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Biochemical and Immunogenic Characterization of Soluble Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Trimers Expressed by Semliki Forest Virus†

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The current lack of envelope glycoprotein immunogens that elicit broadly neutralizing antibody responses remains a major challenge for human immunodeficiency virus type 1 (HIV-1) vaccine development. However, the recent design and construction of stable soluble gp140 trimers have shown that some neutralization breadth can be achieved by using immunogens that better mimic the functional viral spike complex. The use of genetic delivery systems to drive the in vivo expression of such immunogens for the stimulation of neutralizing antibodies against HIV-1 may offer advantages by maintaining the quaternary structure of the trimeric envelope glycoproteins. Here, we describe the biochemical and immunogenic properties of soluble HIV-1 envelope glycoprotein trimers expressed by recombinant Semliki Forest virus (rSFV). The results presented here demonstrate that rSFV supports the expression of stable soluble gp140 trimers that retain recognition by conformationally sensitive antibodies. Further, we show that rSFV particle immunizations efficiently primed immune responses as measured after a single boost with purified trimeric gp140 protein, resulting in a Th1-biased antibody response. This differed from the Th2-biased antibody response obtained after repeated immunizations with purified gp140 protein trimers. Despite this difference, both regimens stimulated neutralizing antibody responses of similar potency. This suggests that rSFV may be a useful component of a viral vector prime-protein boost regimen aimed at stimulating both cell-mediated immune responses and neutralizing antibodies against HIV-1.

Broadly neutralizing antibodies against human immunodeficiency virus type 1 (HIV-1) are rarely elicited during natural infection and to an even lesser extent during vaccination with Env-based immunogens. The primary challenge in the development of a vaccine capable of inducing broadly neutralizing antibodies against HIV-1 lies in the design of the immunogen (6). However, a successful vaccine against HIV-1 will likely need to induce both effective cell-mediated immune responses and broadly neutralizing antibodies. Viral systems are therefore of interest, since they are capable of stimulating both cellular and humoral immune responses. In addition, they can be used to express optimized envelope glycoprotein immunogens in vivo. To obtain potent antibody responses, virus-based vaccines often require subsequent immunizations with recombinant protein. Thus, the characterization of prime-boost regimens, which combine virus-based expression with recombinant protein-based immunogens, is an important area of investigation. The efficacy of neutralizing antibodies in protection against HIV-1 has been demonstrated in passive transfer studies (3, 12, 18, 27, 28, 31, 47). It has also been shown that the in vitro neutralizing capacity of well-characterized antibodies against HIV-1 correlates with their ability to protect against virus challenge in vivo (27). In vitro neutralization assays are therefore important for the screening of new envelope glycoprotein immunogens as well as to evaluate antibody responses elicited by different vaccine vector systems used for immunogen delivery.

Multiple lines of evidence suggest that gp120 and gp41, the HIV-1 envelope glycoproteins, are organized into trimeric spike complexes on the surface of infected cells and infectious virus particles (7, 24, 49). The functional spike is labile, and monomeric gp120 dissociates readily from gp41, resulting in the exposure of nonneutralizing gp120 and gp41 protein surfaces to the immune system (15, 32). Monomeric gp120 has been shown to be a highly flexible molecule (36, 38) which is likely to present many different conformations to the immune system, thereby diverting the immune response away from epitopes found on the functional spike. Other immune-evasion strategies inherent in the spike include a high density of glycans on the accessible outer domain of gp120 and V1/2 loops as well as orientation of the immunodominant gp120 variable loops to shield the conserved, receptor-binding regions of the functional spike (48, 50). Significant efforts have therefore been made to design and construct immunogens that better resemble the functional trimeric envelope glycoprotein complexes, thereby preferentially exposing relevant neutralizing determinants to the immune system (1, 4, 9–11, 20, 42, 43,

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51–53). Several approaches have been taken to generate soluble stable HIV-1 envelope glycoprotein trimers, a majority of which is based upon gp140 molecules that possess both gp120 and the ectodomain of gp41. Frequently, the natural cleavage site between gp120 and gp41 has been rendered defective by site-directed mutagenesis or deletion of sequences to maintain gp120-gp41 association in a covalent manner (11, 51). Soluble gp140 molecules with an intact cleavage site have also been generated (4, 42, 43). In these molecules, the heterodimeric monomeric subunits are stabilized by the introduction of disulfides between gp120 and gp41. A series of reports have shown that the addition of heterologous trimerization domains at the C terminus of the gp41 ectodomain increases the stability and homogeneity of soluble HIV-1 envelope trimers (51–53). Molecules have been constructed by fusing either a trimerization domain derived from the yeast transcription factor (GCN4) or the trimeric motif from the T4 bacteriophage fibritin (FT) to cleavage-defective gp140 molecules to generate YU2gp140(-/GCN4) and YU2gp140(-/FT) constructs, respectively. The trimeric gp140s have been shown to stimulate neutralizing antibodies with greater breadth than YU2gp120, suggesting an advance over the homologous monomeric immunogens (16, 53).

The potential of viral systems to express oligomeric gp140 envelope immunogens and to stimulate neutralizing antibodies against HIV-1 has been investigated in previous studies, most frequently by using systems based on adenoviruses, poxviruses, or alphaviruses (8, 10, 22, 30, 41, 44). However, much remains to be understood about the qualitative and quantitative aspects of the proteins that are expressed in such viral systems as well as the immune responses stimulated by these systems. For example, recombinant virus-based vaccines typically induce a Th1-biased response and it is unclear whether this also allows a sufficient stimulation of humoral immunity. Protein-based regimens are generally the most effective in stimulating antibody responses, since high doses of antigen can be administered. In addition, depending on the adjuvant, protein-based vaccines generally stimulate a Th2-biased immune response, which drives humoral immunity efficiently (17, 46). The nature of the antibody response stimulated by different virus prime-protein boost compared to protein-only regimens has not been thoroughly investigated. To address some of the issues raised above, head-to-head comparisons between selected immunization regimens utilizing matched envelope glycoprotein immunogens are required.

In this study, we used the rSFV vector system to express trimeric envelope glycoproteins derived from the primary isolate YU2. This system allows the packaging of suicidal, single-round infectious viral particles (23) that have been shown to stimulate protective immunity in a number of different infection models (2, 13, 34, 35). The potential of rSFV as a valuable component of a vaccine against HIV-1 has also been demonstrated, but these studies have primarily focused on the ability of rSFV to stimulate $CD8^+$ T-cell responses (21, 33, 37). Here, we have characterized the ability of recombinant Semliki Forest virus (rSFV) particles to express soluble YU2gp140 (-/GCN4) trimers in vitro and compared their expression to monomeric YU2gp120 proteins expressed by rSFV. Further, we investigated the potential of rSFV particles expressing YU2gp140(-/GCN4) to stimulate humoral responses against HIV-1. The availability of a soluble envelope immunogen allowed us to directly compare immunization regimens based on repeated inoculation of purified protein in adjuvant to regimens based on rSFV priming followed by a single boost with purified YU2gp140(-/GCN4) protein in adjuvant.

The results presented in this report show that rSFV expression supports the production of conformationally intact YU2gp140(-/GCN4) molecules that are secreted as homogenous soluble trimers. Immunogenicity experiments were performed with mice and rabbits to study quantitative and qualitative aspects of the YU2gp140(-/GCN4)-directed immune responses (antibody isotypes, $CD4^+$ T-cell responses, and neutralizing antibodies) stimulated by the different immunization regimens. We demonstrate that rSFV-based vaccination stimulated a Th1-biased response, which remained Th1 biased after a boost with recombinant protein in adjuvant. In contrast, and as expected, the immune response was Th2 biased in animals immunized with repeated inoculations of recombinant YU2gp140(-/GCN4) protein in Ribi adjuvant. Interestingly, these qualitative differences did not affect the neutralizing antibody responses, which were comparable using both immunization regimens.

MATERIALS AND METHODS

Cells, genetic constructs, and recombinant viruses. BHK-21 cells (American Type Culture Collection) were cultured in Glasgow minimal essential medium (Invitrogen, Carlsbad, California) supplemented with 5% fetal calf serum (FCS), 10 mM HEPES, 10% tryptose phosphate, 2 mM L-glutamine, penicillin, and streptomycin (Sigma, St. Louis, MO). Sequences encoding YU2gp120, flanked by XmaI/NotI, and YU2gp140(-/GCN4), flanked by XmaI/XhoI, were amplified by PCR from YU2gp120/pCDNA3.1(-) and YU2gp140GCN4/pCDNA3.1(-), respectively (16). The sequences were inserted into pSFV10 (19) to generate pSFV-YU2gp120 and pSFV-YU2gp140(-/GCN4). Single-round infectious rSFV-YU2gp120, rSFV-YU2gp140(-/GCN4), or control rSFV-LacZ particles were produced by cotransfecting BHK-21 cells with in vitro-transcribed vector and split-helper mRNA according to a standard protocol (45). Titration of viral stocks was performed using BHK-21 cells as described previously (19).

Expression and purification of trimeric gp140(-/GCN4) and monomeric gp120 glycoproteins. The trimeric YU2gp140(-/GCN4) proteins were expressed and purified as described previously (16). In brief, 293F, the serum-free medium-adapted cell line, was transfected with the YU2gp140(-/GCN4)/pCDNA3.1(-) expression plasmid in Dulbecco's modified Eagle's medium–10% heat-inactivated fetal bovine serum supplemented with 0.1 mM Glasgow minimal essential medium nonessential amino acid solution (Invitrogen) medium, using LipofectAMINETM 2000 (Invitrogen) per the manufacturer's instructions. At 1 day posttransfection, serum-free 293 SFM II medium (Invitrogen) was added to replace the serum-containing Dulbecco's modified Eagle's medium. Two days after transfection, the cell culture supernatants were collected daily with fresh 293 SFM II medium applied to the cell culture flasks until the sixth day post-transfection. Following collection, the supernatants were centrifuged at 3,500 × *g*, filtered through a 0.22-μm filter, and applied to an immunoglobulin Gb12 (IgGb12) antibody affinity column. After sequentially washing the column with phosphate-buffered saline (PBS)–0.5 M NaCl, pH 7.4, and PBS, pH 7.4, the bound trimeric YU2gp140(-/GCN4) proteins were eluted from the column with 3 M $MgCl_2$–20 mM Tris-HCl, pH 7.4, dialyzed against PBS–0.5 NaCl, pH 7.4, and concentrated with Amicon Ultra 30,000 MWCO centrifugal filter devices (Millipore, Bedford, MA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blue native gel, and gel-filtration analysis were performed on the purified trimeric YU2gp140(-/GCN4) proteins, and the analysis confirmed that the purity of the protein was roughly 95% of homogeneity. Monomeric YU2gp120 protein was prepared similarly by transient transfection of 293 cells and F105 affinity chromatography purification as previously described (16).

Immunoprecipitation and SDS-PAGE. Metabolic labeling was performed according to standard procedures. In brief, 1×10^6 rSFV-YU2gp120- or rSFV-YU2gp140(-/GCN4)-infected BHK-21 cells were pulsed with medium containing [^{35}S]methionine (GE Healthcare, Uppsala, Sweden) for 15 min or 6 h at 14 h

after infection. The labeled protein from the 15-min pulse was subsequently chased for 0.5, 2, or 6 h before the cells and supernatants were harvested. The cells were resuspended in NP-40 lysis buffer (50 mM Tris HCl [pH 7.6], 150 mM NaCl, 2 mM EDTA) and incubated on ice for 15 min, and the soluble fraction was then clarified by centrifugation. The supernatants were precipitated with 0.5× the volume of trichloroacetic acid (TCA) by adding TCA and incubated at −20°C for 30 min. After being washed (three times) in ice-cold acetone, the pellet was dried at room temperature (RT) and then resuspended in SDS-PAGE sample buffer containing 2% β-mercaptoethanol (BME). For immunoprecipitation experiments, supernatants from cells pulsed for 6 h were incubated overnight at 4°C with either 3 μl pooled sera from HIV-1-infected persons (HIVIG) or 1 μg of the monoclonal antibodies (MAbs) IgGb12 or 2G12, kindly provided by James Binley via the IAVI Neutralizing Antibody Consortium. The precipitated protein was incubated with 50 μl of protein G Sepharose (GE Healthcare) and preblocked with 2% bovine serum albumin (BSA) in PBS. After being washed in NP-40 lysis buffer (twice) and PBS (once), the samples were eluted by boiling in SDS-PAGE sample buffer containing 2% BME. Cell lysates, TCA precipitates, and immunoprecipitates were analyzed by SDS-PAGE and autoradiography. For analysis of nonlabeled protein, 1 ml (of a total of 5 ml) 24-hour postinfection supernatants from 1×10^6 BHK-21 cells were immunoprecipitated with IgGb12 as described above, separated on a NuPAGE Novex 3 to 8% Tris-acetate gel (Invitrogen), and subjected to silver staining.

Analysis of envelope glycoprotein oligomers. The oligomeric state of the SFV-expressed proteins was analyzed by running TCA precipitates of supernatants from [^{35}S]methionine-labeled infected cells on a NuPAGE Novex 3 to 8% Tris-acetate gel under nonreducing conditions or by analyzing unlabeled supernatants on blue native gel followed by Western blotting. The blue native gel procedure was performed essentially as previously described (43) with minor modifications. Briefly, samples diluted in 2× sample buffer (100 mM Tris HCl, 100 mM MOPS, 40% glycerol, 0.1% Serva-G, pH 7.7) were analyzed on a NuPAGE Novex 4 to 12% Bis-Tris gel (Invitrogen) at 4°C at 50V for 18 h. The running buffer contained 50 mM Tris HCl–50 mM MOPS, pH 7.7, and 10 mg Serva-G (SERVA Electrophoresis GmbH, Heidelberg, Germany) per 500 ml running buffer was added to the cathode. Thyroglobulin, ferritin, catalase, and aldolase (GE Healthcare) were used as molecular weight markers. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare) as described previously (43). Briefly, the PVDF membranes were soaked in methanol (MeOH) for 30 s before gels and membranes were soaked in transfer buffer (192 mM glycine, 25 mM Tris-HCl, 20% MeOH) for 5 min. Following transfer at 4°C at 100 V, PVDF membranes were destained in 25% MeOH and 10% acetic acid and air dried. Destained membranes were blocked and probed using polyclonal rabbit anti-HIV-1 Env sera [from a rabbit immunized three times with 25 μg purified YU2gp140(-/GCN4) protein] at a 1/500 dilution followed by horseradish peroxidase (HRP)-labeled anti-rabbit IgG (Sigma) at a 1/1,000 dilution. Detection of the envelope glycoproteins was performed using an ECL Western blotting detection system (GE Healthcare) according to manufacturer's instructions.

Mouse and rabbit immunizations. Female BALB/c mice, 6 to 8 weeks old, were obtained from Taconic A/S. The mice were immunized three times with 10 μg YU2gp140(-/GCN4) protein or two or three times with 1×10^7 infectious units (IU) of rSFV-YU2gp140(-/GCN4) followed by a single immunization of 10 μg YU2gp140(-/GCN4) protein. Control mice were immunized two times with 1×10^7 IU rSFV-lacZ followed by a boost with 10 μg of recombinant β-galactosidase (βgal) (Roche Pharmaceuticals, Basel, Switzerland) (see Fig. 4). For mice, the rSFV injections were given subcutaneously (sc) in 0.1 ml PBS and the protein immunizations sc in 0.2 ml monophospholipid A–trehalose dicorynomycolate–cell wall skeleton of tubercule bacillus ribi adjuvant emulsion (Ribi) (Sigma). Sera and spleens for enzyme-linked immunospot (ELISPOT) and ELISA analysis were taken 12 days after the immunizations. New Zealand White rabbits, approximately 3-month-old females, were obtained from HB Lidköpings Kaninfarm (Lidköping, Sweden). The rabbits were immunized three times with 25 μg purified YU2gp140(-/GCN4) protein or twice with 5×10^7 IU rSFV-YU2gp140(-/GCN4) followed by a boost with 25 μg of YU2gp140(-/GCN4) protein. Control rabbits were immunized twice with 5×10^7 IU rSFV-LacZ followed by a boost with 25 μg BSA. For rabbits, the rSFV injections were given in 0.5 ml PBS split between five sites intradermally (id), two sites intramuscularly (im), and one site sc and the protein immunizations were given in 1 ml Ribi split between 10 sites intradermally, 2 sites intramuscularly, and 1 site sc. The rabbits were bled 10 days after each injection. All animals were kept at the animal facility at the Swedish Institute for Infectious Disease Control according to current Swedish animal health regulations.

ELISA. Enzyme-linked immunosorbent assay (ELISA) plates (Immunosorp, Nunc, Denmark) were coated with 100 ng of S2-produced and F105 affinity-purified YU2gp120 as previously described (17) in 100 μl PBS per well at 4°C overnight. The wells were then incubated for 1 h at RT in blocking buffer (PBS–2% dry milk–5% FCS). After being washed three times in wash buffer (PBS–0.2% Tween 20), fivefold serial dilutions (starting at 1/50) of the sera from immunized mice were added in duplicate wells and incubated for 2 h at RT. After washing, the wells were incubated with secondary antibodies diluted 1/5,000 in wash buffer for 1 h at RT; conjugated anti-mouse IgG, anti-mouse-IgG1, anti-mouse IgG2a (Southern Biotech, Birmingham, AL), or anti-rabbit IgG (Sigma) was used as a secondary antibody. The plates were washed, and 100 μl fast o-phenylenediamine dihydrochloride substrate (Sigma) was added for detection of YU2gp120-bound antibodies. Optical density (OD) at 450 nm was read using an ELISA reader. The ELISA endpoint titer was defined as the highest serum dilution at which the mean OD value of duplicate wells minus 2 standard deviations was above the mean value of serum from control animals at the same dilution. The IgG1:IgG2a ratio for each animal was calculated by dividing the OD value from the IgG1 and IgG2a ELISAs at the 1:6,250 serum dilution.

Preparation of responder cells from spleens. The spleens were removed 12 days after the immunization, and single-cell suspensions were prepared by passage through a 100-μm cell strainers (Falcon; BD Biosciences, San Diego, CA). After lysis of the red blood cells (RBC) using RBC lysis buffer (Sigma), the cells were washed, resuspended in complete RPMI 1640 medium, containing 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Sigma), and 20 mM HEPES (Gibco), and counted. An aliquot of the splenocytes was depleted for $CD4^+$ T cells by use of magnetic beads coated with the anti-CD4 MAb L3T4, according to the manufacturer's instructions (Miltenyi Biotec; Bergisch Gladbach, Germany). Total splenocyte cultures and $CD4^+$ T-cell-depleted splenocyte cultures were analyzed by flow cytometry using anti-CD4 and anti-CD3ε antibodies (BD Pharmingen, San Diego, CA) before they were used in ELISPOT analysis.

ELISPOT. ELIIP10SSP plates (Millipore Co., Bedford, MA) were coated with anti-gamma interferon (IFN-γ) AN18, anti-interleukin-2 (IL-2) 1A12, or anti-IL-4 11B11 (all from MabTech, Stockholm, Sweden) in PBS overnight at 4°C. The plates were washed five times with PBS followed by incubation with complete media for 2 h at 37°C to block the plates. Spleen cells from individual mice were added at 2×10^5 cells/well in triplicate and stimulated with medium alone, 0.15 μg concanavalin A (ConA) (Sigma), or 0.5 μg of YU2gp120 protein per well. A 20-h incubation in 5% CO_2 at 37°C were followed by six washes with PBS-Tween (0.05%), biotinylated anti-IFN-γ R4-6A2, anti-IL-2 5H4, or anti-IL-4 BVD6-24G2 (all from MabTech) was added, and the plates were incubated for 2 h at RT. After washing the plates as described above, an avidin-peroxidase complex (ABC kit; Vector Laboratories, Burlingame, CA) was prepared according to the instructions of the manufacturer and added to each well. The plates were incubated for 1 h at RT and were then washed (six times) with PBS. AEC substrate (Sigma) was prepared according to the instructions of the manufacturer and added to the wells. The enzymatic reaction was stopped after 4 min by washing the plates in water. The spots were counted using an ELISPOT reader (Axioplan 2 Imaging; Zeiss) and expressed as number of spots per 10^6 cells. The criterion for a positive YU2gp120-specific response was set to a result equal to or above twice the number of spots obtained after stimulation by medium only.

HIV-1 virus isolates and neutralization assay. Sera from immunized animals were tested for neutralization against six different HIV-1 isolates. All viruses were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. MN (a cell-line-adapted virus), 89.6, and JR-CSF were propagated in peripheral blood mononuclear cells (PBMCs). HxB2, SF162, and YU2 were Env-pseudotyped recombinant viruses that also code for green fluorescent protein (GFP). The HxB2 and YU2 Env expression plasmids were a gift from Dana Gabuzda, Dana-Farber Cancer Institute, Boston, MA. The SF162 Env plasmid was a gift from Leonidas Stamatatos, Seattle Biomedical Research Institute. Virus neutralization assays were performed using PBMC target cells and a flow cytometric assay as previously described (29). This assay detects HIV-1-infected T cells by intracellular staining for HIV-1 p24 Gag antigen (p24-Ag) or by expression of GFP. The recombinant pseudoviruses produce only one round of infection. For PBMC-propagated viruses, a protease inhibitor was used to prevent secondary rounds of virus replication. The percentage of virus neutralization by each immune serum sample was derived by calculating the reduction in the number of p24-Ag (or GFP)-positive cells in the test wells compared to the number of p24-Ag-positive cells in wells containing preimmune serum from the corresponding animal. To control for nonspecific neutralization in rSFV-immunized rabbits, sera from two animals immunized with rSFV-LacZ were analyzed. All serum samples were also assayed for neutralizing activity against a pseudovirus expressing the amphotrophic murine leukemia virus envelope to test for non-HIV-1 specific plasma effects (29).

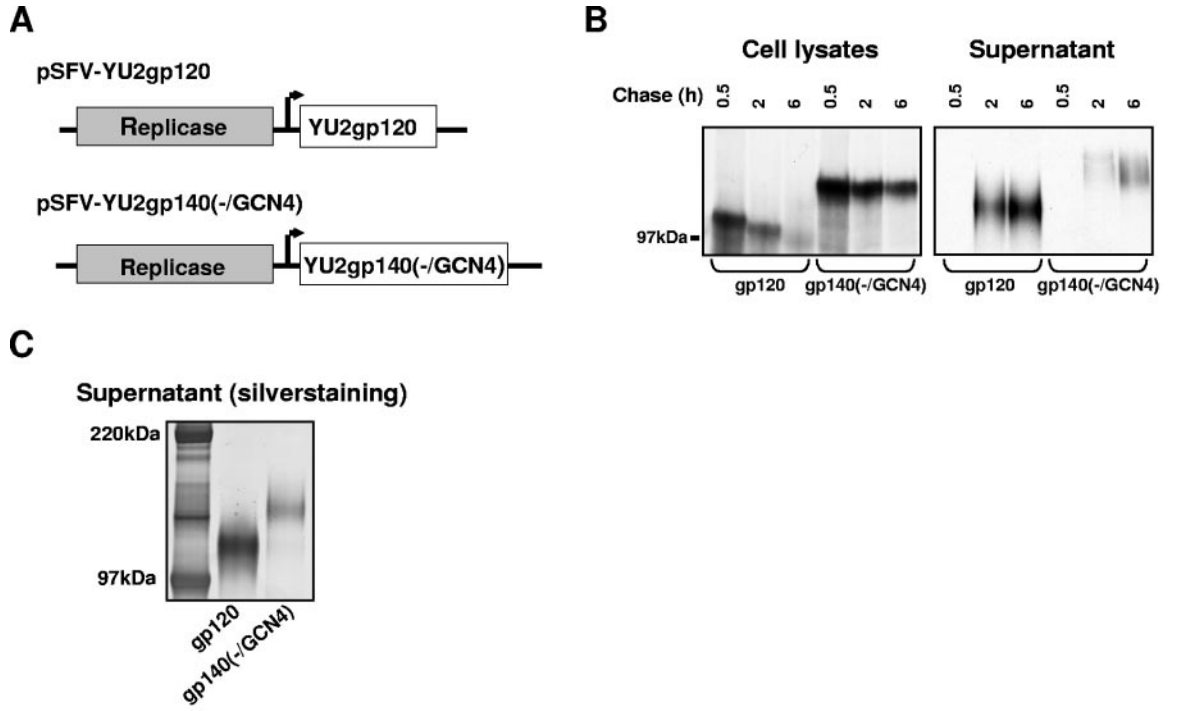

FIG. 1. Recombinant vectors and expression of HIV-1 envelope glycoproteins. (A) A schematic representation of rSFV vectors expressing the YU2gp120 and YU2gp140(-/GCN4) envelope glycoproteins is shown. The envelope genes are inserted downstream of the SFV nonstructural proteins (Replicase). The location of the SFV subgenomic promoter is indicated by an arrow. (B) Pulse-chase analysis of [^{35}S]methionine-labeled BHK-21 cells infected with rSFV-YU2gp120 or rSFV-YU2gp140(-/GCN4) particles. Total labeled proteins at the different chase time points (0.5, 2, and 6 h) in cell lysates (left panel) and in TCA-precipitated supernatants (right panel) were separated by SDS-PAGE, and the proteins were visualized by autoradiography. (C) Supernatants from BHK-21 cells infected for 24 h with rSFV-YU2gp120 or rSFV-YU2gp140(-/GCN4) were immunoprecipitated with MAb IgGb12 and protein G Sepharose and eluted in SDS-PAGE sample buffer containing 2% BME, and the proteins were analyzed by SDS-PAGE and silver staining.

RESULTS

Expression of HIV-1 envelope glycoproteins from rSFV particles. Characterization of rSFV-YU2gp120 (expressing monomeric YU2gp120) was performed side by side with the characterization of rSFV-YU2gp140(-/GCN4) to determine the relative expression levels of the two Env forms. Previous studies have shown that monomeric gp120 derived from other isolates is efficiently produced by rSFV particles (5, 39). BHK-21 cells were infected with rSFV-YU2gp120 and rSFV-YU2gp140(-/GCN4) using a multiplicity of infection of 20 to ensure full infection of the cultured cells. At 10 h following infection, the cultures were pulsed with [^{35}S]methionine-containing medium. At this time point, rSFV replication has induced host cell protein synthesis shutoff, and only proteins expressed from the rSFV vector are labeled. The cells were then incubated for different chase times, and protein expression was analyzed from total cell lysates and from TCA-precipitated culture supernatants by SDS-PAGE. The results shown in Fig. 1 demonstrate that both envelope glycoproteins were expressed and migrated with their expected apparent molecular weights. At the 6-h chase point, almost all YU2gp120 proteins were secreted into the supernatant, while there was still a considerable amount of YU2gp140(-/GCN4) protein in the cell lysate at this time point (Fig. 1B, left panel). Analysis of the supernatants confirmed that YU2gp140 (-/GCN4) proteins were secreted less efficiently than YU2gp120 (Fig. 1B, right panel). To estimate the difference in the amount of secreted YU2gp140(-/GCN4) and YU2gp120 proteins produced by rSFV, supernatants from unlabeled rSFV-YU2gp120- and rSFV-YU2gp140(-/GCN4)-infected cultures were collected after 24 h of infection. The HIV-1 envelope glycoproteins were immunoprecipitated using an excess of IgGb12 MAb and analyzed by reducing SDS-PAGE. The silver-stained gel confirmed that YU2gp120 was secreted more efficiently into the supernatant than YU2gp140(-/GCN4) (Fig. 1C). In a separate experiment, we analyzed known amounts of purified envelope glycoproteins side by side with supernatants from rSFV-infected cells. Based upon this analysis, we estimate that there were approximately fivefold more envelope glycoproteins secreted by rSFV-YU2gp120-infected cells than by rSFV-YU2gp140(-/GCN4)-infected cells (data not shown).

Conformational integrity and oligomeric status of rSFV-expressed YU2gp140(-/GCN4) proteins. The recombinant YU2gp140(-/GCN4) protein expressed in plasmid-transfected cells has been characterized in a series of previous reports (51–53). These reports show that the purified YU2gp140 (-/GCN4) protein from DNA-transfected cells is assembled into relatively homogenous trimers that retain functional receptor-binding elements and are recognized by a panel of conformationally sensitive antibodies. However, infection with rSFV is likely to affect cells differently than plasmid DNA transfection and this may influence the folding and posttrans-

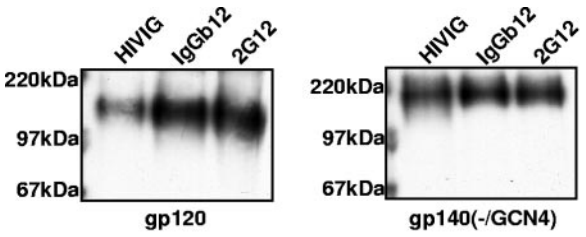

FIG. 2. Conformational integrity of rSFV-expressed envelope glycoproteins. Supernatants from [^{35}S]methionine-labeled BHK-21 cells infected with rSFV-YU2gp120 or rSFV-YU2gp140(-/GCN4) were immunoprecipitated with pooled sera from HIV-1-infected persons (HIVIG) and MAbs IgGb12 and 2G12. The proteins were analyzed by SDS-PAGE.

lational processing of vector-expressed proteins. We therefore investigated whether YU2gp140(-/GCN4) expressed in rSFV-infected cells retained the same biochemical and antigenic properties as described for the recombinant YU2gp140(-/GCN4) protein purified from the supernatant of plasmid-transfected cells. We performed immunoprecipitation experiments using conformationally sensitive MAbs against HIV-1 Env, IgGb12, and 2G12 to assess protein folding. Both YU2gp120 and YU2gp140(-/GCN4) secreted from rSFV-infected cells were recognized by these reagents, suggesting that the proteins retained native folding (Fig. 2). Next, we investigated the oligomeric status of the glycoproteins produced by rSFV expression using two different approaches. First, supernatants from rSFV-YU2gp120- and rSFV-YU2gp140(-/GCN4)-infected BHK-21 cells were [^{35}S]methionine labeled, TCA precipitated, and analyzed under nonreducing conditions by SDS-PAGE (Fig. 3A). This demonstrated that the bulk of gp120 proteins migrated with an apparent molecular weight consistent with monomers (Fig. 3A). A higher-order form was also present, likely representing a small fraction of dimers of YU2gp120, as also observed using DNA transfection or from stable lines (Yuxing Li and Richard Wyatt, unpublished observations). In contrast, YU2gp140(-/GCN4) migrated as two higher-order forms, consistent with a mixture of gp140 dimers and gp140 trimers. Very little gp140 monomer could be detected in the supernatant from rSFV-YU2gp140(-/GCN4)-infected cells.

Since disruption or artifactual formation of higher-order forms cannot be excluded when analyzing proteins in the presence of SDS, we also analyzed supernatants from rSFV-infected cells by blue native gel electrophoresis followed by Western blotting. Blue native gels have previously been shown to allow analysis of oligomeric status of HIV-1 gp140 envelope glycoproteins (42), and we have confirmed that the purified recombinant YU2gp140(-/GCN4) proteins are predominantly trimeric by a combination of gel filtration and blue native gel analysis using Coomassie-stained visualization of the protein bands (data not shown). The results of the blue native Western blot analysis demonstrated that rSFV-YU2gp140(-/GCN4) proteins migrated as a homogenous high-molecular-weight product, consistent with trimers (Fig. 3B), while YU2gp120 predominantly migrated as a monomer. A lesser fraction of the gp120 proteins displayed bands corresponding to higher-order oligomeric forms, the major species being a dimer. We also analyzed the supernatant from rSFV-YU2gp140(-/GCN4)-infected cells (10 μl) side by side with purified recombinant YU2gp140(-/GCN4) protein (30 ng) isolated as described in Materials and Methods (Fig. 3C). This analysis demonstrated that the supernatant of rSFV-YU2gp140(-/GCN4)-infected cells contained a highly homogenous protein product of apparent molecular weight corresponding to that of the major trimeric product present in the purified recombinant YU2gp140(-/GCN4) protein preparation (Fig. 3C). Some

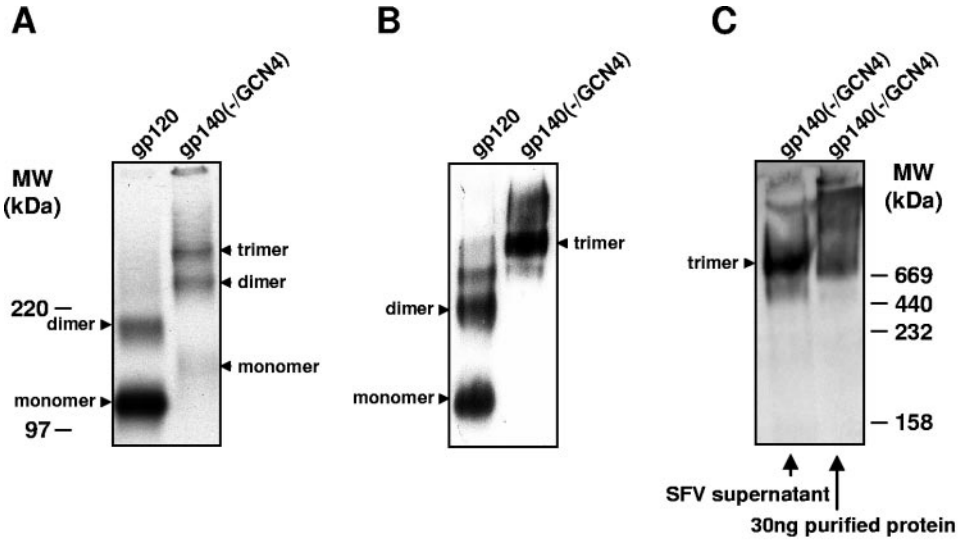

FIG. 3. Oligomeric status of rSFV-expressed envelope glycoproteins. (A) Supernatants from [^{35}S]methionine-labeled BHK-21 cells infected with rSFV-YU2gp120 or rSFV-YU2gp140(-/GCN4) were TCA precipitated and analyzed by SDS-PAGE (3 to 8%) under nonreducing conditions. (B) Unlabeled supernatants (10 μl) from rSFV-YU2gp120 or rSFV-YU2gp140(-/GCN4)-infected BHK-21 cells were analyzed on a blue native gel (4 to 12%), transferred to membranes, and subjected to Western blot analysis. (C) The supernatant (10 μl) from rSFV-YU2gp140(-/GCN4)-infected BHK-21 cells was also analyzed side-by-side with the purified YU2gp140(-/GCN4) protein (30 ng) by blue native/Western blot analysis.

higher-order forms were present in the affinity-purified gp140 protein preparation, likely representing disulfide-linked oligomers of trimers (54), while the supernatants of rSFV-YU2gp140(-/GCN4)-infected cells possessed more homogeneous trimers, as observed in two independent experiments. These data suggest that rSFV expression supports the secretion of highly homogenous stable YU2gp140(-/GCN4) trimers, perhaps more so than protein produced by transient DNA expression and affinity purification.

Study design and immunization schedules. A primary goal of this study was to determine whether qualitatively different antibody responses were elicited by regimens based on homologous protein prime-boost immunizations compared to heterologous viral vector prime-protein boost immunizations. It is well established that viral vectors stimulate strong $CD8^+$ T-cell responses, a potentially valuable property of a vaccine against HIV-1. However, viral vectors also stimulate humoral immune responses and an important consideration is to further characterize their potential to elicit neutralizing antibodies against HIV-1. The ability of rSFV to express correctly folded soluble HIV-1 gp140 trimers with biochemical properties comparable to those of the purified recombinant protein allowed us to perform head-to-head comparisons between immunization regimens based on rSFV-YU2gp140 (-/GCN4) particle vaccination followed by a single boost with recombinant YU2gp140(-/GCN4) protein in Ribi adjuvant and regimens based on repeated injections of purified recombinant protein in Ribi.

We designed immunization experiments using mice and rabbits as outlined in Fig. 4 by the following rationale. Rabbits are preferable compared to mice for analyzing neutralizing activity against HIV-1 due to the larger volumes of sera that can obtained from these animals as well as fewer nonspecific serum effects in HIV neutralization assays (unpublished observations). Rabbits may also produce antibodies with longer CDR3 loops than those produced in mice, and this might improve access to recessed targets on the functional viral spike (40). However, reagents for analyzing other qualitative aspects of the immune response, such as antibody isotype and phenotypic T-cell responses, are currently lacking in the rabbit system, whereas they are available in the mouse system. We therefore performed parallel immunogenicity experiments to analyze the elicitation of Env-binding and neutralizing antibodies in rabbits and to analyze Env-binding antibody isotypes and qualitative T-cell responses in mice elicited by the different immunization regimens. Groups of mice were immunized three times with recombinant YU2gp140(-/GCN4) protein in Ribi adjuvant (Protein only) or two or three times with rSFV-YU2gp140(-/GCN4) particles followed by a single boost with recombinant YU2gp140(-/GCN4) protein in Ribi adjuvant (2×SFV+Protein and 3×SFV+Protein, respectively) (Fig. 4A). Results from a rabbit pilot study indicated that a third rSFV immunization did not boost the antibody response obtained after two rSFV immunizations (see Fig. S1 in the supplementary material). We therefore limited the rabbit study to include the Protein only group and the 2×SFV+Protein group (Fig. 4B).

Analysis of antibody and $CD4^+$ T-cell responses in mice immunized with rSFV prime-protein boost or Protein only regimens. Mice (six per group) were immunized three times

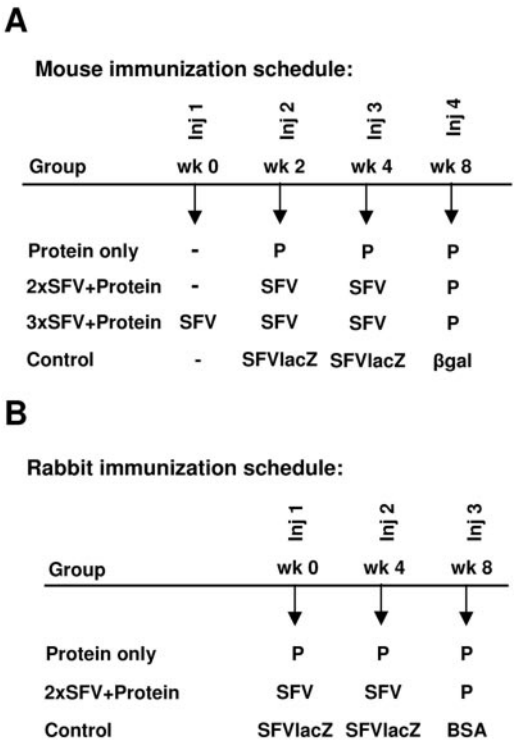

FIG. 4. Immunization schedule. Schematic representation of the immunization regimens used in the mouse and rabbit experiments. (A) Mice (six per group) were immunized three times with 10 μg purified YU2gp140(-/GCN4) protein in Ribi (P) or two or three times with 10^7 IU rSFV-YU2gp140(-/GCN4) particles (SFV), followed by 10 μg purified YU2gp140(-/GCN4) protein in Ribi at the indicated intervals. Four control mice were immunized two times with 10^7 rSFV-lacZ (SFVlacZ) followed by 10 μg βgal in Ribi. (B) Rabbits (four per group) were immunized three times with 25 μg purified YU2gp140 (-/GCN4) protein in Ribi (P) or two times with 5×10^7 IU rSFV-YU2gp140(-/GCN4) particles (SFV), followed by 25 μg or 5 μg purified YU2gp140(-/GCN4) protein in Ribi at the indicated intervals. Two control rabbits were immunized two times with 5×10^7 rSFV-lacZ (SFVlacZ) followed by 25 μg BSA in Ribi.

with purified recombinant YU2gp140(-/GCN4) protein (protein only group) or immunized two or three times with rSFV-YU2gp140(-/GCN4) followed by a boost with YU2gp140 (-/GCN4) protein (2×SFV+Protein and 3 × SFV+Protein groups, respectively), as described in Fig. 4. Total YU2gp120-binding IgG was measured 12 days after each immunization using a standard ELISA, and the end-point antibody titer in each individual mouse was determined (Fig. 5A). The majority of mice in the Protein only group required two immunizations (2×P) to mount a measurable IgG response, and this response was boosted by the third protein immunization (3×P) (Fig. 5A, left panel). No detectable IgG response was obtained after two rSFV-YU2gp140(-/GCN4) immunizations (2×SFV), and only 50% of the mice responded after three rSFV-YU2gp140(-/GCN4) immunizations (3×SFV), suggesting that homologous rSFV immunization was insufficient for inducing antibodies against this antigen (Fig. 5A, middle and right panels). In contrast, when the rSFV-YU2gp140 (-/GCN4)-immunized mice were boosted with recombinant YU2gp140(-/GCN4) protein in Ribi (2×SFV+P and 3×SFV+P), relatively high anti-

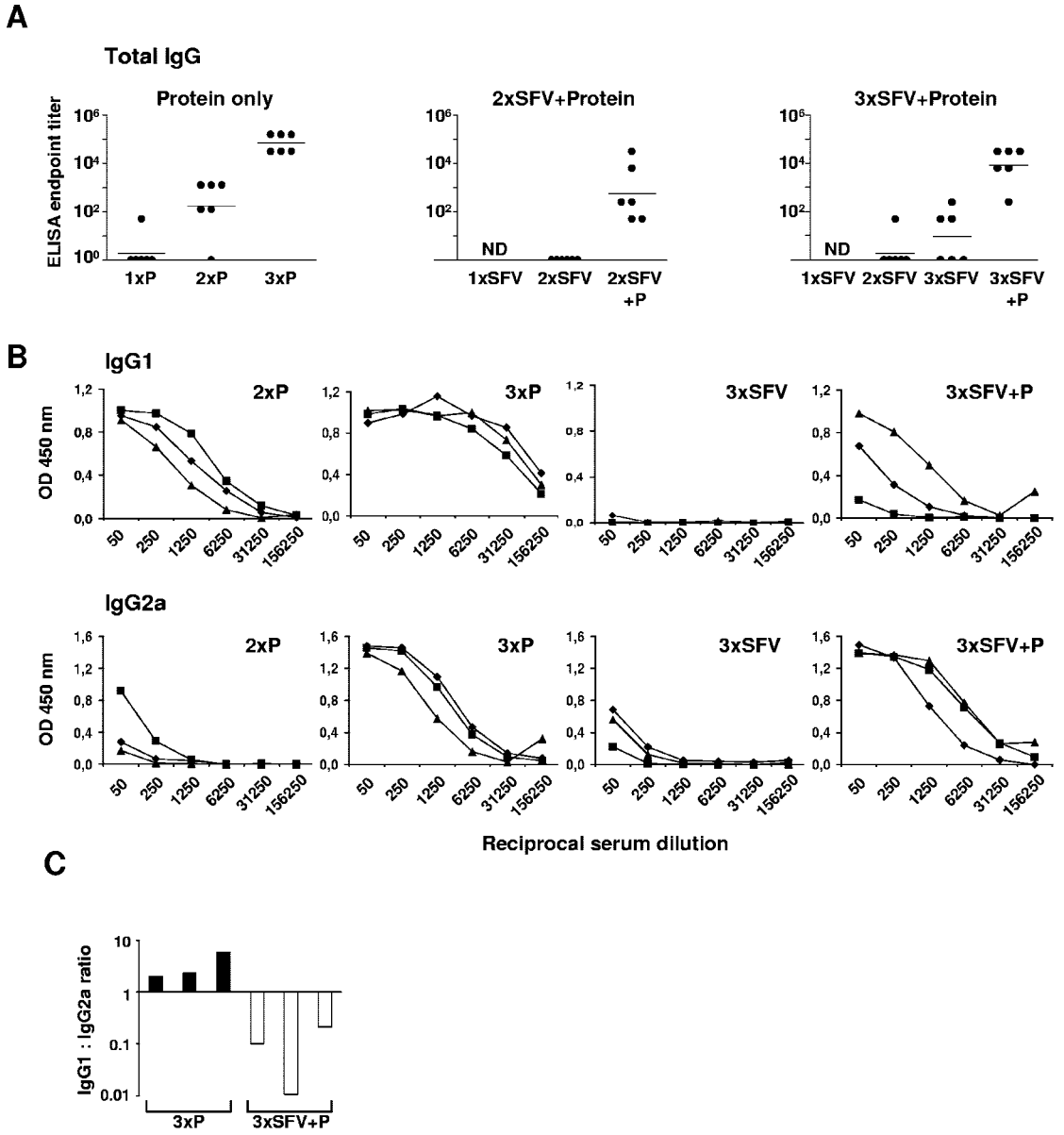

FIG. 5. Envelope-binding antibodies in sera from mice immunized with protein in Ribi or with rSFV particles followed by protein in Ribi. Mice were inoculated three times with 10 μg purified YU2gp140(-/GCN4) protein in Ribi (3×P), two times with 10^7 IU rSFV-YU2gp140(-/GCN4) followed by a single boost with 10 μg protein in Ribi (2×SFV+P), or three times with 10^7 IU rSFV-YU2gp140(-/GCN4) followed by a single boost with 10 μg protein in Ribi (3×SFV+P). (A) IgG ELISA end-point titers of envelope-binding antibodies in sera from individual mice 12 days after each immunization. The bar represents group geometric means; ND indicates not determined. (B) Titration of envelope-binding IgG1 antibodies (upper panels) and IgG2a antibodies (lower panels) before and after the final protein boost in three individual mice each in the Protein only group (2×P and 3×P) and in the 3×SFV+Protein group (3×SFV and 3×SFV+P). (C) IgG1:IgG2a ratios were calculated by dividing the IgG1 and IgG2a OD values for each individual mouse (black bars, 3×P group; white bars, 3×SFV+P group;) at the 1:6,250 serum dilutions.

body titers to gp120 were detected (Fig. 5A, middle and right panels). The geometric mean end-point titer obtained after the protein boost was higher in the 3×SFV+P group than in the 2×SFV+P group, consistent with the higher response measured in the 3×SFV group compared to the 2×SFV groups. A potent priming effect by rSFV was apparent when comparing the 2 × SFV+P and 3×SFV+P groups with the 1×P group (Fig. 5A).

For a more detailed analysis of the antibody responses we measured YU2gp120-specific IgG1 and IgG2a responses in the sera from the three highest responders from the 2×P and 3×P, 3×SFV, and 3×SFV+P groups, respectively (Fig. 5B). There was a strong IgG1 response and a weaker IgG2a response in mice immunized with 2×P and 3×P which was consistent with a Th2-biased antibody response in this group. In contrast, mice immunized with 3×SFV showed a positive IgG2a response but no detectable IgG1 response. Interestingly, the IgG2a response in the 3×SFV group was significantly increased following a boost with recombinant protein (3×SFV+P), while only a weak IgG1 response was stimulated by the protein boost in

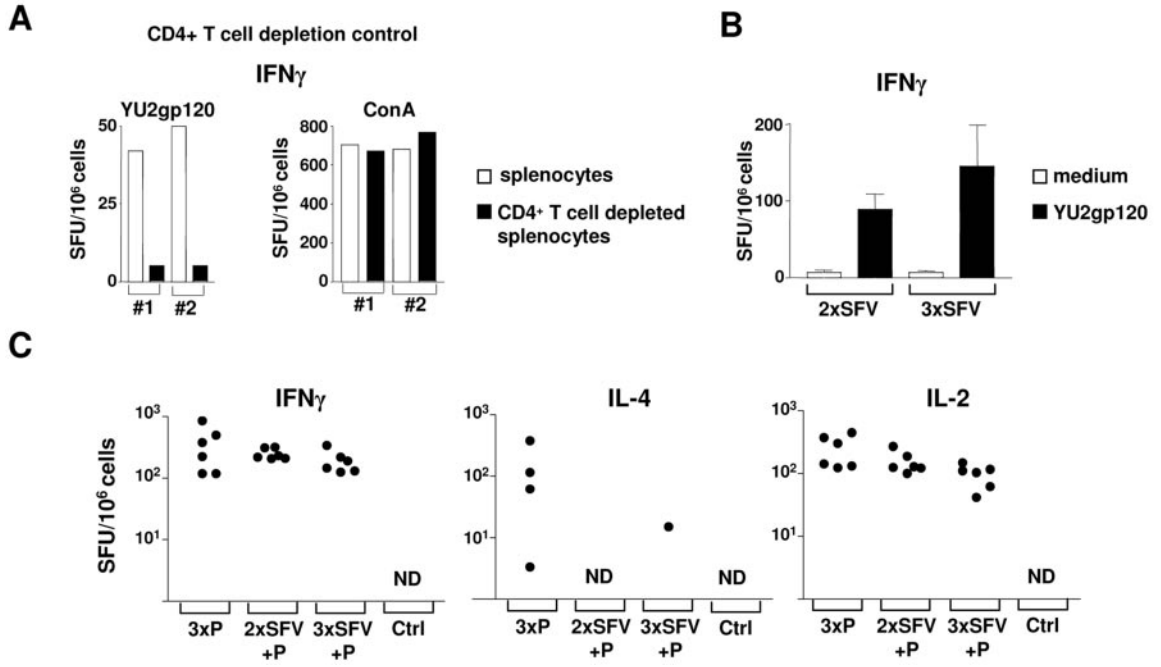

FIG. 6. Analysis of $CD4^+$ T-cell responses in mice immunized with rSFV prime-protein boost or protein only regimens. Recombinant YU2gp120 was used for in vitro stimulation of splenocytes from YU2gp140(-/GCN4)-immunized mice to measure envelope-specific $CD4^+$ T cells responses. Cytokine-producing cells are shown as spot-forming units (SFU)/10^6 cells. (A) $CD4^+$ T-cell depletion control. The contribution of $CD4^+$ T cells in the IFN-γ ELISPOT assay was evaluated by comparing the response in total and $CD4^+$ T-cell depleted splenocyte cultures. Data from representative mice (#1 and #2) stimulated with YU2gp120 or with ConA are shown. (B) Spleen cells from groups of mice immunized two or three times with 10^7 IU rSFV-YU2gp140(-/GCN4) (2×SFV and 3×SFV) were evaluated in an IFN-γ ELISPOT assay after stimulation with medium or with recombinant YU2gp120 protein. Data are presented as group means where the response from each individual mouse is determined as the mean ELISPOT value from triplicate wells. (C) IFN-γ, IL-4, and IL-2 ELISPOT analysis was performed on spleen cells harvested from groups of mice immunized three times with purified YU2gp140(-/GCN4) protein in Ribi (3×P) or three times with 10^7 IU rSFV-YU2gp140(-/GCN4) followed by a single boost with YU2gp140(-/GCN4) protein in Ribi (3×SFV+P). Control mice were immunized two times with rSFV-lacZ followed by a single boost with βgal protein in Ribi (Ctrl). Mean ELISPOT values from triplicate wells stimulated with medium or with YU2gp120 were calculated for each individual mouse. The data are presented as SFU/10^6 cells after subtracting the value obtained in medium-stimulated wells from the value obtained in YU2gp120 stimulated wells. ND (not detected) indicates that no positive responders were detected in a group.

this group. This resulted in inverse IgG1:IgG2a ratios between the groups (Fig. 5C). The ratios in sera from the individual mice were 2.07, 2.34, and 6.13, respectively, in the 3×P group and 0.1, 0.01, and 0.21, respectively, in the 3×SFV+P group. These results demonstrate that the antibody response was strongly influenced by the nature of the prime, with SFV inducing a clear Th1-biased response and recombinant protein in Ribi inducing a clear Th2-biased response.

To further characterize the HIV-1 envelope-specific immune responses stimulated by the different vaccine regimens, we performed ELISPOT analysis on splenocytes collected 12 days after immunization. In these experiments, we used recombinant YU2gp120 protein for in vitro stimulation to preferentially activate HIV-1 envelope-specific $CD4^+$ T cells. IFN-γ ELISPOT assays from total splenocyte cultures and cultures depleted for $CD4^+$ T cells confirmed that this protocol primarily measured $CD4^+$ T cells, since only background levels of activity were observed upon $CD4^+$ T-cell depletion (Fig. 6A). The ConA responses, which acted as a control for cell viability, were similar before and after $CD4^+$ T-cell depletion (Fig. 6A). In these experiments, efficient depletion of $CD4^+$ T cells from the total splenocyte population was confirmed by flow cytometric analysis using anti-CD4 and anti-CD3ε antibodies (data not shown).

We next measured the IFN-γ ELISPOT response in mice immunized two or three times with YU2gp140(-/GCN4) (2×SFV and 3×SFV) to determine whether the third rSFV immunization had boosted the YU2gp120-specific T-cell responses. We found that the IFN-γ ELISPOT response was augmented by the third rSFV immunization, thus providing one possible explanation to the higher antibody responses in the 3×SFV group compared to the 2×SFV group (Fig. 6B). For a broader qualitative assessment of the $CD4^+$ T-cell responses obtained after the final protein boost in the three different immunization groups (3×P, 2×SFV+P, and 3×SFV+P), we performed ELISPOT analysis for IFN-γ-, IL-4-, and IL-2-secreting cells. The results demonstrate that the IFN-γ and IL-2 ELISPOT responses were very similar between the groups (Fig. 6C), while in contrast the IL-4 response differed. Four of six mice gave a positive IL-4 ELISPOT response in the 3×P group compared to zero of six mice in the 2×SFV+P group and one of six mice in the 3×SFV+P group (Fig. 6C). No nonspecific IFN-γ, IL-4, or IL-2 ELISPOT responses were detected in splenocytes from the *lacZ*-immunized control mice.

In agreement with the antibody isotype analysis described above, the analysis of the $CD4^+$ T-cell response supports a more Th2-biased response in mice immunized with only pro-

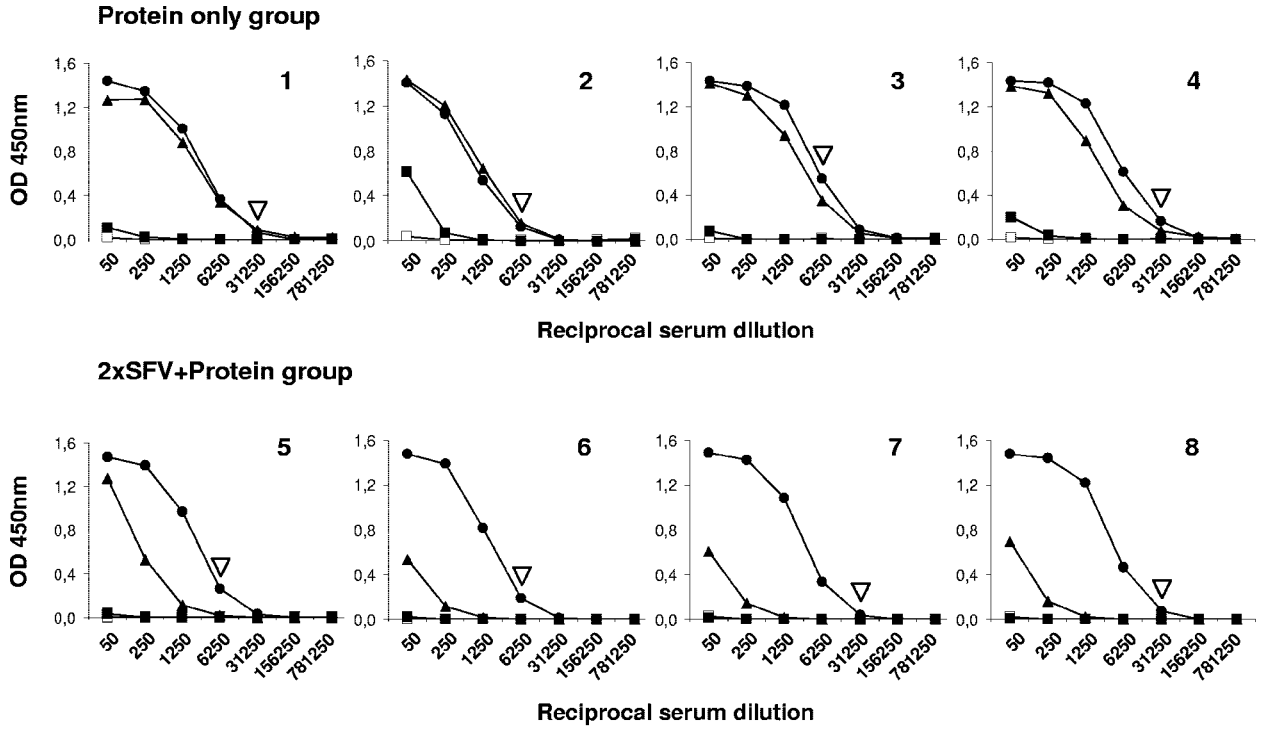

FIG. 7. Envelope-binding antibodies in sera from rabbits immunized with protein in Ribi or with rSFV particles followed by protein in Ribi. Rabbits were inoculated three times with 25 μg purified YU2gp140(-/GCN4) protein in Ribi (Protein only; rabbits 1 to 4) or two times with 5×10^7 IU rSFV-YU2gp140(-/GCN4) followed by a single boost with 25 μg protein in Ribi (2×SFV+Protein; rabbits 5 to 8). The sera from the immunized rabbits, drawn 10 days after each inoculation, were analyzed by fivefold serial dilutions (starting at a 1/50 dilution) for envelope-binding antibodies. Data from individual rabbits at different time points are shown as follows: prebleeds, empty squares; after the first injection, filled squares; after the second injection, triangles; after the third injection, circles. The endpoint titers after three immunizations are marked with open arrowheads.

tein in Ribi adjuvant compared to in mice immunized with SFV followed by a protein boost in Ribi.

HIV-1 neutralizing activity in rabbits after repeated protein immunizations is comparable to rSFV prime followed by protein boost. The ability of rSFV prime-protein boost regimens to stimulate neutralizing antibodies against HIV-1 was evaluated in rabbits. Similarly to the experiments performed in mice, one group of rabbits was immunized three times with recombinant YU2gp140(-/GCN4) protein in Ribi (3×P) and another group of rabbits was immunized two times with rSFV-YU2gp140(-/GCN4) particles followed by one immunization with recombinant protein (2×SFV+P) in Ribi. The immunizations were performed with a 1-month interval, and the doses of protein (25 μg) and rSFV particles (5×10^7) were increased compared to the doses used in the experiments performed in mice.

Prebleed sera from individual rabbits and sera collected 10 days after each immunization in the 3×P and 2×SFV+P immunized groups were analyzed for YU2gp120 binding antibodies using a standard ELISA. High antibody responses were obtained already after two immunizations with recombinant YU2gp140(-/GCN4) protein, and this response was not further boosted by the third protein immunization (Fig. 7, upper panel). Rabbits immunized two times with rSFV-YU2gp140 (-/GCN4) also displayed positive ELISA titers, though this response was lower than that obtained after two immunizations with protein in Ribi. However, when the rSFV-YU2gp140(-/GCN4)-immunized rabbits were boosted with a single injection of recombinant protein the antibody response was augmented to the same level as that obtained after two or three protein immunizations (Fig. 7, lower panel). In both groups, two rabbits had reciprocal endpoint titers of 6,250 and the other two animals had endpoint titers of 31,250. The similar antibody titers obtained after the last immunization in the 3×P and 2×SFV+P groups allowed a direct comparison of the HIV-1 neutralizing activity in these sera. A 1:5-fold dilution of the sera was assayed for neutralizing activity against a panel of viruses, including MN, HXb2, SF162, 89.6, JR-CSF, and YU2. Overall, neutralization activity levels were measured in the sera from rabbits immunized three times with 25 μg purified protein in Ribi that were similar to those measured in sera from rabbits immunized two times with rSFV-YU2gp140(-/GCN4) followed by a boost with 25 μg purified protein in Ribi (Fig. 8). The lack of homologous neutralizing activity against YU2 was somewhat surprising but may be explained by the fact that more than three immunizations were required for homologous neutralization of this relatively resistant isolate in previous studies (16). When the dose of the protein boost after two rSFV particle immunizations was lowered to 5 μg as opposed to 25 μg, the neutralizing capacity against selected viruses was slightly reduced, though the antibody binding reactivity results were not markedly different between these groups (see Fig. S2 in the supplementary material). A more complete dose titration of both the viral particles and the recombinant

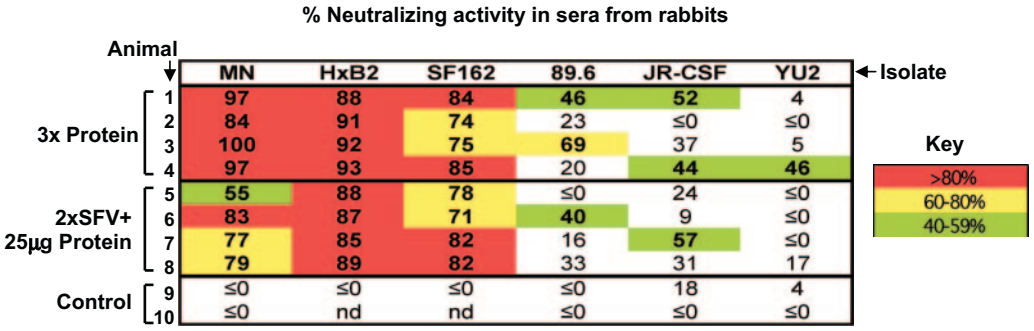

FIG. 8. In vitro neutralizing activity against a panel of HIV-1 isolates in sera from immunized rabbits. Neutralizing activity in 1:5 diluted sera from rabbits immunized three times with 25 μg purified YU2gp140(-/GCN4) protein in Ribi (Protein only; rabbits 1 to 4) or two times with 5×10^7 IU rSFV-YU2gp140(-/GCN4) followed by a single boost with 25 μg protein in Ribi (2 × SFV+Protein; rabbits 5 to 8). Control animals (rabbits 9 and 10) were immunized two times with 5×10^7 IU rSFV-lacZ followed by 25 μg BSA in Ribi. The numbers under each viral isolate represent percentages of neutralization, and color codes demonstrate different levels of neutralization (>80% neutralization, red; 60 to 80% neutralization, yellow; 40 to 59% neutralization, green). Preimmune sera were used to establish baseline-neutralizing activity in each individual rabbit, and these values were subtracted from the values shown. Neutralization equal to or less than zero is indicated as ≤0.

protein used for boosting would be required to determine the effect of vaccine dose on the stimulation of binding and neutralizing antibodies in this and similar immunization regimens.

DISCUSSION

Efforts to develop immunogens capable of stimulating broadly neutralizing antibodies against HIV-1 have so far met with limited success. Structural and biochemical studies of the envelope glycoproteins of HIV-1 are therefore critical for the design of improved immunogens that can be evaluated for their ability to stimulate neutralizing antibodies. As new immunogens are developed, the optimal means to present them to the immune system also needs to be determined. The evaluation of nondenaturing adjuvants and/or genetic expression systems that present correctly folded envelope glycoproteins to B cells is therefore an important vaccine-related area of investigation. Previous work has shown that rSFV is suitable for the expression of complex glycoproteins (23, 25, 26), suggesting that alphavirus-based systems are valuable for the delivery of HIV-1 envelope glycoproteins during vaccination. In addition, regimens using recombinant Venezuelan equine encephalitis virus (another alphavirus) to express envelope glycoproteins have shown promise for the stimulation of neutralizing antibodies against HIV-1 (8, 41).

In this study, we investigated the potential of rSFV particles to express GCN4-stabilized trimeric envelope glycoprotein immunogens from the relatively neutralization-resistant YU2 isolate. We utilized the expression of monomeric gp120 as a means to comparatively assess the in vitro expression of the YU2gp140(-/GCN4) trimers. For the immunogenicity studies we focused on the YU2gp140(-/GCN4) trimers, since these molecules have been shown to elicit neutralizing antibodies of greater breadth than those elicited by monomeric YU2gp120 (53). Initially, we investigated whether rSFV particles could efficiently express correctly folded YU2gp140(-/GCN4) molecules and whether these had the same oligomeric profile as that reported previously for the purified recombinant protein. The biochemical analysis of YU2gp140(-/GCN4) expressed in rSFV-infected cells demonstrated that conformationally intact gp140 trimers were secreted into the culture supernatant. Approximately 3 μg/ml of YU2gp140(-/GCN4) was produced from rSFV-infected cells during a 24-h period, despite a less efficient secretion of YU2gp140(-/GCN4) compared to monomeric YU2gp120. Interestingly, it appeared by native gel analysis that rSFV-YU2gp140(-/GCN4)-infected cells produced a more homogenous trimeric glycoprotein species compared to the purified gp140 molecules produced by transient transfection of 293T cells. Whether this apparent increased trimeric homogeneity is due to the rSFV-driven expression or results from oligomeric heterogeneity during the protein purification process remains to be determined.

Having established that rSFV expression produced native and predominantly trimeric YU2gp140(-/GCN4) glycoproteins, we performed head-to-head comparisons between immunization regimens based on repeated YU2gp140(-/GCN4) protein inoculations and regimens based on rSFV particle-driven expression of the gp140 trimers followed by a single protein boost of the identical gp140 protein. We used mice to investigate whether the two regimens resulted in qualitatively different immune responses. In parallel, we performed immunization studies in rabbits to determine whether the different regimens resulted in different neutralizing activity results against a panel of laboratory-adapted and primary HIV-1 isolates. In the mouse immunogenicity experiments, two inoculations with recombinant protein in Ribi were required for a consistent antibody response, while somewhat surprisingly three immunizations with rSFV-YU2gp140(-/GCN4) yielded responses in only 50% of the mice. Several studies using other antigens have demonstrated potent antibody responses after two immunizations with rSFV (2, 14). The secretion of gp140 trimers from rSFV-YU2gp140(-/GCN4)-infected cells is relatively inefficient; thus, the antigen dose obtained after rSFV-YU2gp140(-/GCN4) immunization may not be comparable with the dose obtained using other antigens expressed from rSFV. However, low secretion levels may not fully explain the poor antibody elicitation, since despite an estimated fivefold-higher secretion level of the rSFV-expressed gp120 monomers compared to the gp140 trimers (Fig. 1C), rSFV-YU2gp120 immunization also results in no or low antibody titers (unpub-

lished observations). This suggests that the YU2 envelope glycoproteins have low intrinsic immunogenicity in mice, as has been previously suggested (17). Despite low antibody titers after rSFV immunization alone, the mice were potently boosted by recombinant YU2gp140(-/GCN4) protein, resulting in similar end-point titers in all groups irrespective of whether the first immunizations were performed with rSFV or with purified protein. However, the different immunization regimens resulted in qualitatively different immune responses both in terms of antibody isotypes and in terms of $CD4^+$ T-cell responses. We found that repeated inoculations with recombinant protein resulted in an IgG1, Th2-biased response, while rSFV immunizations followed by a single protein boost resulted in an IgG2a, Th1-biased response. Interestingly, the Th1-biased response in the rSFV-immunized group remained Th1-biased also after the protein boost. A difference in the T-helper-cell profile between the groups was also apparent by the lack of IL-4-producing $CD4^+$ T cells in the groups of mice immunized with rSFV-YU2gp140(-/GCN4) followed by a single protein immunization compared to the group immunized with protein alone. This supports the conclusion that the type of vaccine used to prime the response influences the response obtained after subsequent boosting. Whether the Th bias induced by virus-based priming would shift upon additional boosting with protein in a Th2-driving adjuvant was not investigated in this study. These are relevant questions to address for future studies that develop virus vector prime followed by protein boost regimens designed to stimulate both cellular and humoral immunity against HIV-1.

Recombinant viral vaccines generally stimulate Th1-driven immune responses, likely because cytokines such as IFN-α, IFN-γ, and Il-12 are induced by viral infection. While Th1-driven immune responses are advantageous for the elicitation of $CD8^+$ T-cell responses, it is less well understood whether a Th1-driven immune response allows the generation of potent antibody responses and, in particular, the generation of neutralizing antibodies against HIV-1. In this study, we addressed this question by analyzing the neutralizing antibody response in rabbits subjected to the different immunization regimens. We were unable to investigate the antibody isotypes and $CD4^+$ T-cell responses in rabbits due to the lack of available reagents; however, we consider it likely that, as in the results obtained with mice, recombinant virus-based vaccines also stimulate a Th1-biased response in rabbits. Our neutralization data demonstrate that the two immunization regimens (repeated protein inoculation versus rSFV priming followed by a single protein boost) stimulate neutralizing antibodies of comparable potency and breadth. Consistent with other studies we were able to efficiently neutralize the two laboratory-adapted isolates, MN and HxB2, and we obtained potent neutralization of the relatively neutralization-sensitive primary isolate, SF162. In contrast, primary viruses, including the homologous YU2 and the heterologous isolates 89.6 and JR-CSF, were poorly neutralized by the sera from almost all rabbits irrespective of immunization regimen. Previous studies using the YU2gp140(-/GCN4) immunogen have reported more-efficient neutralization of these isolates; however, this capacity required up to six immunizations (16).

This is the first report which evaluates the ability of rSFV to prime HIV-1 antibody responses when combined with a recombinant Env protein boost and which examines the neutralizing response elicited by this regimen against a panel of HIV-1 isolates. In addition, this is the first report that directly compares immunization regimens based on repeated inoculations with trimeric gp140 protein and viral delivery of the matched immunogen in combination with a recombinant protein boost. We conclude that rSFV, besides having value to elicit cellular responses as shown elsewhere (2, 14, 21, 37), also demonstrates value as a prime in regimens aimed at stimulating neutralizing antibody responses against HIV-1. Finally, the importance of antibody isotypes and their effector functions in vaccine-induced protection is an area that is largely undefined at the current time. Our results suggest that this issue could be addressed in relevant challenge models by comparing protein-based and virus-based immunization regimens as described here.

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