Cell

Supplemental Information

Rational Design of an Epstein-Barr Virus Vaccine Targeting the Receptor-Binding Site

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Supplemental Experimental Procedures:

Gene synthesis and vector construction

All genes used in the study were human codon optimized (GenScript). The gene encoding *Helicobacter pylori*-bullfrog hybrid ferritin was constructed by fusing residues 2-9 of bullfrog (*Rana catesbeiana*) ferritin lower subunit (UniProt: P07797 with a point mutation at residue 8 (N8Q) to abolish a potential *N*-glycosylation site) to *H. pylori* nonheme ferritin (UniProt: Q9ZLI1, residues 3-167) with mutation at residue 7 (I7E) to recapitulate the conserved salt bridge naturally found in human and bullfrog ferritins (6R and 14E in both human light chain and bullfrog lower subunit ferritins) with 6R of bullfrog ferritin. There is an additional mutation at residue 19 in *H. pylori* ferritin to abolish a potential *N*-glycosylation site. There were extra GS residues at the carboxyl terminus of *H. pylori* ferritin. The secreted encapsulin gene was constructed by fusing a human CD5 signal to *Termotoga maritima* encapsulin (UniProt: Q9WZP2, residues 1-264). The gene encoding EBV strain B95-8 gp350 (UniProt: P03200, residues 1-907) was synthesized and the fragments corresponding to ectodomain (residues 2-860), equivalent to the region used in the crystal structure (Szakonyi et al., 2006) (D_{2H6O}, residues 2-470), D_{123} (residues 2-425) and D_{12} (residues 2-317) were amplified with appropriate primers. These fragments were fused to downstream of a modified bovine prolactin signal sequence (bPRL: MDSKGSSQKG SRLLLLLVVS NLLLPQGVLA) and upstream of the hybrid ferritin with a SG linker to give rise to the gp350-ferritin genes. To construct the gp350-encapsulin genes, gp350 fragments were fused to downstream of encapsulin with a $(SG_3)_2$ linker. To construct soluble gp350 ectodomain and D_{123}, the gene fragments were fused with bPRL and tagged with hexa-histidine at the end. The CR2BS mutant gp350s were made by site-directed mutagenesis (W162N and N164T for glyc162; and additional D208N and E210T for glyc

162/208 to introduce *N*-glycosylation sites at 162 and 208). All genes were then cloned into the CMV/R 8κb (VRC 8405) mammalian expression vector for protein production.

Biosynthesis of recombinant proteins and purification

The expression vectors were transiently transfected into FreeStyle 293F or Expi293F cells (Life Technologies) using 293fectin or ExpiFectamine 293 transfection reagents, respectively (Life Technologies). Four days after transfection, culture supernatants were harvested and cleared. The gp350-based nanoparticles were purified by affinity chromatography using *Galanthus nivalis* agglutinin (GNA, snowdrop lectin) resins (EY Laboratories) followed by size exclusion chromatography with a HiPrep 16/60 Sephacryl S-500 HR column (GE Healthcare) in PBS. The soluble gp350 proteins were purified using Ni sepharose excel resin (GE Healthcare) followed by size exclusion chromatography with a Superdex 200 10/300 GL (GE Healthcare) in PBS.

ELISA

For antigenic characterization, purified nanoparticle and soluble gp350 proteins (25 nM) were coated (100 μl well^{-1}) onto MaxiSorp plates (Nunc) and the wells were probed with anti-gp350 (72A1 and 2L10) or anti-influenza (C179, isotype control) mAbs followed by peroxidase-conjugated secondary antibody (SouthernBiotech). For serum analyses, the plates were coated with gp350 ectodomain at 0.2 μg well^{-1} and probed with serially diluted sera.

Immunoprecipitation

MAbs 72A1, 2L10 and C179 (5 μg) were incubated with purified nanoparticles (5 μg) at ambient temperature for 30 minutes. Protein G Dynabeads (Life Technologies) were then added and incubated for another 30 minutes. Immune complexes were separated, washed and eluted in Laemmli buffer containing reducing agent. Half of each reaction was analyzed by SDS-PAGE followed by InstantBlue staining (Expedeon).

Electron microscopy (EM)

For negative stain EM analysis, samples of about 50 μg ml^{-1} were adsorbed to freshly glow-discharged carbon-coated grids, rinsed with PBS, and stained with 2% ammonium molybdate (pH 7.0). Images were recorded on an FEI T20 microscope with an Eagle CCD camera.

Cryo-EM reconstruction

The gp350 D_{123}-nanoparticles (5 μg μl^{-1}, 3 μl) were applied to holey grids (Quantifoil) and fast-frozen in liquid ethane as described previously (Meng et al., 2013). Cryo-EM images of the D_{123}-ferritin and D_{123}-encapsulin were acquired on an FEI Titan Krios operated at 300 keV and a CM200 FEG microscope operated at 200 keV, respectively. The electron dose for data collection was ~20 e$^-$/Å^2, and the image was defocused by ~2-3 μm. The defocus and the astigmatism of each micrograph were estimated with the program EMAN1 fitctf and further confirmed with the ctfit. Image processing and three-dimensional reconstruction were performed using about 1,000 particles with the EMAN suite of programs (Baker et al., 2010). The final reconstruction was computed and was low-pass filtered to 30 Å in resolution. The EM density of the gp350 in the D_{123}-ferritin was extracted from the difference map between the D_{123}-ferritin and native ferritin using the UCSF Chimera package. The fit model was made using the EMFit program (Rossmann et al., 2001).

Immunization

Animal experiments were carried out in accordance with all federal regulations and NIH guidelines and approved by the Institutional Animal Care and Use Committee. Eight- to 10-week old female BALB/c mice (Charles River Laboratories) were immunized (n = 5) intramuscularly with 5 or 0.5 μg of purified proteins in 100 μl of 50% (v/v) mixture of SAS adjuvant (Sigma) in PBS at weeks 0, 3 and 16. Sera were collected prior to the first dose and periodically after

immunization. For the non-human primate study, 12 cynomolgus macaques (*Macaca fascicularis*) (5.6 ± 1.0 years old, 5.3 ± 1.8 kilograms, 5 males and 3 females) were divided into 3 groups. Monkeys received 50 μg of gp350 ectodomain, 25 μg of gp350 D_{123}-ferritin or D_{123}-encapsulin in SAS adjuvant (total volume of 1.0 ml) intramuscularly in the quadriceps at weeks 0, 4 and 12. Blood samples were collected prior to the first dose and weeks 6, 8 and 14. Plasma was used for neutralization assays.

LIPS assay

The detailed method of gp350 LIPS assay was described previously (Sashihara et al., 2009). Briefly, a fusion protein composed of gp350 and *Renilla* luciferase was incubated with sera for 1 hour at ambient temperature and immunoprecipitated using protein A/G beads (Thermo Scientific). After washing, coelenterazine substrate (Promega) was added and luminescence was counted on a Centro LB 960 Luminometer (Berthold Technologies).

Cell culture

Raji (human Burkitt lymphoma) cells were propagated in RPMI 1640 with complete supplements (10% fetal bovine serum, 100 U ml^{-1} of penicillin, 100 μ g ml^{-1} of streptomycin, and 2 mM L-glutamine). 293/2089 cells (Delecluse et al., 1998) that contain GFP-reporter EBV (B95-8/F) genome were grown in DMEM with complete supplements and hygromycin at 50 μM.

GFP-reporter virus neutralization assay

Neutralization of EBV to B cells has been described previously (Sashihara et al., 2009). Briefly, immune sera were serially diluted and incubated with B95-8/F reporter virus for 2 hours. For protein competition neutralization, sera were incubated with either soluble gp350 WT or glyc162/208 mutant protein (25 μg ml^{-1}) at ambient temperature for 30 minutes prior to mixing with virus. The mixture was added to Raji cells and incubated for 3 days. Cells were fixed in 2%

paraformaldehyde and analyzed with an Accuri C6 flow cytometer (BD Biosciences). The percentage of GFP^+ cells was quantified using BD CSampler software (BD Biosciences). Neutralization antibody titers were expressed as the concentration of serum antibody needed to inhibit viral entry by 50% (IC_{50}) calculated using controls lacking either virus (0%) or serum (100%).

Recombinant vaccinia virus challenge

Full length EBV gp350 was cloned into pRB21, a plasmid encoding vaccinia virus (VV) vp37 which is required for plaque formation (Blasco and Moss, 1995). BSC-1 cells were infected with VV vRB12 (Δ vp37) and subsequently transfected with pRB21 containing EBV gp350. Recombinant virus (rVV-gp350) was rescued and purified by three rounds of plaque purification. Expression of gp350 was confirmed by surface staining of infected cells with mAb 72A1. Challenge stock was purified on a cushion of 36% sucrose by ultracentrifugation (32,900 × g, 80 min) and titrated in BSC-1 cells. The challenge dose was determined in a pilot experiment using $1 \times 10^5 - 1 \times 10^7$ PFU of virus in naïve animals. Immunized mice were challenged intranasally with 1×10^6 PFU of virus (10 μl per nostril). Mice were weighed daily after challenge and euthanized when they lost ≥ 30% of their pre-challenge weight or suffered symptoms.

Surface Plasmon Resonance-based antibody competition

Soluble gp350 WT protein was immobilized on a CM5 sensor chip (GE Healthcare) through amine coupling at pH 4.5. Fifty microliters (100 μg ml^{-1}) of mAb (72A1, 2L10 or C179) were then flowed over the chip at 30 μl min^{-1} before injecting 50 μl of the serum samples (1/50 dilution) at 30 μl ml^{-1} on a Biacore 3000 instrument (GE Healthcare). The chip was regenerated by two injections (50 μl) of 3M $MgCl_2$ after each run. Percent inhibition (PI) of serum antibodies

to bind gp350 by 72A1 or 2L10 was calculated by an equation: PI = 100 – [(maximum RU of 72A1- or 2L10-saturated curve/maximum RU of C179-saturated curve) × 100].

Statistical analysis

P values were generated by one-way ANOVA using the Prism 5 program (GraphPad Software)

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