP-1 species (arrowheads) are shown to the right of the blot. The asterisks (*) mark the migration of the precipitating antibody. Migration of protein standards (in kDa) are shown on the left of all blots.

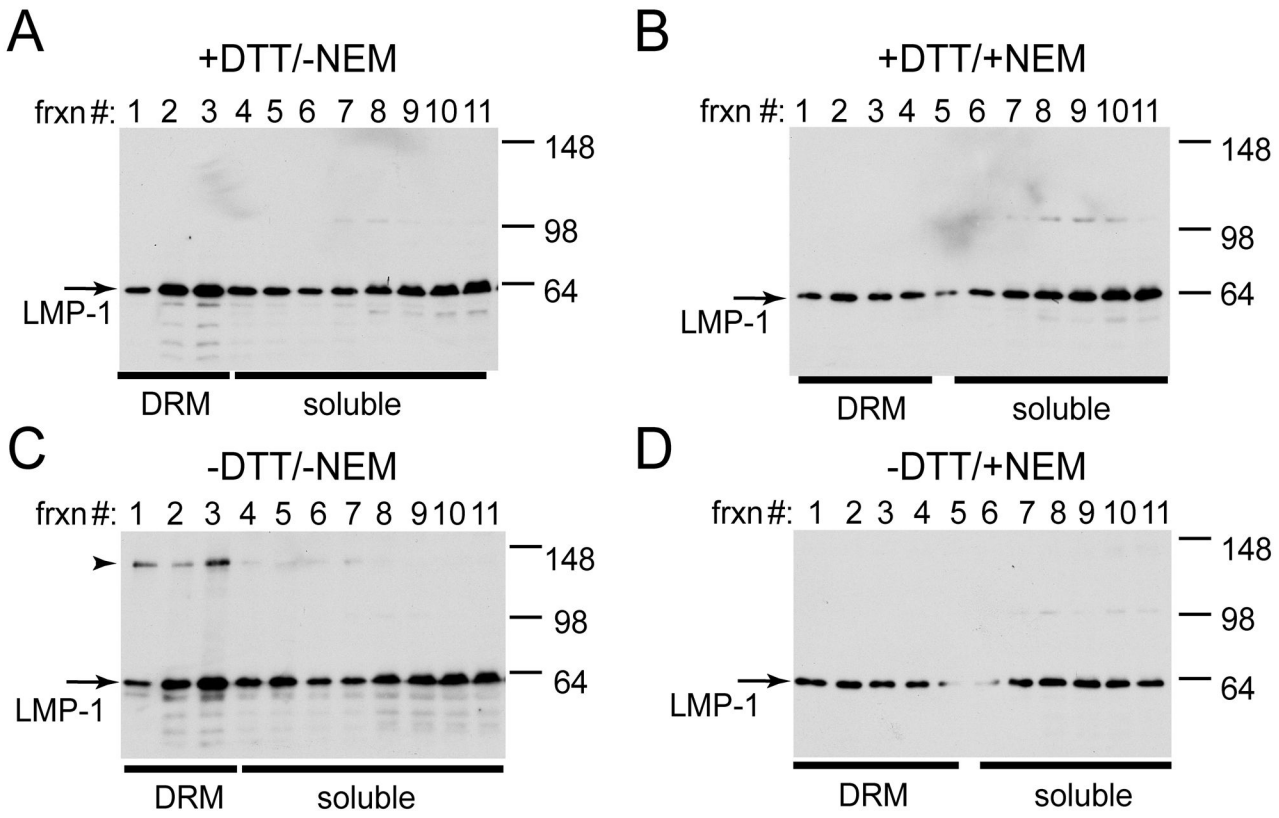

Fig. 10.

Dimeric LMP-1 is restricted to lipid rafts. 721 cells were pretreated with (B and D) or without (A and C) 20 mM NEM for 60 minutes and then homogenized in MNE/0.2% Triton X-100 and fractionated by flotation through sucrose step gradients. Fractions were taken from the top of the gradient (fraction 1, top of gradient) and mixed with nonreducing SDS-sample buffer and heated to 85°C, with (1 and 2) or without (3 and 4) 50 mM DTT, for 15 minutes. Samples were resolved in 10% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1. Detergent-resistant membranes (DRMs) float to the top of the gradient whereas detergent soluble material is found in the lower fractions (soluble). Fractions containing DRMs were identified by Western blotting for Lyn kinase, which localizes exclusively to DRMs (not shown). The distinction between DRMs and soluble material is noted beneath each blot and gradient fraction numbers are noted above. The migration of protein standards (in kDa) is noted to the right of each blot, the arrows point to the position of monomeric LMP-1 and the arrowhead points to the position of dimeric LMP-1. The faint ~98 kDa band in B is a cross-reacting cellular protein recognized by the rabbit polyclonal anti-LMP-1 antibody.

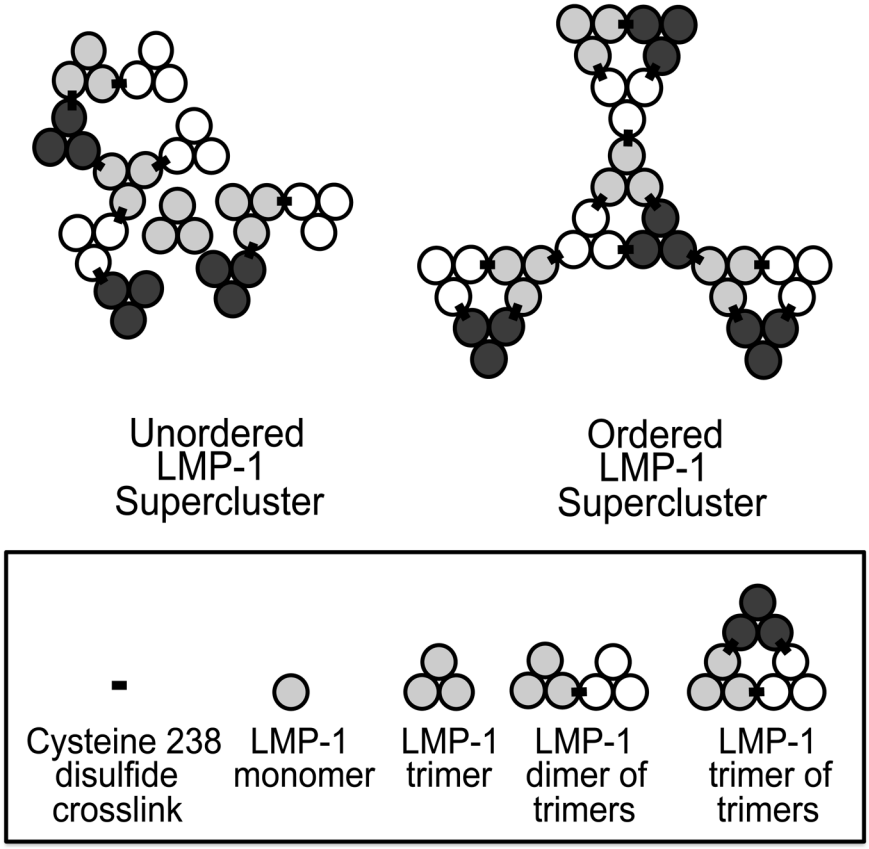

Fig. 11.

Model illustrating the hypothetical relationship between LMP-1 trimerization, multimerization, and formation of LMP-1 disulfide-linked dimers. Hypothetical relationship between LMP-1 monomers, “core” LMP-1 trimers and multimers of the core LMP-1 trimer (superclusters). Circles represent LMP-1 monomers and each trimer of the multimer is distinguished by shading. The black line marks the position of disulfide-crosslinks between cysteine 238 residues in adjacent trimers (in multimers of trimers). Cysteine 238 of adjacent trimers (in the multimer) can be crosslinked by oxidation or by addition of membrane permeable sulfhydryl crosslinkers, thus giving rise to covalently linked dimers upon solubilization in SDS-sample buffer under nonreducing conditions. Dimeric LMP-1 species are stabilized by chemical crosslinking (with BMH, EGS or TMEA) and high molecular weight native complexes are resolved by BN-PAGE, the latter of which we propose represents the multimerized LMP-1 “supercluster”. Core LMP-1 trimers could potentially aggregate in a variety of configurations that lead to multimerization in high molecular weight superclusters. Shown in the figure are two hypothetical configurations of multimerized core trimers, each representing an extreme of possible levels of organization: a less ordered aggregate of trimers (left) and a highly ordered supercluster built by trimeric interactions of core LMP-1 trimers (right). We predict that either of these configurations would localize in detergent resistant membrane fractions (lipid raft) microdomains because the LMP-1 crosslinked dimer is found only in the DRM fractions of sucrose gradients and the larger high molecular weight native complexes are enriched in those fractions (Fig. 5,8). Other arrangements with varying degrees of order, or combinations of different arrangements are equally possible.