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A novel tetrameric gp350¹⁻⁴⁷⁰ as a potential Epstein-Barr virus vaccine

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Abstract

Infectious mononucleosis and B-cell transformation in response to infection with Epstein-Barr virus (EBV) is dependent upon binding of the EBV envelope glycoprotein gp350 to CD21 on B-cells. Gp350-specific antibody comprises most of the EBV neutralizing activity in the serum of infected patients, making this protein a promising target antigen for a prophylactic EBV vaccine. We describe a novel, tetrameric gp350-based vaccine that exhibits markedly enhanced immunogenicity relative to its monomeric counterpart. Plasmid DNA was constructed for synthesis, within transfected CHO cells, of a tetrameric, truncated (a.a. 1-470) gp350 protein (gp350¹⁻⁴⁷⁰). Tetrameric gp350¹⁻⁴⁷⁰ induced ~20-fold higher serum titers of gp350¹⁻⁴⁷⁰-specific IgG and >19-fold enhancements in neutralizing titers at the highest dose, and was >25-fold more immunogenic on a per-weight basis than monomeric gp350¹⁻⁴⁷⁰. Further, epidermal immunization with plasmid DNA encoding gp350¹⁻⁴⁷⁰ tetramer induced 8-fold higher serum titers of gp350¹⁻⁴⁷⁰-specific IgG relative to monomer. Tetrameric gp350¹⁻⁴⁷⁰ binding to human CD21 was >24-fold more efficient on a per-weight basis than monomer, but neither tetramer nor monomer mediated polyclonal human B-cell activation. Finally, the introduction of strong, universal tetanus toxoid (TT)-specific CD4+ T-cell epitopes into the tetrameric gp350¹⁻⁴⁷⁰ had no effect on the gp350¹⁻⁴⁷⁰-specific IgG response in naïve mice, and resulted in suppressed gp350¹⁻⁴⁷⁰-specific IgG responses in TT-primed mice. Collectively, these data suggest that tetrameric gp350¹⁻⁴⁷⁰ is a potentially promising candidate for testing as a prophylactic EBV vaccine, and that protein multimerization, using the approach described herein, is likely to be clinically relevant for enhancing the immunogenicity of other proteins of vaccine interest.

Keywords

EBV; vaccine; antibody; multimer; CD21; mononucleosis

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1. Introduction

EBV is a major, global source of morbidity and mortality, implicated in Burkitt lymphoma, nasopharyngeal carcinoma, infectious mononucleosis, a subset of Hodgkin's disease, and lymphoproliferative disease in immunosuppressed patients [1, 2]. In the developing world, subclinical EBV infection and seroconversion typically occurs in infancy, whereas in developed countries it is more likely contracted in adolescence. Infectious mononucleosis typically occurs only in this latter group [2]. Gp350, an EBV envelope protein, binds to CD21 on B cells for cellular entry [3, 4], an event that is critical for infectivity and B-cell neoplastic transformation [1]. Adsorption of EBV immune sera with gp350, eliminates most of the viral neutralizing activity [5], emphasizing the importance of gp350 as a vaccine target.

Immunization of rabbits with monomeric gp350 protein in adjuvant has been shown to elicit EBV-neutralizing antibody [6]. Studies in non-human primates using gp350-based vaccination strategies have shown protection against EBV-induced lymphoma and EBV replication [7–14]. In humans, a single dose of gp350/220 expressed by vaccinia virus (VV) was given to 1–3 year olds who were EBV-seronegative. These children developed neutralizing antibodies to EBV and a subset were protected from infection [15]. More recently, Phase I/II studies were conducted in which EBV-seronegative adults were immunized with a recombinant monomeric gp350 protein in alum+/-monophosphoryl lipid A [16, 17]. Following 3 doses, up to 82% of subjects had detectable neutralizing serum anti-gp350 antibody. The vaccine demonstrated an efficacy of 78% in preventing infectious mononucleosis but not in preventing asymptomatic EBV infection. Finally, an additional phase I trial of recombinant monomeric gp350 protein in alum given to children with chronic kidney disease demonstrated only a minority of subjects developing detectable neutralizing serum anti-gp350 titers [18]. Collectively, these studies support the feasibility of using gp350 as a target antigen for a prophylactic EBV vaccine, but suggest the desirability of a vaccine more potent than that afforded by a monomeric gp350 protein.

Monomeric proteins are relatively weak immunogens relative to proteins that are expressed in a multimeric manner or that are aggregated [19–24]. However, approaches to multimerize proteins in a defined and cost-effective manner for vaccine use, with direct validation of a resultant increase in immunogenicity, have been limited. In this study, we constructed and expressed a plasmid DNA in which two identical gp350 sequences are separated by a (Gly₄Ser₁)₃ linker to allow for proper protein folding [25], followed by a *Saccharomyces cerevisiae* GCN4 leucine zipper sequence [26, 27] to mediate self-association of the gp350 dimer, and thus formation of a tetrameric gp350 protein. The gp350 utilized was truncated, consisting of the first 470 amino acids (gp350¹⁻⁴⁷⁰) that contain the CD21 binding site recognized by the neutralizing mAb, 72A1 [28, 29]. We directly compared the immunogenicity of tetrameric and monomeric gp350¹⁻⁴⁷⁰, on a per-weight basis, using both DNA and protein immunization approaches.

2. Materials and methods

2.1 Construction of plasmids for production of monomeric and tetrameric gp350¹⁻⁴⁷⁰

Gp350 monomer construct—A gp350 cDNA encoding amino acids 1-470 was cloned by PCR [30], inserted into the pENTR/SD/D-TOPO cloning vector (Invitrogen, Grand Island, NY) and verified by sequencing. Monomer construct was created by PCR amplification using the following primers: *GFI*: 5' - GCCACCATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCA GGTTCCTACTGG TGACGCGGCCAGCCGCGCCAGGCGCGCGCCGTACGAAGCTCGCCCTT-3' and

GR6: 5'-TCAATGGTGGTGGTGGTGGTGGGATACAGTGGGGCCTGT-3'. *GFI* contained an IgGκ leader sequence and *GR6* encoded a His₆ tag. The PCR product was cloned into the pOptiVEC-TOPO vector (Invitrogen), and verified by sequencing.

Gp350 tetramer construct with TT epitopes—Two separate gp350 units: gp350F1R1 and gp350F2R5, were made by PCR and subsequently ligated. Primer sets: *GFI* (above) and *GR1*: 5'-CCATCGATGGCTAGCTAGCGGTGGATACAGTGGGGCCTGT-3' were used to create Gp350F1R1. *GR1* contained NheI and ClaI restriction sites. The PCR product was cloned into the pOptiVEC-TOPO vector, and was verified by sequencing. Gp350F2R5 contained sequences encoding the (Gly₄Ser)₃ linker, the universal tetanus toxoid (TT)-specific CD4⁺ T-cell epitopes P₂ and P₃₀ [31], a leucine zipper [26, 27] and a His₆ tag, and was created by 3 rounds of PCR, adding the coding sequences sequentially. The PCR product was cloned into the PCRII-TOPO vector (Invitrogen), and was verified by sequencing. The plasmids gp350F1R1 and gp350F2R5 were digested with NheI and ClaI, the fragments containing gp350 were gel-purified and ligated with T4 DNA ligase, followed by transformation of “*Top10F*”-*E. coli* (Invitrogen). Two clones were selected for further study following verification by sequencing.

Gp350¹⁻⁴⁷⁰ tetramer construct without TT epitope—The plasmid was constructed using a similar approach as described above, but the sequences encoding P₂ and P₃₀ were deleted.

2.2 CHO culture in hollow fiber bioreactors and purification of recombinant gp350¹⁻⁴⁷⁰ proteins

Chinese hamster ovary (CHO) cells (clone DG44, Invitrogen) were stably transfected with the gp350 constructs and cultured in optiCHO serum free medium (Invitrogen). After methotrexate selection, CHO cells were loaded into “Fibercell” cartridges (FiberCell Systems, Frederick, MD) and supernatants were concentrated for affinity purification using a cobalt column (Thermo Scientific). Proteins were analyzed by PAGE, Coomassie blue staining and Western blot using anti-gp350 mAbs (clones 72A1 and 2L10). In addition, analysis of monomeric and tetrameric gp350 by reverse phase HPLC demonstrated ~93% purity of each protein preparation.

2.3. Mice

Female BALB/c mice (NCI, Frederick, MD or Harlan Laboratories, Indianapolis, IN) were respectively used at 7–10 weeks (protein immunizations) and 4–6 weeks (DNA vaccinations). These studies were conducted in accordance with the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, NRC), and were approved by USUHS and U. Washington Institutional Animal Care and Use Committees.

2.4 Antigens, immunizations, and measurement of serum antigen-specific IgG titers

Pneumococcal polysaccharide, type 14 (PPS14) was purchased from ATCC (Manassas, VA). Gp350¹⁻⁴⁷⁰-PPS14 and TT-PPS14 were synthesized as described [32]. Alum (Alhydrogel 2%) was obtained from Brenntag Biosector (Denmark). Mice were immunized i.p. using 13 µg of alum +/- 25 µg 30mer stimulatory CpG-ODN [33]. Mice were vaccinated by particle-mediated epidermal delivery (PMED) in the abdominal skin using the PowderJect-XR-1 DNA vaccine delivery system [34]. Immunizations consisted of two tandem deliveries of 0.5 mg, 1–3 µm-diameter gold particles coated with 1.0 µg DNA vaccine (total dose of 4.0 µg DNA) [34]. DNA vaccines were administered at a helium pressure of 350 psi. Sera were obtained from the tail vein. Serum titers of IgG specific for

gp350¹⁻⁴⁷⁰, TT, or PPS14 were measured by ELISA under conditions described previously [35].

2.5 Measurement of serum gp350¹⁻⁴⁷⁰-specific neutralizing antibody

25 μ l of mouse serum from naïve or immunized mice, were incubated with DyLight-633 (Thermo Scientific)-labeled gp350¹⁻⁴⁷⁰ monomer (final 1 μ g/ml). 5x10⁵ human erythroleukemia cells expressing human CD21 (clone CR2M1 α) [36] were resuspended in the serum/ gp350¹⁻⁴⁷⁰ monomer mixture for 30 min on ice, washed, and fixed in 4% paraformaldehyde. A standard curve was generated using varying concentrations of 72A1 mAb (final concentrations of 1-256 μ g/ml). Cells were then analyzed on a BD LSRII Flow Cytometer Cell Analyzer.

2.6 Binding of monomeric and tetrameric gp350¹⁻⁴⁷⁰ proteins to human CD21

CR2M1 α cells were incubated for 30min on ice with gp350¹⁻⁴⁷⁰ monomer or tetramer (0.05–30 μ g/ml), washed 3x with 0.5% BSA-PBS and incubated further with mouse anti-gp350 mAb (2L10, Thermo Scientific) for 30 min. 2L10 mAb binds to a non-neutralizing epitope on gp350, distinct from the neutralizing epitope recognized by 72A1 [28, 29]. Cells were then washed 3x in 0.5% BSA-PBS, followed by incubation with DyLight-633-labeled goat anti-mouse IgG (Thermo Scientific). Cells were fixed in 4% paraformaldehyde and analyzed on a BD LSRII Flow Cytometer Cell Analyzer.

2.7 Statistics

Serum titers of antigen-specific Ig were expressed as the geometric means $\pm$ SEM of the individual serum titers. Significance was determined by the Student *t* test. *p*-values of ≤ 0.05 were considered statistically significant.

3. Results

3.1. Tetanus toxoid is a more potent carrier protein than gp350¹⁻⁴⁷⁰ for a conjugate vaccine

Robust antibody responses require strong B- and T-cell epitopes, although the latter are undefined for gp350. We compared gp350¹⁻⁴⁷⁰ and tetanus toxoid (TT) for their relative potency as T-cell-inducing carriers for a conjugate vaccine containing pneumococcal polysaccharide (PPS14). BCR-mediated recognition of the polysaccharide component of the conjugate vaccine by specific B cells, and the subsequent internalization of the covalent polysaccharide-protein conjugate, results in presentation of peptides, derived from the carrier protein, to CD4⁺ T cells that then provide help for the polysaccharide-specific IgG response [37]. Mice were immunized and boosted with gp350¹⁻⁴⁷⁰-PPS14 or TT-PPS14. Serum titers of PPS14-specific IgG were significantly higher using TT-PPS14 relative to gp350¹⁻⁴⁷⁰-PPS14 using either 1 or 5 μ g of conjugate (Fig. 1), strongly suggesting that TT contained more potent CD4⁺ T-cell epitopes.

3.2. DNA plasmid and production of a tetrameric gp350¹⁻⁴⁷⁰ protein containing TT-specific CD4⁺ T-cell epitopes

Protein multimerization confers increased immunogenicity [19–24]. We designed a DNA construct encoding tetrameric gp350¹⁻⁴⁷⁰ that incorporated two universal human TT-specific CD4⁺ T-cell epitopes (P₂ and P₃₀) [31] (Fig. 2A). An IgG light chain leader sequence was introduced 5' for protein secretion, followed by two identical gp350¹⁻⁴⁷⁰ sequences separated by a (Gly₄Ser₁)₃ linker to allow protein folding [25]. P₂ and P₃₀ were introduced 3' to the second gp350¹⁻⁴⁷⁰ followed by a leucine zipper sequence [26, 27] for homodimerization. A 3' His₆ tag was included for purification. DNA encoding only monomeric gp350¹⁻⁴⁷⁰ was produced for comparison.

CHO cells were transfected with one of the two constructs and secreted gp350^{I-470} was purified and detected by SDS-PAGE (denaturing) using Coomassie blue staining, and SDS-PAGE and PAGE (native) using 72A1 mAb that recognizes the CD21-binding epitope [38] (Fig. 2B) or 2L10 mAb (non-CD21-binding epitope) [data not shown]. For the tetramer, SDS-PAGE (leucine zipper disrupted) and PAGE, yielded single bands of ~200 kD and 350–400 kD, respectively, using either mAb, whereas monomeric gp350^{I-470} was detected as a ~100 kD band on either gel. These data thus confirmed the secretion of a tetrameric gp350^{I-470}. 10-fold more 72A1 mAb was required for comparable detection on SDS-PAGE versus PAGE, likely reflecting gp350^{I-470} denaturation in the former.

3.3 Tetrameric gp350^{I-470} is markedly more immunogenic than monomeric gp350^{I-470} protein, even in the presence of a strong adjuvant, and following DNA vaccination

Mice were injected and boosted i.p. with tetrameric or monomeric gp350^{I-470} in alum. Tetrameric versus monomeric gp350^{I-470} induced 18-fold higher secondary serum gp350^{I-470}-specific IgG titers at the highest dose (25 µg) [Fig. 3A]. Of note, tetrameric gp350^{I-470} at 1.0 µg induced 7-fold higher titers than 25 µg of monomer, thus exhibiting >25-fold higher immunogenicity. This enhanced gp350^{I-470}-specific IgG response to tetrameric gp350^{I-470} required both priming and boosting with tetramer (Supplementary Fig. 1).

Mice were next immunized with tetrameric or monomeric gp350^{I-470} using alum + CpG-ODN, resulting in 21-fold and 54-fold enhancements in gp350^{I-470}-specific IgG titers in response to 25 µg of tetrameric and monomeric gp350^{I-470}, respectively, relative to the alum-adjuvanted groups (Fig. 3B). Nevertheless, tetrameric gp350^{I-470} in alum + CpG-ODN still induced 11-fold higher gp350^{I-470}-specific IgG titers relative to monomer (25 µg dose), with 1 µg of tetrameric gp350^{I-470} inducing responses comparable to mice receiving 25 µg of monomer. Thus, tetrameric gp350^{I-470} is markedly more immunogenic even using a relatively strong adjuvant.

Plasmid DNA encoding tetrameric and monomeric gp350^{I-470} was directly introduced into the epidermis on microscopic gold particles [39, 40] with boosting at 4 weeks. Whereas primary immunization with either plasmid induced minimal gp350^{I-470}-specific IgG titers (Fig. 3C), upon boosting, a strong secondary gp350^{I-470}-specific IgG response to the DNA encoding tetrameric gp350^{I-470} was elicited that was 8-fold higher ($p=0.0004$) than monomer. As illustrated in Fig. 3D, gp350^{I-470} protein in alum elicited primarily IgG1. Addition of CpG-ODN boosted gp350^{I-470}-specific IgG1 and further induced gp350^{I-470}-specific IgG2b and IgG2a. DNA vaccination induced gp350^{I-470}-specific IgG1 and IgG2a comparable to and lower (~3-fold), respectively, to that observed for gp350^{I-470} protein in alum + CpG-ODN, with no detectable IgG2b (Fig. 3D). Minimal titers of gp350^{I-470}-specific IgG3 were produced in any groups.

One mechanism underlying the enhanced gp350^{I-470}-specific IgG response to tetrameric gp350^{I-470} could be enhanced priming of gp350^{I-470}-specific CD4⁺ T cells. To study this, we primed mice with either monomeric or tetrameric gp350^{I-470} in alum + CpG-ODN for 14 d followed by in vitro restimulation with the respective proteins and measurement of the percentage of CD4⁺ T cells expressing cytoplasmic IL-4, IL-5, or IFN- γ by flow cytometry. As illustrated in Fig. 3E, both monomeric and tetrameric gp350^{I-470} primed CD4⁺ T cells for a recall response for all 3 cytokines, but with significantly higher numbers in each case for tetrameric gp350^{I-470}.

3.4. Immunization with tetrameric gp350¹⁻⁴⁷⁰ protein induces markedly higher levels of neutralizing gp350¹⁻⁴⁷⁰-specific Ig relative to monomer

To measure the amount of neutralizing antibody in sera from gp350¹⁻⁴⁷⁰-immunized mice, we utilized an erythroleukemia cell line transfected with human CD21 (CR2M1 α). Monomeric gp350¹⁻⁴⁷⁰ was labeled with DyLight-633 and then mixed with undiluted pre-immune sera or sera from mice that were primed and boosted with 25 μ g of tetrameric or monomeric gp350¹⁻⁴⁷⁰ in alum. The mixtures were incubated with CR2M1 α cells and analyzed by flow cytometry. A standard curve was also generated using varying amounts of the neutralizing 72A1 mAb instead of immune sera [38]. As illustrated in Fig. 4, pre-immune sera contained <4.0 μ g/ml of 72A1 mAb-equivalents of neutralizing activity. Monomeric gp350¹⁻⁴⁷⁰ induced 13 μ g/ml of neutralizing activity, whereas tetramer induced 253 μ g/ml, a 19-fold greater level of activity than monomer. This difference closely mirrored the difference observed for total serum titers of gp350¹⁻⁴⁷⁰-specific IgG (see Fig. 3).

3.5 The TT-specific T-cell epitopes in tetrameric gp350¹⁻⁴⁷⁰ do not contribute to the gp350¹⁻⁴⁷⁰-specific IgG response

Immunization with alum-adjuvanted tetrameric gp350¹⁻⁴⁷⁰ containing TT epitopes primed for TT-induced IL-4 and IL-5 secretion by CD4⁺ T-cells upon in vitro restimulation (Supplementary Fig. 2A). However, initial in vivo priming with whole TT protein in alum, resulted in an inhibition of the IgG anti-gp350¹⁻⁴⁷⁰ response following immunization with tetrameric, but not monomeric, gp350¹⁻⁴⁷⁰ (Supplementary Fig. 2B), consistent with previous studies [41–43]. We thus wished to determine the extent, if any, to which the TT epitopes contributed to the more potent immunogenicity of tetrameric versus monomeric gp350¹⁻⁴⁷⁰ in naïve mice. To accomplish this we constructed and validated a new DNA plasmid in which the sequence encoding the TT-specific T-cell epitopes were deleted (referred to as “tetramer^{tt}”). Mice were immunized with 25 or 1.0 μ g of monomer, tetramer, or tetramer^{tt} in alum and boosted in a similar fashion on d21. As illustrated in Fig. 5A, the tetramer and tetramer^{tt} induced dose-dependent gp350¹⁻⁴⁷⁰-specific IgG responses that were not significantly different from each other, but that were each ~25-fold higher than that elicited by monomer at each of the two doses. Similarly, tetramer and tetramer^{tt} elicited a similar gp350¹⁻⁴⁷⁰-specific neutralizing antibody response at the 25 μ g dose, that was each >40-fold higher than that elicited by monomer (Fig. 5B). Finally, immunization with plasmid DNA encoding tetramer and tetramer^{tt} elicited gp350¹⁻⁴⁷⁰-specific IgG responses that were similar, but ~8-fold higher than that observed using plasmid DNA encoding monomeric gp350¹⁻⁴⁷⁰ (Fig. 5C). These data strongly suggest that the marked enhancement in the gp350¹⁻⁴⁷⁰-specific IgG response to tetrameric versus monomeric gp350¹⁻⁴⁷⁰ is based exclusively on protein multimerization, and not to the provision of stronger T-cell epitopes.

3.6. Tetrameric gp350¹⁻⁴⁷⁰ binds more avidly to human CD21 than monomer

We next wished to compare the efficiency of binding of tetrameric versus monomeric gp350¹⁻⁴⁷⁰ to human CD21. Of note, gp350 does not bind to mouse CD21 [44]. To accomplish this, CR2M1 α cells expressing human CD21, were incubated with increasing concentrations of unlabeled monomer or tetramer (0.05–30 μ g/ml), followed by unlabeled 2L10 mAb (mouse IgG1 anti-gp350). This mAb binds to gp350 at a site distinct from the CD21-binding site, and hence is not blocked upon gp350/CD21 binding. This was followed by staining with DyLight-633-labeled goat anti-mouse IgG and analysis by flow cytometry. Incubation of CR2M1 α cells with increasing concentrations of monomeric and tetrameric gp350¹⁻⁴⁷⁰ resulted in a dose-dependent progressive increase in MFI staining in both cases (Fig. 6). Of note, staining using 1.25 μ g/ml of tetrameric gp350¹⁻⁴⁷⁰ resulted in an MFI equivalent to 30 μ g/ml of monomeric gp350¹⁻⁴⁷⁰, suggesting an ~24-fold greater binding avidity of tetramer versus monomer, a similar difference to that observed for induction of

gp350^{I-470}-specific IgG and neutralizing antibody in response to tetramer versus monomer. These data suggest that BCR binding to tetrameric gp350^{I-470} by gp350^{I-470}-specific B-cells will similarly be of higher avidity and may account, at least in part, for its greater immunogenicity in vivo. Of note, neither tetrameric nor monomeric gp350^{I-470} alone induced polyclonal activation of purified peripheral blood human B-cells in vitro, as evidenced by flow cytometric analysis of CD69, CD25, and CD86 expression, (Supplementary Fig. 3) and B-cell size (data not shown).

4. Discussion

Multimerization resulted in marked enhancement of gp350^{I-470}-specific IgG responses, either following direct immunization with plasmid DNA or with protein in adjuvant. Tetrameric gp350^{I-470} bound more efficiently to human CD21 but did not polyclonally activate human B-cells. Thus, a tetrameric gp350^{I-470} may be superior to monomeric gp350^{I-470} [15–18] for a human EBV prophylactic vaccine. Multimerization of proteins/peptides enhances immunogenicity, including a polyglutamine tail that mediates aggregation [20], covalent attachment of proteins to dextran [24], glutathione S-transferase (GST) fusion proteins with multiple peptides [19], and covalent attachment of peptides onto virus-like particles [22]. Increased immunogenicity may reflect efficient activation of complement, enhanced BCR binding, signaling, and enhanced B-cell uptake and presentation of protein-derived peptide to CD4⁺ T-cells and/or enhanced trapping by follicular dendritic cells (FDC) [23, 45]. The advantages of our approach include: recombinant technology to create multimeric proteins with batch-to-batch consistency, proper conformational folding, minimal aggregation, and the creation of multi-epitope constructs.

EBV gp350 binds to human, but not mouse, CD21 [44]. CD21 expressed on B-cells and FDC binds C3d that in association with antigen, promotes immunogenicity [25, 46, 47]. This occurs via co-crosslinking of BCR and CD21, leading to synergistic B-cell signaling [48], and trapping of antigen via CD21 on FDC to promote germinal centers [49]. Gp350 could potentially substitute for C3d as an adjuvant, by promoting human B-cell signaling via BCR/CD21 co-crosslinking [50]. C3d-mediated immunopotentiality, required at least 2 copies of C3d per antigen molecule. Thus, in humans, gp350^{I-470}-specific B-cells binding tetrameric gp350^{I-470} via a single BCR would have access to 2–3 gp350^{I-470} molecules for CD21 binding, for synergistic B-cell signaling and enhanced presentation of gp350^{I-470} peptides to CD4⁺ T cells. Tetrameric gp350^{I-470} should also bind more avidly to CD21 on FDC. Thus, tetrameric gp350^{I-470} in humans, unlike mice may possess intrinsic adjuvanting activity. Importantly, tetrameric gp350^{I-470} did not polyclonally activate human B-cells, obviating concerns for non-specific immune stimulation. The molecular strategy described herein for creating tetrameric gp350^{I-470} could also be applied to potentially boost humoral immune responses to other proteins of vaccine interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. Antigen multimerization can markedly enhance antigenicity.
2. Novel methods for efficient production of multimeric antigens have vaccine relevance.
3. A multimeric protein vaccine is produced using a peptide spacer and leucine zipper.
4. Multimeric EBV protein, gp350, is markedly more immunogenic than monomeric gp350.
5. This method for multimerization of proteins has potential broad applications.

Figure 1. Tetanus toxoid is a more potent carrier protein than gp350^{I-470} for a pneumococcal polysaccharide conjugate vaccine

Mice were immunized i.p. with gp350^{I-470}-PPS14 or TT-PPS14 at 1 or 5 μg/mouse (5 mice per group) in alum + CpG-ODN, and boosted in a similar fashion on day 14. PPS14-specific IgG titers were measured by ELISA from sera obtained on day 21. *Significance, $p < 0.05$ between gp350^{I-470}-PPS14 and TT-PPS14.

Figure 2. DNA plasmid and production of a tetrameric gp350¹⁻⁴⁷⁰ protein containing TT-specific CD4⁺ T-cell epitopes
 (A). Diagram of DNA plasmid for production of tetrameric gp350¹⁻⁴⁷⁰ containing TT-specific CD4⁺ T-cell epitopes. (B). Coomassie blue-stained SDS-PAGE gel [denatured] (left). SDS-PAGE [denatured] (center) and PAGE [native] (right) gels developed with 72A1 mAb.

Figure 3. Tetrameric gp350¹⁻⁴⁷⁰ is markedly more immunogenic than monomeric gp350¹⁻⁴⁷⁰ protein, even in the presence of a strong adjuvant, and following DNA vaccination (A, B). Mice (5 per group) were immunized i.p. with the indicated doses per mouse, of monomeric or tetrameric gp350¹⁻⁴⁷⁰ in (A) alum or (B) alum + CpG-ODN, and boosted in a similar manner on day 21. (C) Mice (7 per group) were immunized in the abdominal skin with two tandem deliveries of 0.5 mg 1–3 µm-diameter gold particles coated with 1.0 µg DNA vaccine for a total dose of 4.0 µg DNA. Mice were boosted in a similar manner on week 4. Serum titers of gp350¹⁻⁴⁷⁰-specific IgG were determined by ELISA on the indicated days. *Significance, $p < 0.05$ between tetramer and monomer (A-C). (D) Sera from tetramer-immunized mice: Sera obtained in “3A” and “3B” (day 35, 25 µg dose) and “3C” (week 6) were analyzed for serum titers of IgG isotypes. (E) Mice (5 per group) were immunized i.p. with 25 µg of monomeric or tetrameric gp350¹⁻⁴⁷⁰ in alum + CpG-ODN. Spleen cells were harvested from each mouse on day 14 and separately cultured for 5h in medium containing either monomeric or tetrameric gp350¹⁻⁴⁷⁰ (50 µg/ml) as indicated. Golgi Stop was added 1 h after initiation of culture. Cells were then washed, fixed, and permeabilized, stained for cytoplasmic IL-5, IL-4, or IFN-γ, and analyzed by flow cytometry. The percentage of gated CD4⁺ cells staining positively for each cytokine is illustrated. *Significance, $p < 0.05$ between difference groups.

Figure 4. Immunization with tetrameric gp350^{I-470} protein induces markedly higher levels of neutralizing gp350^{I-470}-specific Ig relative to monomer

25 μ l of pooled mouse serum (5 mice each) from naïve or immunized mice (day 35, 25 μ g monomeric or tetrameric gp350^{I-470}/mouse in alum, see Fig. 3A), was incubated with DyLight-633-labeled gp350^{I-470} monomer (1 μ g/ml final concentration). CR2M1 α cells were then stained with these mixtures and analyzed by flow cytometry. Various concentrations of 72A1 mAb (neutralizing gp350^{I-470}-specific IgG) were used instead of serum to create a standard curved for quantitation.

Figure 5. The TT-specific T-cell epitopes in tetrameric gp350¹⁻⁴⁷⁰ do not contribute to the gp350¹⁻⁴⁷⁰-specific IgG response in naïve mice

(A). Mice (5 per group) were immunized with monomeric gp350¹⁻⁴⁷⁰ protein, tetrameric gp350¹⁻⁴⁷⁰ containing TT epitopes (“tetramer”), or tetrameric gp350¹⁻⁴⁷⁰ without TT epitopes (“tetramer^{-tt}”) at either 25 µg or 1.0 µg per mouse in alum, and boosted in a similar manner on day 21. Serum titers of gp350¹⁻⁴⁷⁰-specific IgG were measured by ELISA.

*Significance, $p < 0.05$ between tetramer and monomer; #Significance, $p < 0.05$ between tetramer^{-tt} and monomer. (B) gp350¹⁻⁴⁷⁰-specific neutralization titers of sera from “A” (25 µg/mouse, day 35 as described in Fig. 5); *Significance, $p < 0.05$ between tetramer or tetramer^{-tt} versus monomer. (C) DNA immunization with plasmids encoding monomer, tetramer, and tetramer^{-tt} as described in Fig. 3C; *Significance, $p < 0.05$ between tetramer or tetramer^{-tt} versus monomer.

Figure 6. Tetrameric gp350¹⁻⁴⁷⁰ binds more avidly to human CD21 than monomer
 CR2M1α cells were incubated for 30 min on ice with gp350¹⁻⁴⁷⁰ monomer or tetramer (0.05–30 μg/ml), washed, then incubated further with 2L10 mAb (mouse IgG1 anti-gp350 mAb) for 30 min. Cells were then washed, followed by staining with DyLight-633-labeled goat anti-mouse IgG. Cells were analyzed by flow cytometry. Broken line: CR2M1α cells without gp350¹⁻⁴⁷⁰; solid line: with gp350¹⁻⁴⁷⁰.