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Expression of an immunogenic Ebola immune complex in *Nicotiana benthamiana*

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Summary

Filoviruses (Ebola and Marburg viruses) cause severe and often fatal hemorrhagic fever in humans and non-human primates. The US Centers for Disease Control identify Ebola and Marburg viruses as "category A" pathogens (defined as posing a risk to national security as bioterrorism agents), which has lead to a search for vaccines that could prevent the disease. Because the use of such vaccines would be in the service of public health, the cost of production is an important component of their development. The use of plant biotechnology is one possible way to cost-effectively produce subunit vaccines. In this work, a geminiviral replicon system was used to produce an Ebola immune complex (EIC) in *Nicotiana benthamiana*. Ebola glycoprotein (GP1) was fused at the C-terminus of the heavy chain of humanized 6D8 IgG monoclonal antibody, which specifically binds to a linear epitope on GP1. Co-expression of the GP1-heavy chain fusion and the 6D8 light chain using a geminiviral vector in leaves of *Nicotiana benthamiana* produced assembled immunoglobulin, which was purified by ammonium sulfate precipitation and protein G affinity chromatography. Immune complex formation was confirmed by assays to show that the recombinant protein bound the complement factor C1q. Size measurements of purified recombinant protein by dynamic light scattering and size exclusion chromatography also indicated complex formation. Subcutaneous immunization of BALB/C mice with purified EIC resulted in anti-Ebola virus antibody production at levels comparable to those obtained with a GP1 virus-like particle. These results show excellent potential for a plant-expressed EIC as a human vaccine.

Keywords

Ebola virus; immune complex; Nicotiana benthamiana; plant vaccine; geminiviral replicon system

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Introduction

Recombinant protein expression systems are widely used in the biotechnology industry to produce enzymes, protein hormones, monoclonal antibodies, and antigenic proteins for subunit vaccines. Currently available commercial products are produced by fermentation using bacterial suspensions or cultures of insect, yeast or mammalian cells. Recently there has been substantial progress in the use of green plants as a platform for production of pharmaceutical proteins (Arntzen, 2008; Rybicki, 2010). Advantages in using plants that facilitate industrial adaptation include rapid time frames for production of gram quantities of new proteins for preclinical testing, the ability of plants to produce large and complex proteins with correct processing and assembly into multi-subunit complexes, and recent advances in plant biotechnology allowing the production of glycoproteins with human-like glycosylation patterns (Dieryck et al., 1997; Huang et al., 2009; Huang et al., 2010; Stein et al., 2009; Castilho A, 2010; Huang et al., 2010). In addition, cost advantages of plant-made pharmaceutical proteins relate to capital cost avoidance (plant production *vs*. fermentation) for new manufacturing facilities.

Recombinant protein production in plants can be achieved utilizing two different systems; stable genetic transformation or transient gene expression. The establishment of stable transgenic plants is a time-consuming, with the major limitation of relatively low levels of protein expression (Newell, 2000; Gleba et al., 2005). For transient expression, the plant viral vectors can be used to amplify gene copy number leading to much higher protein expression in comparison to stable transformation. Various types of RNA viruses have been used to create plant viral expression vectors such as tobacco mosaic virus (TMV), potato virus X (PVX), cowpea mosaic virus (CPMV), and alfalfa mosaic virus (AIMV) (Yusibov et al., 2006). In an effort to improve the efficiency of the traditional viral vector, a deconstructed tobacco mosaic virus (TMV)-based system called magnICON was created (Gleba et al., 2004; Marillonnet et al., 2004; Gleba et al., 2007). The magnICON vector system yielded excellent results with different proteins including enzymes, antibodies, antigens, cytokines, and hormones (Gleba et al., 2005).

DNA viruses such as the geminiviruses can be used as plant viral vectors to express recombinant protein. Geminiviruses have a single stranded DNA genome that replicates in the nucleus of host cells by a rolling circle replication mechanism using a double stranded DNA intermediate (Stenger et al., 1991; Gutierrez, 1999). The viral replication initiation protein (Rep) initiates the replication cycle by binding to a specific viral DNA sequence in the long intergenic region (LIR) and cleaving it, which promotes rolling circle replication mediated by host cell DNA polymerase and other factors. Rep then ligates the single stranded DNA products to form a circular viral DNA (Laufs et al., 1995). For recombinant protein expression, the geminiviral coat protein and movement protein can be replaced with the gene of interest. After Rep protein is expressed, the replicon containing the gene of interest is amplified, and the double stranded DNA intermediates act as transcription templates, leading to high amounts of mRNA and subsequent recombinant protein (Mor et al., 2003; Huang et al., 2009; Huang et al., 2010).

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Ebola virus, the causative agent of Ebola hemorrhagic fever, is a negative sense, single stranded RNA virus belonging to family Filoviridae. The shape of the virus is filamentous in form and approximately 80 nm in diameter, but it demonstrates no uniform length (Ellis et al., 1978). There are 4 distinct species of Ebola virus: Zaire, Sudan, Ivory Coast, and Reston, with Zaire Ebola virus causing the highest mortality rate among all Ebola viruses, up to 90% (Wilson et al., 2001). Currently, there are no vaccines or antiviral drugs approved for prevention or treatment of this disease in humans. However, there are some new vaccine candidates, which have demonstrated protection in nonhuman primates against lethal challenge with Ebola virus, including Ebola Venezuelan equine encephalitis virus replicon particles (VRP) (Pushko et al., 2000), Ebola virus-like particle (Warfield et al., 2007), recombinant vesicular stomatitis virus-based Ebola vaccine (Geisbert et al., 2008; Geisbert et al., 2009; Qiu et al., 2009), adenovirus-based vaccine (Richardson et al., 2009) and a prime-boost strategy with DNA encoding glycoprotein of the Zaire strain followed with recombinant adenovirus encoding the same antigen (Hensley et al.; Sullivan et al., 2000). Moreover, a DNA vaccine encoding for Ebola glycoprotein (GP) has been shown to be both safe and immunogenic in humans (Martin et al., 2006).

Ebola GP is a glycoprotein containing both N- and O-linked carbohydrate (Feldmann et al., 1991; Feldmann et al., 1994). GP is a type I transmembrane protein that forms homotrimers, which create a spike on the viral envelope (Malashkevich et al., 1999; Yang et al., 2000). It can mediate viral binding and entry into host cell (Lee et al., 2008). GP consists of 2 subunits, the extracellular GP1 and membrane anchored GP2, linked by disulfide bonds (Sanchez et al., 1998; Manicassamy and Rong, 2009). GP1 is responsible for receptor binding and GP2 mediates membrane fusion. Transient expression of GP in cultured mammalian cells causes cytopathic effects including cell rounding and detachment (Chan et al., 2000; Simmons et al., 2002). GP can down-regulate several cell surface molecules such as major histocompatibility complex class I (Sullivan et al., 2005) and integrin (Simmons et al., 2002). Since Ebola GP is the protein that mediates infection and is the major antigen (Wilson et al., 2000), it is a critical target for vaccine development. Ebola GP or segments thereof can be expressed in recombinant systems such as *E. coli* (Das et al., 2007), insect cells (Mellquist-Riemenschneider et al., 2003; Ye et al., 2006), and mammalian cells (Melito et al., 2008). However, these systems are not optimal, and in order to reduce its toxicity on the host cell, GP1 expression in mammalian cells was regulated by an ecdysone inducible system (Melito et al., 2008).

Recombinant immune complexes were originally expressed in tobacco plants via fusion of tetanus toxin fragment C (TTFC) to the heavy chain of a TTFC-binding IgG and co-expression with its light chain (Chargelegue et al., 2005). The TTFC immune complexes were shown to bind to C1q, Fc receptor gamma RIIa (FcγRIIa), and antigen presenting cells. Mice immunized with the recombinant TTFC immune complexes showed much higher antibody titers than those immunized with TTFC alone. This study demonstrated the recombinant immune complex as a strong vaccine candidate and led us to pursue a similar strategy with Ebola GP1.

In this study, we used the geminiviral replicon system derived from bean yellow dwarf virus (Huang et al., 2010) to produce Ebola immune complexes (EIC) in *Nicotiana benthamiana*.

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We fused Ebola GP1 at the C-terminus of the heavy chain of humanized 6D8 monoclonal antibody (mAb) (Huang et al., 2010), which specifically binds to the 6D8 linear epitope on GP1. When the fusion protein was co-expressed with the light chain of 6D8 mAb, IgG molecules assembled, leading to immune complex formation. EIC were expressed, purified, and used to immunize mice, showing that plant expressed EIC represent a viable vaccine candidate for humans.

Results

Transient Expression of recombinant Ebola immune complex in leaves

We produced EIC by co-expression of a heavy chain-GP1 fusion protein (H2GP1) with the light chain (K3). We compared EIC expression in *N. benthamiana* leaves using geminiviral replicons. The expression of viral Rep protein (C1/C2 gene) is required for amplification of the replicon (Laufs et al., 1995; Huang et al., 2010). The Rep cassette is contained in the complimentary sense orientation in the light chain vector pBYK3R (Fig. 1). The expression cassettes, driven by the dual-enhancer CaMV 35S promoter, are placed between the long intergenic region (LIR) and short intergenic region (SIR) in the viral-sense orientation, replacing the viral movement and coat protein genes. In the case of dual replicon vector pBYRH2GP1kdK3, the heavy chain-GP1 fusion and light chain cassettes are placed within different replicons oriented in tandem. In all cases we also co-expressed the gene silencing inhibitor p19 from tomato bushy stunt virus using the non-replicating expression vector pPSp19. We attempted Ebola GP1 protein expression without heavy chain fusion in plant leaves using pBYR6HGP1kd, which has a 6His tag at the N-terminus (Fig. 1). Ebola GP1 expressed from pBYR6HGP1kd produced strong necrosis in leaves, but fusing GP1 to 6D8 mAb reduced the toxicity of GP1 (Fig. 2). Due to the extensive necrosis, were unable to obtain unfused GP1 in sufficient yield for immunization experiments.

We compared the protein expression levels using pBYK3R co-delivered with pBYH2GP1 or pBYH2GP1kd (encoding H2GP1 with SEKDEL at C-terminus), and extracting four days after agroinfiltration. We assayed by ELISA to measure human IgG, and found that the SEKDEL construct yielded ~3-fold higher expression (Fig. 3a). Thus, we used the SEKDEL construct in the dual replicon vector pBYRH2GP1kdK3, which provided somewhat higher expression than co-delivery of the two separate vectors, up to ~50μg IgG per g leaf mass (Fig. 3a). Therefore, we used pBYRH2GP1kdK3+p19 in subsequent experiments to produce and characterize EIC. A time course of EIC expression on different days after infiltration showed that the optimal harvest time was 4 days after infiltration (Fig. 3b).

C1q Binding assay

C1q is a protein component of the complement cascade of the mammalian innate immune system. C1q binds to immune complexes with a higher affinity than to antibody alone (Krieger et al., 1985). We used the C1q binding assay to confirm immune complex formation in plant leaf extracts. Varying dilutions of leaf extracts expressing 6D8 mAb (pBYH2kd+pBYK3R) or EIC (pBYRH2GPkdK3) at equivalent IgG concentrations were incubated with C1q, which was immobilized on the microtiter plate wells. The data (Fig. 4) show that EIC leaf extracts produced substantially higher OD signals than the mAb alone,

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which is consistent with previous observations (Krieger et al., 1985). These data indicate the formation of immune complexes when EIC was produced in *N. benthamiana*.

Purification and Characterization of Ebola immune complex

To purify the immune complex, we used ammonium sulfate precipitation and protein G affinity chromatography. These two steps removed the great majority of the endogenous leaf proteins, including the major plant protein Rubisco, as shown by reducing SDS-PAGE (Fig. 5, lane 3). We estimate that the EIC preparation was ~90% pure based on visual inspection of the Coomassie stained gels (lane 8 in Fig. 5 and Fig. 6b). We observed that EIC extracted directly from leaves in SDS sample buffer (reducing condition) displayed a single band at 130kDa when probed with anti-heavy chain (Fig. 6a). The purified EIC examined on Coomassie-stained gels comprised 5 different protein species with apparent molecular weights of 130, 110, 55, 50, and 25kDa (Fig. 6b). Western blot showed that anti-human heavy chain bound to the largest 4 of the 5 species (Fig.6c), whereas anti-GP1 linear epitope 6D8 (Wilson et al., 2000) bound to only the 130kDa protein (Fig. 6e). Anti-human kappa chain antibody bound to the protein at 25kDa (Fig. 6d), which is the expected size of kappa chain. A gel run under non-reducing condition and probed with monoclonal antibody 13C6, which binds a conformational epitope (Wilson et al., 2000), showed a signal at high molecular mass (Fig. 6f), which is consistent with a fully assembled immunoglobulin structure. This confirmed the correct folding of GP1 in the H2-GP1 fusion protein expressed in *N. benthamiana*. The presence of multiple protein species that contain heavy chain sequence (Fig. 6c) indicates proteolytic degradation during extraction and purification, since the smaller bands were not present when leaves were extracted directly in SDS sample buffer (Fig. 6a). Nonetheless, we conclude that the full length EIC is assembled *in planta* and the GP1 showed correct folding.

Molecular sizing of the Ebola RIC

We assessed the diameter size of the purified EIC preparation using a Zetasizer Nano-ZS instrument (Malvern Instruments, UK), which measures the hydrodynamic diameter using dynamic light scattering. For these measurements, the purified EIC, plant-made 6D8 mAb, and human IgG (Southern Biotech, AL) were diluted in PBS pH7.5 to a concentration 0.1mg/ml. The data shows that the average diameter of EIC was ~20nm (Fig. 7a), whereas plant-made 6D8 mAb and standard human IgG were ~10nm. The diameter of the antibody standard measured by Malvern Instruments was also 10nm (http://www.malvern.com/common/downloads/campaign/MRK880-01.pdf), which was consistent with our result. Samples were also tested at a lower concentration of 10μg/ml, with the same results (data not shown). Moreover, size exclusion chromatography was used to confirm the complex formation. The mouse IgG and plant-made 6D8 mAb (150 kDa) eluted at 18 min and bovine thyroglobulin (669 kDa) eluted at 15 min (Fig. 7b). The EIC eluted over a broad region, but had peaks at 11 min and 15-16 min. Therefore, the size of EIC eluted at 11 min is bigger than 669 kDa, which suggested there are more than 2 molecules in the complex (1 molecule is ~310 kDa). The peak at 15-16 min probably represents the dimer, and material eluting between 11-15 min may indicate larger oligomers.

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Immunization study

To examine the immunogenicity of the plant-produced EIC, we immunized mice by subcutaneous injection, on days 0, 21, 42, and 63 with of 10μg purified EIC (measured as IgG equivalent). We used VRP-GP1 (provided by John Dye, USAMRIID, Ft. Detrick, MD) as the positive control (Pushko et al., 2000). We used four doses because three doses of either EIC or VRP-GP1 yielded low titres. Serum antibody responses were evaluated by ELISA using irradiated Ebola virus as the capture antigen. Three weeks after the last dose, a geometric mean titer (GMT) antibody level in the plant-made EIC group was comparable to the VRP-GP1 group (Fig. 8). Using Kruskal-Wallis Test (nonparametric one-way ANOVA), the P value for comparison of EIC and VRP-GP1 GMT values is >0.05, indicating no significant difference. A placebo PBS-immunized group showed no anti-Ebola response and was arbitrarily placed at a GMT value of 1.0. These results indicate that plant-made EIC is immunogenic in mice to an extent comparable to VRP-GP1. It should be noted that VRP-GP1 is an alphavirus-based particle and contains an RNA genome that undergoes a single round of replication in the host dendritic cells and macrophages (Pushko et al., 2000).

Discussion

In this work, *N. benthamiana* plants were used as a bioreactor for the geminiviral replicon system to produce EIC. We chose to use this viral vector, which uses a rolling circle replication mechanism with a double stranded DNA intermediate, because it has distinct advantages for the simultaneous expression of two (or, potentially more) different proteins. For the EIC, the efficient assembly of the complex requires synchrony in expression of the H2GP1 fusion and the mAb 6D8 light chain, and the geminiviral vector allows efficient and non-competing amplification of two different replicons delivered together (Huang et al., 2010). DNA amplification with geminiviral vectors enhanced expression at least 5-fold over non-replicating vectors using the same expression cassette (Huang et al., 2009), and we observed similar enhancement for EIC expression (data not shown). We considered using vectors based upon RNA viruses, but recognized that until recently it has been difficult to efficiently express multi-component proteins like mAbs with these vectors. Co-delivery of RNA viral vectors built on the same virus backbone always results in early segregation and subsequent preferential amplification of one of the vectors in one cell – a common scenario of “competing replicons” (Dietrich and Maiss, 2003; Hull and Plaskitt 1970). We recognize that this problem can be overcome by utilizing two sets of vectors derived from non-competing viruses; e.g., an IgG was produced using tobacco mosaic virus (TMV) and potato virus X (PVX) vectors, with each virus driving the expression of separate protein subunits (Giritch et al., 2006). While this has been an elegant example of overcoming the competing replicon constraint for one class of heterodimeric proteins, for other proteins of more dramatic size variation (such as the EIC), a search for appropriate “paired” RNA virus vectors would need to be identified by trial and error. We reasoned that a DNA vector, containing one copy of each gene to be expressed in the EIC heterodimeric complex, might be a more direct and efficient strategy to express the immune complex. This hypothesis was borne out in our experiments.

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Geminiviral replicon systems have been used for plant production of several recombinant proteins, including GUS protein (Mor et al., 2003), GFP (Kim et al., 2007), and capsid proteins of hepatitis B and Norwalk viruses (Huang et al., 2009). It was alsoused to produce an IgG mAb, which efficiently assembled and retained its specific antigen binding property (Huang et al., 2010). In the present study, we used the geminiviral replicon system to produce a heavy chain-GP1 fusion protein, which has a molecular mass of ~130kDa. The viral Rep protein can be supplied either *in trans* (Mor et al., 2003) or in the geminiviral replicon, linked with the gene of interest (Huang et al., 2010). We demonstrated that the single vector pBYRH2GP1kdelK3 yielded higher expression than co-delivery of two separate geminiviral replicons for H2-GP1 and K chains (Fig. 3). Comparison of constructs with or without the ER retention signal SEKDEL showed substantially higher expression with SEKDEL.

The immune complex resulted from the expression of a fusion protein in which the antigen GP1 was linked to the heavy chain of the mAb 6D8 and co-expressed with the 6D8 light chain (K3). When the heavy and light chains assemble, immune complexes can be formed when the 6D8 antigen-combining sites at the N-termini encounter the GP1 6D8 epitope near the C-terminus of another molecule; this process is diagrammatically described in Fig. 9. Immune complexes have several immunological functions, including C1q binding and Fcγ receptor binding, which enhance its ability to be taken up by antigen presenting cells (Bachmann et al., 1994; Heyman, 2000; de Jong et al., 2006). Previous studies also demonstrated that the binding of immune complex via Fc receptor to dendritic cells (DC) induced DC maturation and promoted the efficient presentation of peptides on major histocompatibility complex class I and II (Regnault et al., 1999; Bajtay et al., 2006). The enhanced endocytosis, the improved antigen presentation, and thereby enhanced T cell activation may create the potential for immune complexes to be used as effective self-adjuvanting vaccines. C1q is a member of the complement cascade, which enhances the ability of the immune system to clear pathogens. It is known to bind to immune complexes with higher affinity than monomeric IgG (Krieger et al., 1985). In this study, we demonstrated that the EIC binds to C1q significantly better than antibody alone. Moreover, the EIC particle size measured by dynamic light scattering indicated immune complex formation. The diameters of standard human IgG and plant-derived 6D8 mAb were measured at 10nm, which is similar to that reported for IgG by Malvern Instruments, UK (http://www.malvern.com/common/downloads/campaign/MRK880-01.pdf). EIC presented a diameter of 20nm, which strongly suggests the formation of immune complex. Although the structure of Ebola GP1 is unknown, it is reasonable that 20nm diameter of EIC would accommodate approximately 4 Ag-Ab fusion molecules. Moreover, size-exclusion chromatography data (Fig. 7b) were consistent with this interpretation, showing a range of complex sizes greater than 2 molecules.

After purification, the protein G affinity purified material showed 5 protein species when analyzed under reducing conditions and stained with Coomassie which (Fig. 4, bands at 130, 110, 55, 50, and 25kDa), which indicate proteolytic cleavages in the fusion protein. The calculated approximate size of the full-length glycosylated GP1-heavy chain fusion is ~130kDa, equivalent to the largest band observed. Based on amino acid sequence, the H2-

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GP1 fusion protein is expected to be ~100kDa. However, because the GP1 sequence contains 15 potential N-glycosylation sites that may be differentially utilized, the molecular mass could increase by up to ~30kDa. Thus, it is possible that the 110kDa protein represents a glycosylation variant, but it may also represent a fragment resulting from proteolysis at a site proximal to the GP1 C-terminus. The presence of H-chain species (indicated by positive reaction in Western blots, Fig. 5) at 55 and 50kDa suggests that some proteolytic degradation of the H2-GP1 occurred during the purification process, despite the use of protease inhibitors. Since extraction of leaves directly in reducing SDS sample buffer yielded only the 130kDa species (Fig. 5a), the full length fusion protein appeared to be stable *in planta*, suggesting that the observed degradation occurred only after extraction and processing under non-denaturing conditions. We observed a direct relationship between the extent of degradation in crude extracts and the time they were kept at 4°C (data not shown), which further supports the idea of post-extraction instability. Thus, there is potential to increase yields of EIC by the use of more appropriate protease inhibitors, and decreasing the processing time involved in purification.

It is important to note that our data indicate that the GP1 protein folded correctly to produce authentic antigen. Western blotting with 13C6 mAb, which recognizes a conformational epitope of Ebola GP1 (Wilson et al., 2000), showed a signal at the expected size under non-reducing condition (Fig. 5f). This observation indicates that the plant expressed Ebola GP1 is folded correctly when assembled into a high molecular weight IgG complex. A further indication that the EIC contains authentic GP1 antigen is provided by the mouse immunization data. EIC delivered subcutaneously elicited antibodies (Fig. 8) that were specific to whole Ebola virus. The antibody titres in mice immunized with EIC were comparable to, although somewhat less than, those achieved using the VRP-GP1 positive control, which showed complete protection of the mice and guinea pigs from Ebola challenge (Pushko et al., 2000). Since VRP-GP1 is an alphavirus vector, it undergoes limited replication in antigen presenting cells of the immunized host, thus creating viral replicon RNA that can act as a powerful adjuvant through toll-like receptor signaling (Alexopoulou et al., 2001). We did not use any adjuvant for the EIC antigen in this study, but in yet unpublished work we found a significant effect using adjuvants, which yielded mouse serum antibody titres at levels that protected mice from Ebola virus challenge (Phoolcharoen et al., manuscript in preparation). The titre of Ebola-specific antibody may not always correlate with viral neutralizing activity, but protection from viral challenge indicates vaccine efficacy. Thus, the antibody response to plant-derived EIC shown here is a significant observation that indicates strong potential for future development of an EIC-based vaccine.

It is important to note that the purified EIC used for immunization was partially degraded, and that complex formation was likely impaired due to loss of part or all of the GP1 protein (Figs. 5 and 6). We estimate that each dose contained ~5μg of GP1 protein, which was roughly estimated from the immunoblots. This suggests that a much better result could be obtained by inhibition of protease activity during the purification, which would enhance the yield and facilitate higher dosing. Furthermore, the presence of unfused IgG in the purified material likely increased the potential for immunodominance of the platform, which could limit the immunogenicity of the GP1 component. We detected substantial levels of antibody

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against human IgG in the serum of our immunized mice (data not shown). Thus, future development of this strategy will put strong effort into protease inhibition.

Our results are consistent with those of a previous study (Chargelegue et al., 2005), which showed that TTFC immune complex produced from *Nicotiana tabacum* enhanced immune response and acted as "self adjuvant" when immunized into mice. In that study, a mouse IgG was fused with TTFC, and the resulting TTFC immune complexes bound to mouse dendritic cells and induced significantly higher anti-TTFC IgG in mice compared to TTFC antigen alone. Our finding of high titres of anti-human IgG antibodies in the immunized mice is not surprising, since we used the humanized 6D8 antibody in the immune complex, in order to use EIC as a vaccine in humans. Therefore, it is possible that anti-human IgG responses in the mice interfered with optimal anti-GP1 immune responses. We predict that use of EIC in humans will induce a stronger anti-Ebola immune response, due to the potential for targeting the GP1 to antigen presenting cells.

We were unable to produce unfused GP1 in sufficient quantities for immunization, due to the extreme leaf toxicity that it produced (Fig. 2). Since there is no commercial source of recombinant Ebola GP1, we could not directly determine anti-GP1 specific IgG. Previous studies indicated that transient expression of Ebola GP caused toxicity in mammalian cells including cell rounding and detachment (Chan et al., 2000; Simmons et al., 2002). Our observations of leaf toxicity upon GP1 expression in *N. benthamiana* are consistent with the effects observed in mammalian cells. Interestingly, when GP1 was fused to antibody forming immune complex, the toxicity of GP1 to leaves was reduced compared to unfused GP1 (Fig. 2). Since unfolded protein response can contribute to cell death response (Urade, 2007), fusion of GP1 to IgG H-chain may allow more efficient folding in the ER, perhaps by recruitment of ER chaperones and the limitation of an ER stress response.

In conclusion, this study provides evidence that EIC can be produced in *N. benthamiana* using the geminiviral replicon system, accompanied by folding of GP1 into an immunogenic complex that contains a conformation-specific epitope. The GP1 component of EIC is immunogenic in mice, eliciting antibody levels comparable to the "gold standard" VRP-GP1. Future work will focus on minimizing proteolytic degradation during purification, and enhancing the vaccine efficiency of plant-produced EIC by optimization of dosage and use of adjuvants that enhance cell-mediated as well as humoral immune responses.

Experimental Procedures

Design of the construct for producing Ebola immune complex

The geminiviral vector pBYK3R was previously described (Huang et al., 2010). We designed a plant-optimized DNA sequence encoding Ebola GP1 based upon the GenBank Accession AY354458, using codons that are preferred in tobacco, and removing spurious mRNA processing signals (Geyer et al., 2010). The designed sequence is deposited in the GenBank (Accession HM136775). The GP1 coding sequence was fused via a $(G_4S)_3$ linker to the C-terminus of a gene encoding the humanized 6D8 monoclonal antibody (mAb) H2 (Huang et al., 2010). The linker was added to the 3′ end and an XbaI site created at the 5′ end of the 6D8-H2 gene by end-tailoring PCR using the primers H2-Xba-F 95′-

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GGTCTAGAACaATGGGATGGTCTTGCATC) and 6D8H2-G4S-Bam (5′-GGGGATCCACCTCCGCCTGAACCGCCTCCACCTGATCCGCCACCTCCTTTACCCGGAGACAAGGAGAG). A BamHI site was created at the 5′ end of the GP1 gene by PCR using the primer GP1-Bam (5′-gGGGATCCATCCCACTTGGAGTTATTC), and the 6D8-H2-$((G_4S)_3$ and GP1 genes fused via the BamHI site. The 6D8-H2y$((G_4S)_3$-GP1 fusion was inserted into the geminiviral vector pBYR1 (a derivative of pBYK3R that has unique XbaI, KpnI, and SacI sites for insert cloning) via XbaI and SacI sites. The hexapeptide SEKDEL was added to the C-terminus of the GP1 sequence by end-tailoring with the reverse primer GP1-SEKDEL-Kpn-R (5′-CCGGTACCTTAAAGCTCATCCTTCTCTGAACGCCTAGTTCTTCGTCC) and the modified gene inserted into pBYR1 via XbaI and KpnI sites. H2gp1 is inserted in the geminivector between LIR and SIR. The light chain of 6D8 mAb or K3 is expressed in another geminivector with Rep protein. When H2GP1 and K3 were expressed in tobacco, this forms immune complex because the variable region of 6D8 mAb can specifically bind to 6D8 epitope in gp1. The dual replicon vector pBYRH2GP1kdK3, containing the fused replicons from pBYH2GP1kd and pBYK3R, was constructed by ligating 3 fragments: pBY-HL(6D8).R (Huang et al., 2010)/XbaI-SacI, pBYH2GP1kd/XbaI-HindIII, and pBYH2GP1kd/HindIII-SacI. Ebola GP1 alone was expressed by co-infiltrating with pBYR6HGP1kd and pPSP19. pBYR6HGP1kd was constructed by fusing the GP1-SEKDEL coding sequence from pBYH2GP1kd (BamHI-SacI) with the soybean vspA N-terminal signal peptide (NcoI-BamHI) from pBTI201.4 (Judge et al., 2004), which had been modified by addition of a 5′ sequence to encode the amino acids M-A-S-S and the 3′ sequence encoding a 6,His tag, by PCR using the mutagenic primers aS-MASS (5′-GATCCATGGCTTCCTCTAAGGTCCTTGTTTTCTTCG) and 6H-Bam-R (5′, CGGGGATCCgTGaTGATGGTGATGGTGTC). The two fragments were ligated with pBYR1 (digested NcoI-SacI) to make pBYR6HGP1kd. GFP was expressed by using pBYGFP.R (Huang et al., 2009) co-infiltrating with pPSp19.

Plant inoculation and protein expression

Nicotiana benthamiana plants were inoculate with *Agrobacterium* by needle infiltration on the lower surface of leaves. The agroinfiltration procedure was performed as previously described (Huang et al., 2009). For geminivector constructs, final OD600 is 0.25. Plants were maintained in growth chamber. The leaves were harvested on day 2, 4, 6, and 8 after infiltration for expression time-course experiments. For other experiments, the leaves were harvested on day 4 after infiltration. Soluble proteins were extracted by grinding the leaves with Fastprep (Bio101) machine in 1ml of extraction buffer (phosphate-buffered saline (PBS) pH7.5, leupeptin, and 0.1% Tween-20) per 0.1mg of leaves. After centrifugation at 13,000 rpm for 5 minutes, the supernatant was retained to be analyzed by ELISA and Western blot.

Sandwich ELISA protocol for Ebola immune complex quantification

Ebola immune complex in plant extract was quantified by ELISA. Goat anti human IgG was diluted 1:1000 in phosphate-buffered saline (PBS pH7.5; 50ul per well) was bound to 96-well polyvinylchrolide microtiter plates overnight at 4°C. The plate was blocked with 5% skim milk in PBS for 2 hours 37°C. After washing the wells one time with PBS containing

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0.05% Tween 20 (PBST), samples (50ul per well) diluted in 1% skim milk in PBST were added to wells and incubated 1 hour at 37°C. The wells were washed three times with PBST and incubated with goat anti human kappa-horseradish peroxidase (HRP) conjugate diluted 1:4000 in 1% skim milk in PBST for 1 hour at 37°C. Plates were developed with TMB substrate (Pierce, IL) for 5 min at 23°C. The reaction was ended by addition of equal volume of 1M H2SO4, and the absorbance was read at 450 nm.

SDS-PAGE and Western blot

Plant protein crude extracts and human IgG were denatured by boiling in SDS-PAGE sample buffer and separated on 4-15% gradient polyacrylamide gels. Proteins were either visualized by Coomassie blue staining or electrophoretically transferred to polyvinlidene difluoride (PVDF) membrane (Amersham, NJ). To detect human heavy chain, the membrane was probed with goat anti-human IgG-HRP conjugated (Southern biotech, AL) diluted at 1:5000 in 1% skim milk in PBST. To detect human light chain, the membrane was probed with goat anti-human kappa-HRP conjugated (Southern biotech, AL) diluted at 1:10000 in 1% skim milk in PBST. To detect Ebola GP1, the membrane was incubated with mouse anti-6D8 (antibody against linear 6D8 epitope in GP1) or mouse anti-13C6 (antibody against conformational 13C6 epitope) diluted 1:10000 in 1% skim milk in PBST and goat anti-mouse IgG-HRP conjugated diluted 1:10000 in 1% skim milk in PBST. The membranes were developed by chemiluminescence using ECL plus detection reagent (Amersham, NJ).

C1q binding assay

50μl of 7.5μg/ml human complement C1q in PBS was coated on the plate overnight at 4°C. After washing with PBST for 2 times, the plate was blocked with 5% skim milk in PBST for 2 hours at 37°C. The plate was washed with PBST 1 time. Plant extracts containing 25ng equivalent amounts of human IgG from leaves expressing 6D8 mAb or EIC (determined by ELISA) were serial diluted, added into the plate and incubated overnight at 4°C. After washing for 3 times with PBST, the plate was incubated with 1:4,000 goat anti human kappa-HRP in 1% skim milk in PBST for 1 hour at 37°C. The plate was developed with TMB substrate and read OD450.

Protein Purification

Infiltrated tobacco leaves were homogenized by using a blender with extraction buffer (PBS + protease inhibitor tablet from Sigma, MO). Crude extract was filtered through Miracloth and centrifuged at 17700g for 15 minutes. Ammonium sulfate was added into the supernatant to 35% saturation (194 grams per 1 liter of the solution) and mixed with stirrer at 4°C for 1 hour. The solution was centrifuged at 17700g for 15 minutes and the pellet was discarded. Then ammonium sulfate was added to the supernatant to 60% saturation (151 grams per 1 liter of the solution) and mixed with stirrer at 4°C for 1 hour. The solution was centrifuged at 17700g for 15 minute and the pellet was dissolved with the extraction buffer. The solution was filtered with 0.2-micron filter and Protein G bead (Pierce, IL) was added into the filtered solution. The protein extract and the Protein G bead were rotated at 4°C for 1 hour. Then it was loaded into the column and the resin allowed to settle. The protein G column was washed with PBS, pH7.5 and eluted with 50mM citric acid pH2.5. After the protein was eluted from the column, 1M Tris-base was added to neutralize to a final pH of

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7.5. The purified protein was filtered through 0.2 micron filter and concentrated with Amicon ultra-4 Centrifugal Filter Units-30kDa (Millipore, MA).

Estimation of complex size by dynamic light scattering

The diameter size was measured by Dynamic Light Scattering using Zetasizer Nano-ZS instrument (Malvern Instruments, UK). The purified plant-made Ebola immune complex, purified plant-made 6D8 mAb, and human IgG (Southern biotech, AL) were diluted in PBS to the concentration 0.1mg/ml and added into the disposable polystyrene cuvette for Zetasizer measurement.

Size exclusion chromatography

A sample volume of 20 μl was loaded onto BioSep SEC-S4000 column, 600X7.8 mm (Phenomenex, Torrance, CA). The proteins were eluted with PBS, pH 7.3, at a flow rate 1 ml/min. A chromatogram was recorded measuring the UV absorbance at 280 nm. Bovine thyroglobulin (Sigma, St. Louis, MO) and mouse IgG (SouthernBiotech, Birmingham, AL) were used as protein markers.

Mice immunization

Female BALB/C mice were subcutaneously immunized by purified Ebola immune complex from tobacco leaves. Ebola immune complex (10μg, equivalent to ~5μg of Ebola GP1) was injected into the mice. VRP-GP1 (1.6×10^8 replicon per mouse) delivered subcutaneously was used as the positive control (provided by William Pratt, USAMRIID), and PBS was used as the negative control. The mice were immunized on days 0, 21, 42, and 63. Individual preimmune serum was collected before the first immunization, day 0. Blood samples were obtained 3 weeks after last immunization.

Serum antibody analysis

The specific serum IgG response was determined by end point titer ELISA. Polyvinyl chloride 96-well ELISA plates were coated with 50ul of irradiated Ebola virus (provided by John Dye, USAMRIID) diluted 1:1000 in PBS and incubated at 4°C overnight. Plates were blocked with 5% skim milk in PBST at 23°C for 2 hours. Subsequently, the plates were incubated with the serum diluted in 1%skim milk in PBST for 1 hour at 37°C and then HRP-conjugated goat anti-mouse IgG for 1 hour at 37°C. The plates were developed with TMB substrate and read OD450. Endpoint titre was reported as the reciprocal of the highest dilution that had an absorbance value ≥0.02 (two times of OD value for pre-immune serum) above the background (absorbance of the well lacking the serum). The geometric mean titre data were subjected to statistical analysis using the Kruskal-Wallis test (nonparametric one-way ANOVA).

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References

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. Nature. 2001; 413:732. [PubMed: 11607032]

Arntzen CJ. Plant science. Using tobacco to treat cancer. Science. 2008; 321:1052–1053. [PubMed: 18719274]

Bachmann MF, Kundig TM, Hengartner H, Zinkernagel RM. Regulation of IgG antibody titers by the amount persisting of immune-complexed antigen. Eur J Immunol. 1994; 24:2567–2570. [PubMed: 7925584]

Bajtay Z, Csomor E, Sandor N, Erdei A. Expression and role of Fc- and complement-receptors on human dendritic cells. Immunol Lett. 2006; 104:46–52. [PubMed: 16448704]

Castilho A,SR, Stadlmann J, Grass J, Jez J, Gattinger P, Kunert R, Quendler H, Pabst M, Leonard R, Altmann F, Steinkellner H. In planta protein sialylation through over-expression of the respective mammalian pathway. J Biol Chem. 2010

Chan SY, Ma MC, Goldsmith MA. Differential induction of cellular detachment by envelope glycoproteins of Marburg and Ebola (Zaire) viruses. J Gen Virol. 2000; 81:2155–2159. [PubMed: 10950971]

Chargelegue D, Drake PM, Obregon P, Prada A, Fairweather N, Ma JK. Highly immunogenic and protective recombinant vaccine candidate expressed in transgenic plants. Infect Immun. 2005; 73:5915–5922. [PubMed: 16113311]

Das D, Jacobs F, Feldmann H, Jones SM, Suresh MR. Differential expression of the Ebola virus GP(1,2) protein and its fragments in E. coli. Protein Expr Purif. 2007; 54:117–125. [PubMed: 17383893]

de Jong JM, Schuurhuis DH, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Ossendorp F, Toes RE, Verbeek JS. Murine Fc receptors for IgG are redundant in facilitating presentation of immune complex derived antigen to CD8+ T cells in vivo. Mol Immunol. 2006; 43:2045–2050. [PubMed: 16513171]

Dietrich C, Maiss E. Fluorescent labelling reveals spatial separation of potyvirus populations in mixed infected Nicotiana benthamiana plants. J Gen Virol. 2003; 84:2871–2876. [PubMed: 13679622]

Dieryck W, Pagnier J, Poyart C, Marden MC, Gruber V, Bournat P, Baudino S, Merot B. Human haemoglobin from transgenic tobacco. Nature. 1997; 386:29–30. [PubMed: 9052777]

Ellis DS, Simpson IH, Francis DP, Knobloch J, Bowen ET, Lolik P, Deng IM. Ultrastructure of Ebola virus particles in human liver. J Clin Pathol. 1978; 31:201–208. [PubMed: 641193]

Feldmann H, Will C, Schikore M, Slenczka W, Klenk HD. Glycosylation and oligomerization of the spike protein of Marburg virus. Virology. 1991; 182:353–356. [PubMed: 2024471]

Feldmann H, Nichol ST, Klenk HD, Peters CJ, Sanchez A. Characterization of filoviruses based on differences in structure and antigenicity of the virion glycoprotein. Virology. 1994; 199:469–473. [PubMed: 8122375]

Geisbert TW, Geisbert JB, Leung A, Daddario-DiCaprio KM, Hensley LE, Grolla A, Feldmann H. Single-injection vaccine protects nonhuman primates against infection with marburg virus and three species of ebola virus. J Virol. 2009; 83:7296–7304. [PubMed: 19386702]

Geisbert TW, Daddario-Dicaprio KM, Geisbert JB, Reed DS, Feldmann F, Grolla A, Stroher U, Fritz EA, Hensley LE, Jones SM, Feldmann H. Vesicular stomatitis virus-based vaccines protect nonhuman primates against aerosol challenge with Ebola and Marburg viruses. Vaccine. 2008; 26:6894–6900. [PubMed: 18930776]

Geyer BC, Kannan L, Cherni I, Woods RR, Soreq H, Mor TS. Transgenic plants as a source for the bioscavenging enzyme, human butyrylcholinesterase. Plant Biotechnol J. 2010; 8:1–14.

Giritch A, Marillonnet S, Engler C, van Eldik G, Botterman J, Klimyuk V, Gleba Y. Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. Proc Natl Acad Sci USA. 2006; 103:14701–14706. [PubMed: 16973752]

Gleba Y, Marillonnet S, Klimyuk V. Engineering viral expression vectors for plants: the ‘full virus’ and the ‘deconstructed virus’ strategies. Curr Opin Plant Biol. 2004; 7:182–188. [PubMed: 15003219]

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Gleba Y, Klimyuk V, Marillonnet S. Magnifection--a new platform for expressing recombinant vaccines in plants. Vaccine. 2005; 23:2042–2048. [PubMed: 15755568]

Gleba Y, Klimyuk V, Marillonnet S. Viral vectors for the expression of proteins in plants. Curr Opin Biotechnol. 2007; 18:134–141. [PubMed: 17368018]

Gutierrez C. Geminivirus DNA replication. Cell Mol Life Sci. 1999; 56:313–329. [PubMed: 11212359]

Hensley LE, Mulangu S, Asiedu C, Johnson J, Honko AN, Stanley D, Fabozzi G, Nichol ST, Ksiazek TG, Rollin PE, Wahl-Jensen V, Bailey M, Jahrling PB, Roederer M, Koup RA, Sullivan NJ. Demonstration of cross-protective vaccine immunity against an emerging pathogenic Ebolavirus Species. PLoS Pathog. 6:e1000904. [PubMed: 20502688]

Heyman B. Regulation of antibody responses via antibodies, complement, and Fc receptors. Annu Rev Immunol. 2000; 18:709–737. [PubMed: 10837073]

Huang Z, Chen Q, Hjelm B, Arntzen C, Mason H. A DNA replicon system for rapid high-level production of virus-like particles in plants. Biotechnol Bioeng. 2009; 103:706–714. [PubMed: 19309755]

Huang Z, Phoolcharoen W, Lai H, Piensook K, Cardineau G, Zeitlin L, Whaley KJ, Arntzen CJ, Mason HS, Chen Q. High-level rapid production of full-size monoclonal antibodies in plants by a single-vector DNA replicon system. Biotechnol Bioeng. 2010; 106:9–17. [PubMed: 20047189]

Hull R, Plaskitt A. Electron microscopy on the behavior of two strains of alfalfa mosaic virus in mixed infections. Virology. 1970; 42:773–776. [PubMed: 5483266]

Judge NA, Mason HS, O'Brien AD. Plant cell-based intimin vaccine given orally to mice primed with intimin reduces time of *Escherichia coli* O157:H7 shedding in feces. Infect. Immun. 2004; 72:168–175. [PubMed: 14688094]

Kim KI, Sunter G, Bisaro DM, Chung IS. Improved expression of recombinant GFP using a replicating vector based on Beet curly top virus in leaf-disks and infiltrated Nicotiana benthamiana leaves. Plant Mol Biol. 2007; 64:103–112. [PubMed: 17294255]

Krieger G, Kneba M, Bolz I, Volling P, Wessels J, Nagel GA. Binding characteristics of three complement dependent assays for the detection of immune complexes in human serum. J Clin Lab Immunol. 1985; 18:129–134. [PubMed: 3878887]

Laufs J, Jupin I, David C, Schumacher S, Heyraud-Nitschke F, Gronenborn B. Geminivirus replication: genetic and biochemical characterization of Rep protein function, a review. Biochimie. 1995; 77:765–773. [PubMed: 8824773]

Lee JE, Fusco ML, Hessell AJ, Oswald WB, Burton DR, Saphire EO. Structure of the Ebola virus glycoprotein bound to an antibody from a human survivor. Nature. 2008; 454:177–182. [PubMed: 18615077]

Malashkevich VN, Schneider BJ, McNally ML, Milhollen MA, Pang JX, Kim PS. Core structure of the envelope glycoprotein GP2 from Ebola virus at 1.9-A resolution. Proc Natl Acad Sci U S A. 1999; 96:2662–2667. [PubMed: 10077567]

Manicassamy B, Rong L. Expression of Ebolavirus glycoprotein on the target cells enhances viral entry. Virol J. 2009; 6:75. [PubMed: 19505320]

Marillonnet S, Giritch A, Gils M, Kandzia R, Klimyuk V, Gleba Y. In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by Agrobacterium. Proc Natl Acad Sci U S A. 2004; 101:6852–6857. [PubMed: 15103020]

Martin JE, Sullivan NJ, Enama ME, Gordon IJ, Roederer M, Koup RA, Bailer RT, Chakrabarti BK, Bailey MA, Gomez PL, Andrews CA, Moodie Z, Gu L, Stein JA, Nabel GJ, Graham BS. A DNA vaccine for Ebola virus is safe and immunogenic in a phase I clinical trial. Clin Vaccine Immunol. 2006; 13:1267–1277. [PubMed: 16988008]

Melito PL, Qiu X, Fernando LM, deVarennes SL, Beniac DR, Booth TF, Jones SM. The creation of stable cell lines expressing Ebola virus glycoproteins and the matrix protein VP40 and generating Ebola virus-like particles utilizing an ecdysone inducible mammalian expression system. J Virol Methods. 2008; 148:237–243. [PubMed: 18242720]

Mellquist-Riemenschneider JL, Garrison AR, Geisbert JB, Saikh KU, Heidebrink KD, Jahrling PB, Ulrich RG, Schmaljohn CS. Comparison of the protective efficacy of DNA and baculovirus-

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derived protein vaccines for EBOLA virus in guinea pigs. Virus Res. 2003; 92:187–193. [PubMed: 12686428]

Mor TS, Moon YS, Palmer KE, Mason HS. Geminivirus vectors for high-level expression of foreign proteins in plant cells. Biotechnol Bioeng. 2003; 81:430–437. [PubMed: 12491528]

Newell CA. Plant transformation technology. Developments and applications. Mol Biotechnol. 2000; 16:53–65. [PubMed: 11098468]

Pushko P, Bray M, Ludwig GV, Parker M, Schmaljohn A, Sanchez A, Jahrling PB, Smith JF. Recombinant RNA replicons derived from attenuated Venezuelan equine encephalitis virus protect guinea pigs and mice from Ebola hemorrhagic fever virus. Vaccine. 2000; 19:142–153. [PubMed: 10924796]

Qiu X, Fernando L, Alimonti JB, Melito PL, Feldmann F, Dick D, Stroher U, Feldmann H, Jones SM. Mucosal immunization of cynomolgus macaques with the VSVDeltaG/ZEBOVGP vaccine stimulates strong ebola GP-specific immune responses. PLoS One. 2009; 4:e5547. [PubMed: 19440245]

Regnault A, Lankar D, Lacabanne V, Rodriguez A, Thery C, Rescigno M, Saito T, Verbeek S, Bonnerot C, Ricciardi-Castagnoli P, Amigorena S. Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. J Exp Med. 1999; 189:371–380. [PubMed: 9892619]

Richardson JS, Yao MK, Tran KN, Croyle MA, Strong JE, Feldmann H, Kobinger GP. Enhanced protection against Ebola virus mediated by an improved adenovirus-based vaccine. PLoS One. 2009; 4:e5308. [PubMed: 19390586]

Rybicki EP. Plant-made vaccines for humans and animals. Plant Biotechnol J. 2010

Sanchez A, Yang ZY, Xu L, Nabel GJ, Crews T, Peters CJ. Biochemical analysis of the secreted and virion glycoproteins of Ebola virus. J Virol. 1998; 72:6442–6447. [PubMed: 9658086]

Simmons G, Wool-Lewis RJ, Baribaud F, Netter RC, Bates P. Ebola virus glycoproteins induce global surface protein down-modulation and loss of cell adherence. J Virol. 2002; 76:2518–2528. [PubMed: 11836430]

Stein H, Wilensky M, Tsafrir Y, Rosenthal M, Amir R, Avraham T, Ofir K, Dgany O, Yayon A, Shoseyov O. Production of bioactive, post-translationally modified, heterotrimeric, human recombinant type-I collagen in transgenic tobacco. Biomacromolecules. 2009; 10:2640–2645. [PubMed: 19678700]

Stenger DC, Revington GN, Stevenson MC, Bisaro DM. Replicational release of geminivirus genomes from tandemly repeated copies: evidence for rolling-circle replication of a plant viral DNA. Proc Natl Acad Sci U S A. 1991; 88:8029–8033. [PubMed: 1896448]

Sullivan NJ, Sanchez A, Rollin PE, Yang ZY, Nabel GJ. Development of a preventive vaccine for Ebola virus infection in primates. Nature. 2000; 408:605–609. [PubMed: 11117750]

Sullivan NJ, Peterson M, Yang ZY, Kong WP, Duckers H, Nabel E, Nabel GJ. Ebola virus glycoprotein toxicity is mediated by a dynamin-dependent protein-trafficking pathway. J Virol. 2005; 79:547–553. [PubMed: 15596847]

Warfield KL, Swenson DL, Olinger GG, Kalina WV, Aman MJ, Bavari S. Ebola virus-like particle-based vaccine protects nonhuman primates against lethal Ebola virus challenge. J Infect Dis. 2007; 196(Suppl 2):S430–437. [PubMed: 17940980]

Wilson JA, Bosio CM, Hart MK. Ebola virus: the search for vaccines and treatments. Cell Mol Life Sci. 2001; 58:1826–1841. [PubMed: 11766882]

Wilson JA, Hevey M, Bakken R, Guest S, Bray M, Schmaljohn AL, Hart MK. Epitopes involved in antibody-mediated protection from Ebola virus. Science. 2000; 287:1664–1666. [PubMed: 10698744]

Yang ZY, Duckers HJ, Sullivan NJ, Sanchez A, Nabel EG, Nabel GJ. Identification of the Ebola virus glycoprotein as the main viral determinant of vascular cell cytotoxicity and injury. Nat Med. 2000; 6:886–889. [PubMed: 10932225]

Ye L, Lin J, Sun Y, Bennouna S, Lo M, Wu Q, Bu Z, Pulendran B, Compans RW, Yang C. Ebola virus-like particles produced in insect cells exhibit dendritic cell stimulating activity and induce neutralizing antibodies. Virology. 2006; 351:260–270. [PubMed: 16678231]

Yusibov V, Rabindran S, Commandeur U, Twyman RM, Fischer R. The potential of plant virus vectors for vaccine production. Drugs R D. 2006; 7:203–217. [PubMed: 16784246]

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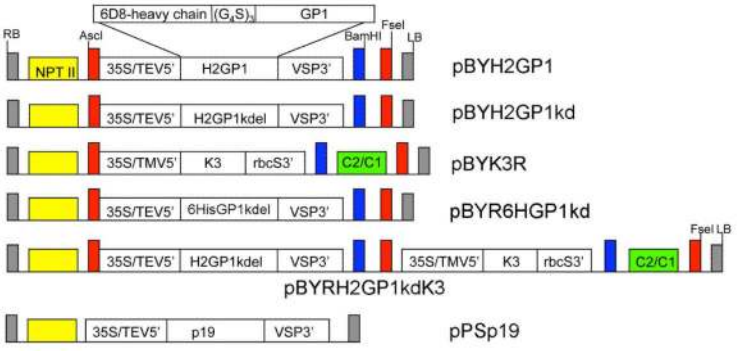

Fig. 1.

Schematic representation of the T-DNA region of the vectors used in this study. 35S/TEV5′: CaMV 35S promoter with tobacco etch virus 5'UTR; VSP3′: soybean vspB gene 3′ element, NPTII (yellow box): expression cassette encoding nptII gene for kanamycin resistance, LIR (red box) : long intergenic region of BeYDV genome, SIR (blue box): short intergenic region of BeYDV genome, C2/C1 : BeYDV ORFs C1 and C2 which encode for replication initiation protein (Rep) and RepA, LB and RB : the left and right borders of the T-DNA region.

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Fig. 2.

Typical phenotype of leaves on day 5 expressing 6D8 mAb (1), Ebola GP1 (2), Ebola immune complex (EIC) (3), and GFP (4). *N. benthamiana* leaves were co-infiltrated with pBYH2kdel+pBYK3R+p19 for 6D8 mAb expression (1), pBYR6HGP1kdel+p19 for Ebola GP1 expression (2), pBYRH2GP1kdelK3+p19 for EIC expression (3), and pBYGFP.R+p19 for GFP expression (4) at final OF600 = 0.25. The leaves were photographed on day 5 after infiltration.

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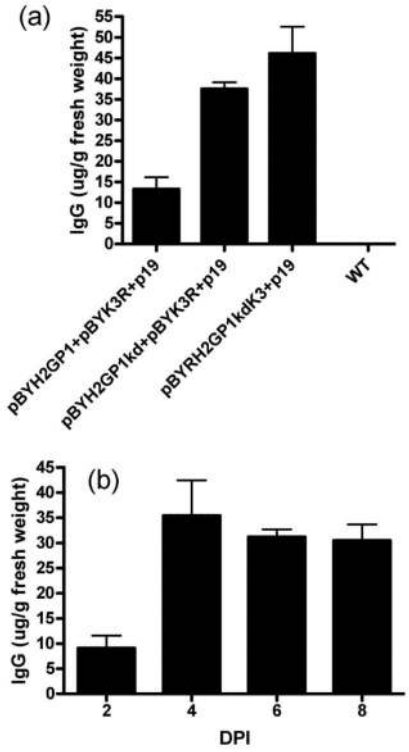

Fig. 3.

Expression of Ebola immune complex in *N. benthamiana* plants. a. Protein expression of Ebola immune complex compared among different constructs. *N. benthamiana* leaves were co-infiltrated with pBYH2GP1+pBYK3R+p19, pBYH2GP1kdel+pBYK3R+p19, and pBYRH2GP1kdelK3R+p19 at final OD600=0.25. The leaves were harvested 4 days after infiltration and extracted for ELISA to quantify IgG (Experimental Procedures) b. Protein expression level at different times after agroinfiltration using pBYRH2GP1kdK3 with pPSp19. The leaves were harvested on days 2, 4, 6, and 8 dpi. Data are means ± SD of samples from three independent infiltration experiments.

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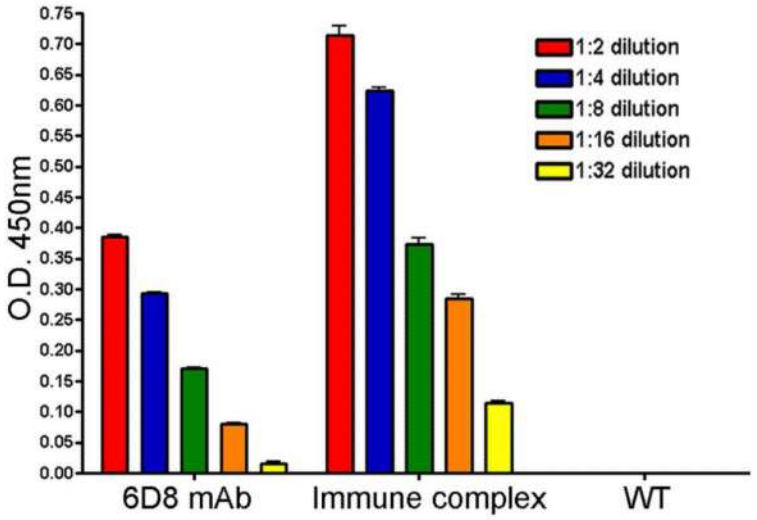

Fig. 4.

C1q binding of crude plant extracts. Crude extracts were quantified for the amount of IgG by ELISA. Samples were then adjusted to 100 ng/ml IgG, and serial 2-fold dilutions from 1:2 to 1:32 were made. Different dilutions of crude extracts were incubated in wells containing immobilized C1q. Detection with HRP labeled goat anti-human IgG yielded OD450 measurements. Data are means ± SD of samples from three independent infiltration experiments.

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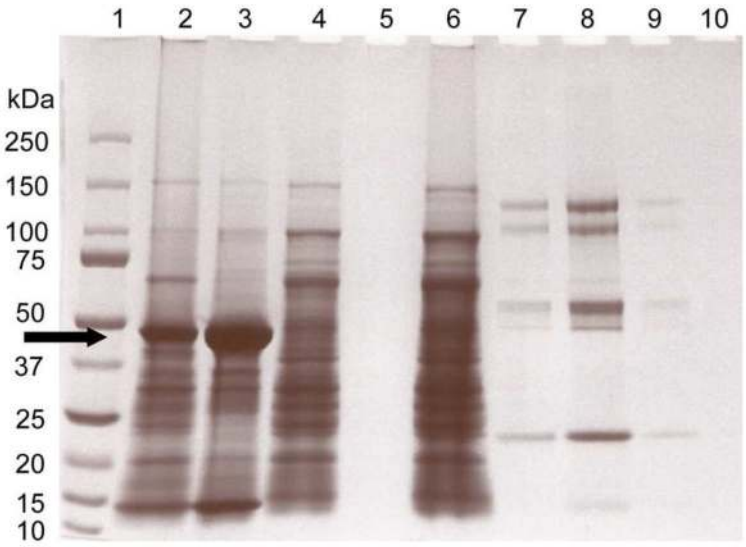

Fig. 5.

Purification of Ebola immune complex from *N. benthamiana* leaves. Infiltrated leaves were extracted, and EIC purified and analyzed by SDS-PAGE under reducing conditions. Lane 1: Protein ladder; lane 2: clarified crude leaf extract; lane 3: leaf proteins removed by 35% ammonium sulfate precipitation; lane 4: 60% ammonium sulfate precipitate resuspended for Protein G chromatography; lane 5: 100% ammonium sulfate precipitate; lane 6: protein G flow-through fraction; lanes 7-10: sequential elution fractions from Protein G chromatography. The black arrow indicates rbcL protein.

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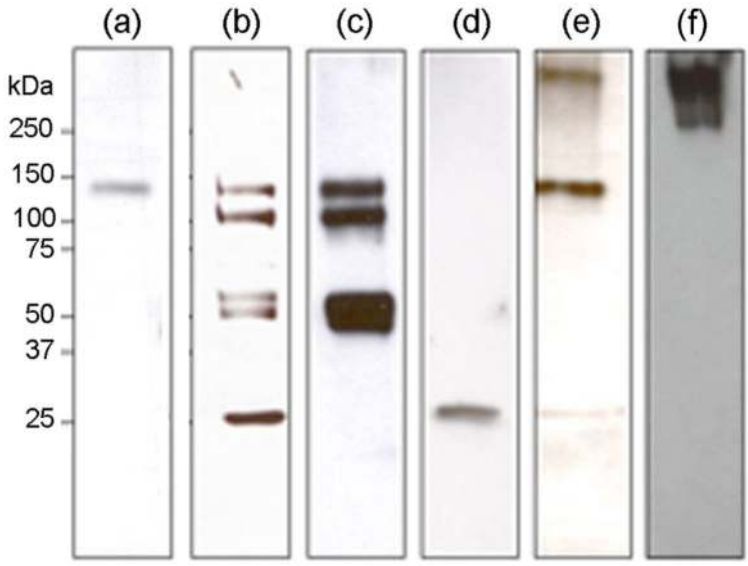

Fig. 6.

Western blotting of EIC. a. Crude extract from *N. benthamiana* leaf agroinfiltrated with pBYH2GP1kdel, pBYK3R and pPS19. The 100 mg leaf sample was harvested 4 dpi and extracted with 500μl SDS sample buffer containing DTT, and 20μl was loaded on the gel. Western blot was probed with anti human IgG heavy chain. b-f. Purified EIC from leaves agroinfiltrated with pBYH2GP1kdelK3 and pPS19. b. Coomassie stained SDS-PAGE reducing gel; c. Reducing western blot detected with anti-human IgG heavy chain; d. Reducing western blot detected with anti human kappa chain; e. Reducing western blot detected with anti-linear epitope 6D8 mAb; f. Non-reducing western blot detected with anti-conformational epitope 13C6.

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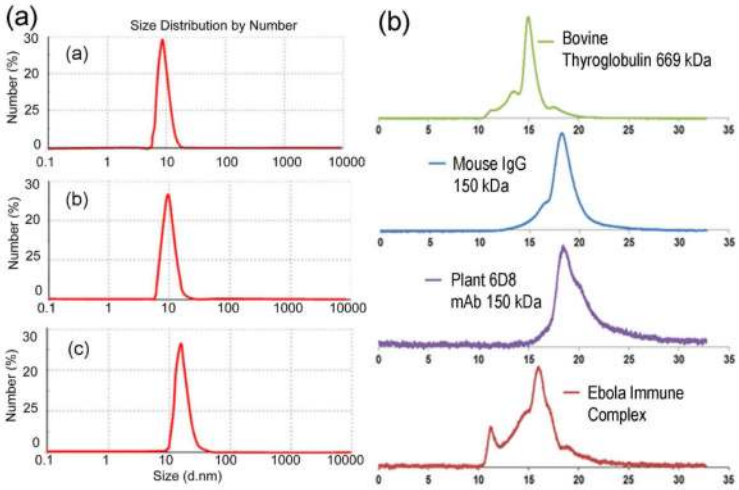

Fig. 7.

Size measurement confirmed the complex formation. a. Hydrodynamic diameters of EIC or 6D8 mAb determined by dynamic light scattering using Zetasizer Nano-ZS instrument (Malvern Instruments, UK). The abscissa indicates the diameter in nm, and the ordinate indicates the relative number of molecules at that size, comparing human IgG, plant-expressed 6D8 mAb, and plant-expressed EIC. b. Size exclusion chromatography was used to determine the size of the immune complex. EIC was loaded onto BioSep SEC-S4000 (Phenomenex, USA) and eluted with PBS, pH7.3. The elution time in min is shown on the abscissa, and the A_{280} was continuously monitored (colored curves). Bovine thyroglobulin (Sigma, USA), mouse IgG (SouthernBiotech, USA), and plant-expressed 6D8 mAb were used as protein markers.

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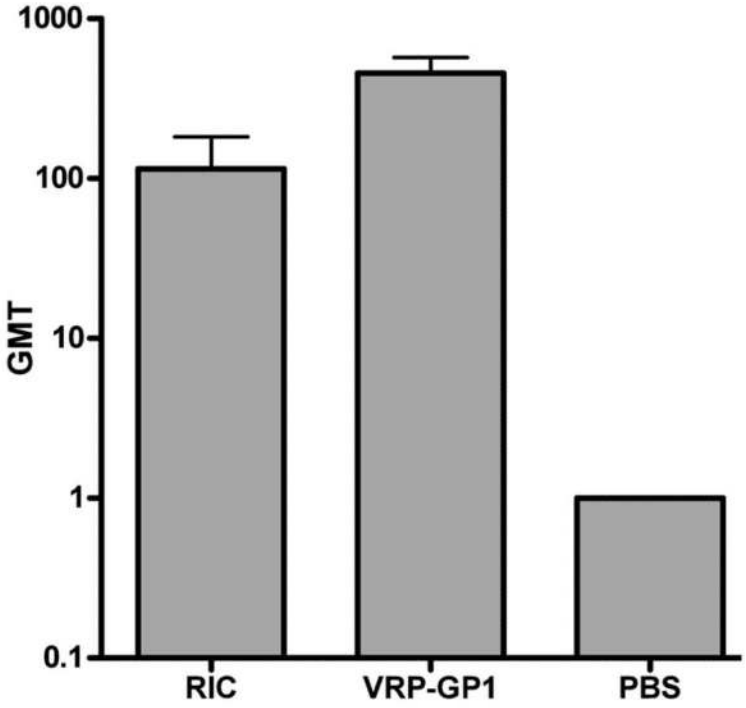

Fig. 8.

Anti-Ebola IgG responses in mice immunized with EIC. Groups of 7 mice were immunized subcutaneously with EIC, VRP-GP1, or PBS. The immunizations were given on days 0, 21, 42, and 63. The serum was collected on day 84 and assayed for anti-Ebola IgG by ELISA, using gamma-irradiated Ebola virus as the capture antigen. The data are presented as Geometric Mean Titer (GMT) ± the standard deviation for each group of mice.

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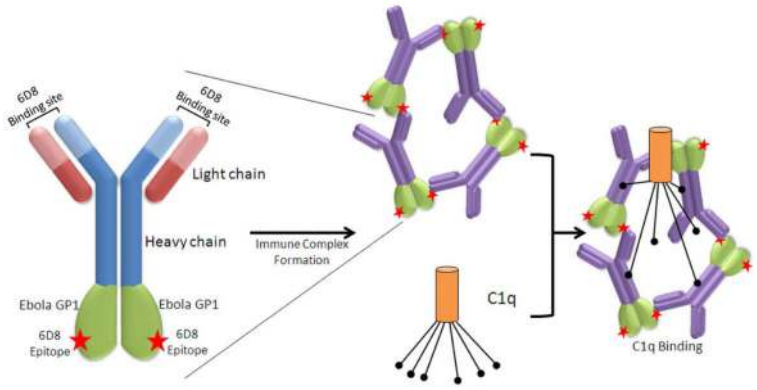

Fig. 9.

Diagram illustrating the possible structure of the recombinant IgG and its assembly to form EIC. At left, the 6D8 H2-GP1 fusion protein (blue H2 chain, green GP1) is assembled with the light chain (red) to form a chimeric IgG-GP1. The 6D8 epitope on GP1 is shown as a red star. The epitope binding site at the top of the molecule can bind to the 6D8 epitope displayed on other chimeric molecules, which results is complex assembly (middle, with IgG component shown in purple). The complement component C1q can bind to the Fc region of IgG molecules that are bound to antigen (right).