

Specific HIV gp120-cleaving Antibodies Induced by Covalently Reactive Analog of gp120*

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We report the results of efforts to strengthen and direct the natural nucleophilic activity of antibodies (Abs) for the purpose of specific cleavage of the human immunodeficiency virus-1 coat protein gp120. Phosphonate diester groups previously reported to form a covalent bond with the active site nucleophile of serine proteases (Paul, S., Tramontano, A., Gololobov, G., Zhou, Y. X., Taguchi, H., Karle, S., Nishiyama, Y., Planque, S., and George, S. (2001) *J. Biol. Chem.* 276, 28314–28320) were placed on Lys side chains of gp120. Seven monoclonal Abs raised by immunization with the covalently reactive analog of gp120 displayed irreversible binding to this compound (binding resistant to dissociation with the denaturant SDS). Catalytic cleavage of biotinylated gp120 by three monoclonal antibodies was observed. No cleavage of albumin and the extracellular domain of the epidermal growth factor receptor was detected. Cleavage of model peptide substrates occurred on the C-terminal side of basic amino acids, and K_m for this reaction was ~200-fold greater than that for gp120 cleavage, indicating Ab specialization for the gp120 substrate. A hapten phosphonate diester devoid of gp120 inhibited the catalytic activity with exceptional potency, confirming that the reaction proceeds via a serine protease mechanism. Irreversible binding of the hapten phosphonate diester by polyclonal IgG from mice immunized with gp120 covalently reactive analog was increased compared with similar preparations from animals immunized with control gp120, indicating induction of Ab nucleophilicity. These findings suggest the feasibility of raising antigen-specific proteolytic antibodies on demand by covalent immunization.

Promiscuous cleavage of small peptide substrates is a heritable function of Abs¹ encoded by germ line gene variable domains (for review, see Ref. 1). Peptide bond cleaving Abs with specificity for individual polypeptides have been identified in

patients with autoimmune (1) and alloimmune disease (2). Specific monoclonal Abs and Ab light chain subunits displaying proteolytic activities can be raised by routine immunization with polypeptides (3, 4). Under ordinary circumstances, however, adaptive maturation of the catalytic activity may not be a favored event. B cell clonal selection occurs by sequence diversification of genes encoding the Ab variable domains followed by selective binding of the antigen to cell surface Abs with the greatest affinity, which drives proliferation of the B cells (5). Catalysis entails chemical transformation of the antigen and release of products from the Ab, which may cause cessation of B cell proliferation when the catalytic rate exceeds the rate of transmembrane signaling necessary to stimulate cell proliferation.

Originally developed as irreversible inhibitors of conventional serine proteases, haptenic phosphonate esters are reported to bind the nucleophilic sites of natural proteolytic Abs covalently (6, 7). The haptenic phosphonates could potentially serve as covalently reactive analogs (CRAs) for inducing the synthesis of Abs with improved nucleophilicity. To the extent that Ab nucleophilicity is rate-limiting in proteolysis, its enhancement may permit more rapid peptide bond cleavage, *i.e.* if the subsequent steps in the catalytic reaction cycle (hydrolysis of the acyl-Ab complex and product release) do not pose significant energetic hurdles (see Fig. 1). The innate character of Ab nucleophilic reactivity is the central element of this approach, and there is no requirement for *de novo* formation of chemically reactive sites over the course of variable domain sequence diversification. Most previous attempts to program the structure of catalytic sites in Abs in comparison have relied on noncovalent stabilization of the oxyanionic transition state (*i.e.* by immunization with transition state analogs; Refs. 8 and 9). An Ab with esterase activity (10) and another with aldolase activity (11) utilize covalent catalytic mechanisms, but the relationship of these activities to innate Ab nucleophilicity is unclear.

An ideal antigen-specific proteolytic Ab may be conceived to combine traditional noncovalent binding interactions in the ground state of the Ab-antigen complex with nucleophilic attack on the peptide backbone. The ground state interactions are desirable to obtain specificity for individual polypeptide antigens. No impediments for catalysis are presented by the stable ground state complexes, provided the noncovalent interactions are carried over into the transition state complex and are properly coordinated with nucleophilic attack at the reaction center. In theory, synthesis of antigen-specific proteolytic Abs could be induced by an analog that presents a mimetic of the chemical reaction center in the context of classical antigenic epitopes available for noncovalent binding interactions. If the reaction proceeds by a lock-and-key stereochemical mech-

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¹ The abbreviations used are: Ab, antibody; mAb, monoclonal Ab; Bt, biotin; CRA, covalently reactive antigen analog; MCA, methylcoumarinamide; VIP, vasoactive intestinal peptide; HIV, human immunodeficiency virus; exEGFR, extracellular domain of enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay.

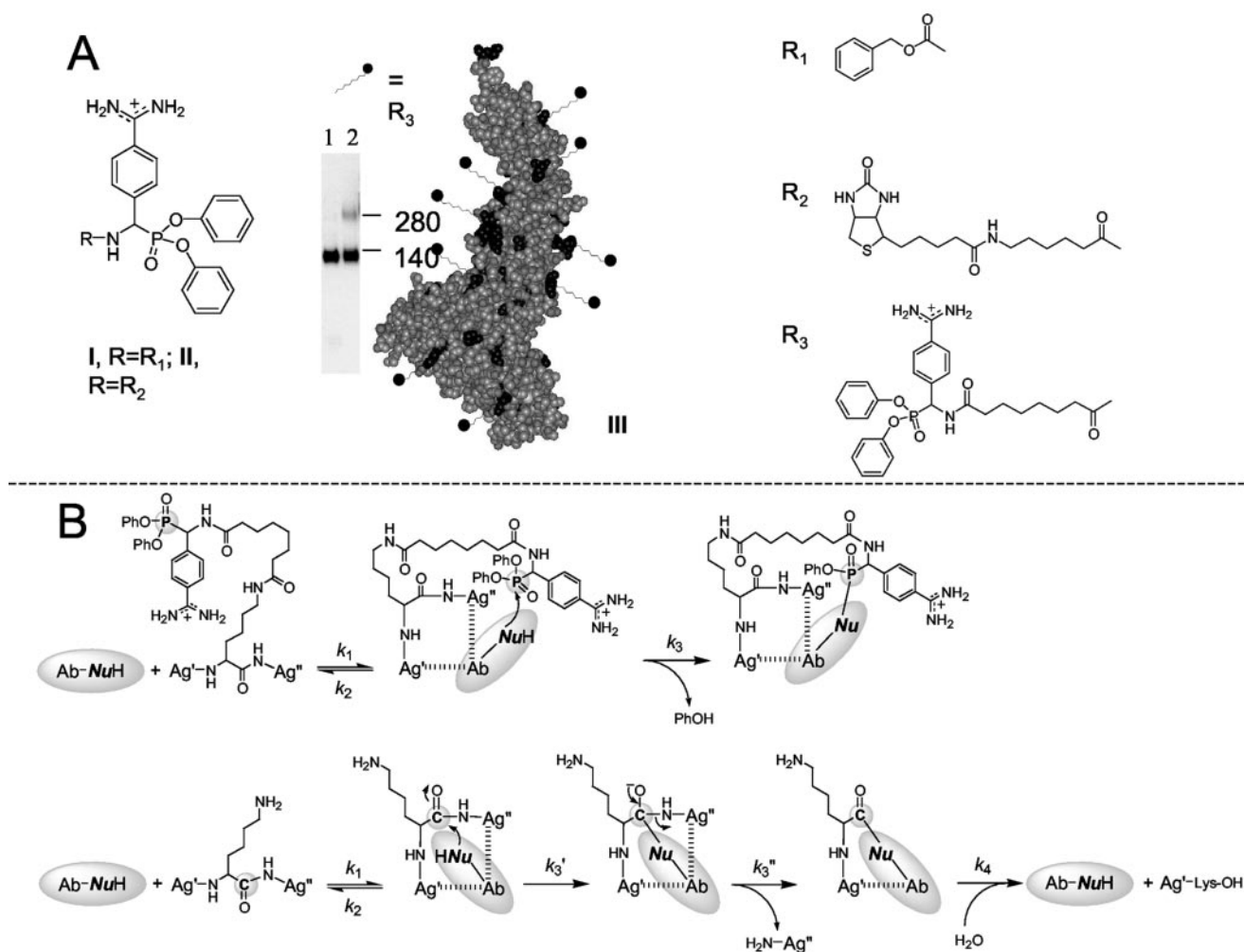


FIG. 1. CRA structures (A) and their reaction with Abs (B). III is a schematic representation of gp120 with R3 substituents at Lys residues. Left of **III** are streptavidin-peroxidase stained blots of SDS-electrophoresis gels showing biotinylated **III** containing 4 mol (lane 1) and 14 mol (lane 2) of phosphonate diester groups/mol gp120. **B**: Nu, nucleophile; Ag⁺-Lys-OH, N-terminal antigen fragment; NH₂-Ag⁺, C-terminal antigen fragment; $k_{\text{cat}} = k_3' + k_3''$. A catalytic Ab forms the initial noncovalent complex by conventional epitope-paratope interactions. The active site nucleophile attacks the carbonyl carbon of the scissile bond in Ag²⁺ (substrate) to form the tetrahedral transition-state complex. The C-terminal antigen fragment is released, and the acyl-Ab complex is formed. Hydrolysis of the acyl-Ab complex results in release of the N-terminal antigen fragment and regeneration of the catalytic Ab. The reaction with phosphonate-containing Ag²⁺ recapitulates the interactions in the ground and transition state Ab-Ag²⁺ complexes (noncovalent binding at peptide epitopes and nucleophilic attack by the Ab), but unlike the acyl-Ab intermediate, the phosphonyl-Ab adduct is a stable product. A potential weakness is that immunogen **III** does not contain structural features favoring synthesis of Abs capable of rapid hydrolysis of the acyl-Ab intermediate and product release (bottom reaction scheme).

anism, the mimetic must be located precisely at the position of the intended scissile bond in the backbone of the polypeptide antigen. In the instance of large proteins, locating the mimetic within the protein backbone is outside the range of present-day synthetic technologies. A potential solution is to place the mimetic group at amino acid side chains using chemical linker techniques. An Ab nucleophile that recognizes the side chain mimetic could facilitate proteolysis if it enjoys sufficient conformational freedom to approach the polypeptide backbone of the substrate and form the acyl-Ab complex (see Fig. 1).

We describe here the characteristics of Abs induced by a CRA of the HIV-1 coat protein gp120 (gp120-CRA) consisting of phosphonate diester groups located in Lys side chains of the protein. Enhanced serine protease-like nucleophilic reactivity of the Abs was observed. One monoclonal Ab cleaved gp120 slowly and specifically, it displayed preference for cleavage on the C-terminal side of Lys/Arg residues, and the catalytic reaction was susceptible to CRA inhibition. These findings are the first indications that Abs with proteolytic activity specific for individual proteins can be raised on demand.

MATERIALS AND METHODS

Hapten, gp120-CRAs, and Biotinylated Proteins—Synthesis of hapten CRAs **I** and **II** (see Fig. 1) and their characterization by electrospray ionization-mass spectroscopy and elemental analyses have been described previously (12). For preparation of gp120-CRA **III**, the precursor diphenyl-*N*-[*O*-(3-sulfosuccinimidyl)suberoyl]amino-(4-amidinophenyl)methanephosphonate (**IV**) was synthesized by mixing a solution of diphenylamino(4-amidinophenyl)methanephosphonate (79 mg, 0.13 mmol) in *N,N*-dimethylformamide (2 ml) containing *N,N*-diisopropylethylamine (0.11 ml, 0.63 mmol) and bis(sulfosuccinimidyl)suberate disodium salt (150 mg, 0.26 mmol; Pierce) for 2 h. **IV** was obtained by reversed-phase high performance liquid chromatography (12) and lyophilized to give a colorless powder (yield 54%, 50 mg; *m/z* 715 (MH⁺) by electrospray ionization mass spectroscopy). **IV** (1.1 mg) was reacted with electrophoretically pure gp120 (0.5 mg; Immunodiagnostic Inc., MN strain, purified from baculovirus expression system) in 5 ml of 10 mM HEPES, 25 mM NaCl, 0.1 mM CHAPS, pH 7.5 buffer (2 h, 25 °C). Excess **IV** was removed by gel filtration (Micro Bio-Spin 6 disposable column, Bio-Rad), and the concentration of free amines in the initial protein and CRA-derivitized protein was measured using fluorescamine (13). The density of labeling was varied as needed from 4.0 to 32.6 mol of CRA/mol of gp120 by varying the concentration of **IV**. Preparation of gp120 labeled at Lys residues with biotin (Bt-gp120) was

by similar means using 6-biotinamido-hexanoic acid *N*-hydroxysuccinimide ester (Sigma). The reaction time and reactant concentrations were controlled to yield biotin/gp120 molar ratios 0.8–1.9. Unreacted biotinylation reagent was removed using a disposable gel filtration column in 50 mM Tris-HCl, 100 mM glycine, 0.1 mM CHAPS, pH 7.8. The biotin content was determined using 2-(4'-hydroxyazobenzene)benzoic acid (14). Total protein measurements were done using the BCA method (Pierce kit). Biotinylated **III** was prepared from Bt-gp120 as described for **III**. With increasing incorporation of the hapten groups, biotinylated **III** tended to form dimers and trimers evident in SDS electrophoresis gels as bands at ~240 and 380 kDa (nominal mass of monomer gp120, 120 kDa). Biotinylated **III** at hapten density similar to the non-biotinylated **III** employed as immunogen (23 mol/mol of gp120) contained the monomer, dimer, and trimer species at proportions of 50, 21, and 29%, respectively. Protein-CRAs were lyophilized and stored at -20 °C until used. Bt-gp120 was stored at -70 °C in 50 mM Tris-HCl, pH 8.0, 0.1 M glycine, 0.1 mM CHAPS. Storage of **I** and **II** was at -70 °C as 10 mM solutions in *N,N*-dimethylformamide. The extracellular domain of EGFR (exEGFR) obtained from Dr. Maureen O'Connor (15) was biotinylated as described for gp120 (0.9 mol of biotin/mol of exEGFR).

Antibodies—mAbs were prepared from female MRL/MpJ-Fas^{pr} mice (The Jackson Laboratory, Bar Harbor, ME; 4–5 weeks) immunized with gp120-CRA **III** (23 mol of phosphonate diester/mol of gp120). The mice were injected intraperitoneally on days 0, 14, and 28 days with gp120-CRA **III** (11 µg) in Ribi adjuvant (monophosphoryl lipid A + trehalose dicorynomolate emulsion; Sigma) followed by a fourth intravenous booster without adjuvant on day 55. Blood was obtained from the retroorbital plexus over the course of the immunization schedule. Three days after the final injection, hybridomas were prepared by fusion of splenocytes with myeloma cell line (NS-1; Ref. 3). After identification of wells secreting the desired Abs by ELISA, monoclonal cell lines were prepared by two rounds of cloning by limiting dilution. Monoclonal IgG was prepared from tissue culture supernatants containing mAbs (200 ml) by affinity chromatography on immobilized protein G (3). Control mAbs (anti-VIP clone c23.5 and anti-yellow fever virus antigen clone CRL 1689; ATCC) and serum IgG were purified similarly. The IgG preparations were electrophoretically homogeneous, determined by silver staining of overloaded IgG and immunoblotting with specific Abs to mouse IgG (3). Additional immunizations of female BALB/c mice (Jackson; 4–5 weeks) with gp120 or gp120-CRA were carried out similarly. mAb heavy and light chain isotypes were determined by ELISA as described (3).

ELISA—Maxisorp 96-well microtiter plates (Nunc) were coated with gp120 or gp120-CRA (40–100 ng/well) in 100 mM bicarbonate buffer, pH 8.6. Routine ELISAs were carried out as described (16). For assay of irreversible binding, the Abs were allowed to bind the plates, and the wells were treated for 30 min with 2% SDS in 10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween 20, pH 7.4 (PBS-Tween) or PBS-Tween without SDS (control wells for measurement of total binding). The wells were then washed three times with PBS-Tween, and bound IgG was determined as usual using a peroxidase conjugate of goat anti-mouse IgG (Fc-specific; Sigma). Observed values of binding were corrected for nonspecific binding in wells containing nonimmune IgG or nonimmune mouse serum ($A_{490} < 0.03$). Percent residual binding in SDS-treated wells was computed as (A_{490} , SDS-treated wells) × 100/ $(A_{490}$, PBS-Tween-treated wells).

Electrophoresis of Ab-CRA Complexes—Irreversible binding of biotinylated CRAs by purified IgG was determined by denaturing electrophoresis (6). Briefly, the reaction mixtures were incubated at 37 °C in 50 mM Tris-HCl, 0.1 M glycine, pH 8.0. SDS was added to 2%, and the mixtures were boiled (5 min) and then subjected to SDS-PAGE (4–20%, Bio-Rad, or 8–25% Phast gels, Amersham Biosciences). After electroblotting onto nitrocellulose membranes (0.22 µm, Bio-Rad), the membranes were blocked with 5% skim milk in PBS-Tween and processed for detection of IgG or biotin using peroxidase-conjugated goat anti-mouse IgG (Sigma) or peroxidase-conjugated streptavidin, respectively. Imaging and quantification were using x-ray film (Eastman Kodak Co.) with Unscan-it software (Silk scientific, Orem, UT) or a Fluoro-STM MultiImager (Bio-Rad). Biotinylated bovine serum albumin (11 mol of biotin/mol of bovine serum albumin; Sigma) was employed to construct a standard curve (0.06–1.5 pmol of biotin/lane).

Hydrolysis Assays—Biotinylated proteins were incubated with IgG in 50 mM Tris-HCl, 0.1 M glycine, 0.1 mM CHAPS, pH 8, at 37 °C, the reaction was terminated by addition of SDS to 2%, and the samples were boiled (5 min) and then analyzed by reducing SDS-gel electrophoresis (4–20%, Bio-Rad). Biotin containing protein bands in blots of the gel were identified and quantified as in the preceding section. In some blots, reaction products were identified by immunoblotting using

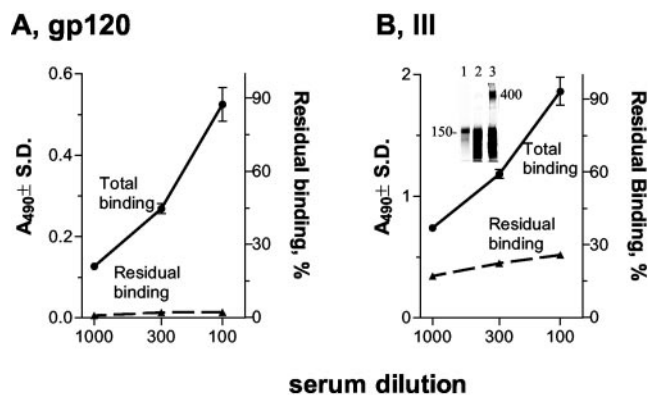


FIG. 2. Irreversible **III binding by polyclonal Abs.** A, immobilized gp120. B, immobilized **III**. Shown are ELISA values for binding of polyclonal Abs in serum of mice hyperimmunized with **III** (pooled sera, $n = 4$ mice). Binding of nonimmune mouse serum was negligible (A_{490} of 1:100 nonimmune serum in 0.001 (A) and -0.002 (B)). Residual and total binding represent A_{490} values in wells treated with and without SDS, respectively. *Inset*, anti-**III** stained blot of SDS-electrophoresis gels showing **III** (0.3 µM) treated for 48 h with nonimmune IgG (lane 2, 0.1 µM) and anti-**III** IgG (lane 3, 0.1 µM). Large Ab-containing adducts are evident at ~400 kDa in lane 3. Lane 1 is a shorter exposure of lane 2 showing a well defined 150-kDa band at the position of the smear evident in overexposed lanes 2 and 3.

peroxidase-conjugated goat anti-gp120 Abs (Fitzgerald, Concord, MA; catalog #60-H14) (16). N-terminal sequencing of protein bands from electrophoresis gels was done as described previously (17). Hydrolysis of peptide-MCA substrates (Peptide International, Louisville, KY or Bachem Biosciences, King of Prussia, PA) was determined in 96-well plates by fluorimetric detection of aminomethylcoumarin (Varian Cary Eclipse; λ_{ex} 360 nm, λ_{em} 470 nm) with authentic aminomethylcoumarin as standard (6). Cleavage of [Tyr^{10,125}]VIP by mAb c23.5 was measured as the radioactivity rendered soluble in trichloroacetic acid (17). Kinetic parameters for cleavage of increasing concentrations of peptide-MCA substrates were determined from the Michaelis-Menten equation, $v = (V_{max}[S])/(K_m + [S])$. Because of the expense of studying gp120 cleavage at large concentrations of the protein, K_d (~ K_m) and k_{cat} for this reaction were obtained from the general quadratic equation (17) $[CS]^2 - [CS]([C_t] + [S_t] + K_d) + [C_t][S_t] = 0$, where $[C_t]$ and $[S_t]$ are the total concentrations of catalyst and substrate, and $[CS]$ is the catalyst-substrate concentration. The method consists of calculation of $[CS]$ at a series of assumed K_d values. The assumed K_d value yielding the best fit (by linear regression) between the observed reaction velocity and $[CS]$ represents the experimentally determined K_d . k_{cat} is computed as the slope of the observed velocity versus $[CS]$ plot.

RESULTS

gp120-CRA Design and Validation—Synthesis of hapten CRAs **I** and **II** (Fig. 1) and their covalent reactivity with naturally occurring proteolytic Abs has been described previously (6, 7). The electrophilic phosphonate mimics the peptide bond carbonyl group susceptible to nucleophilic attack, the positively charged amidino group adjacent to the phosphonate diester serves as a mimic of Lys/Arg P1 residues at which cleavage by germ line-encoded proteolytic Abs is observed (6), and the biotin group in **I** permits sensitive detection of Ab-phosphonate adducts. gp120-CRA **III** contains phosphonate diester groups in spatial proximity with antigenic epitopes presented by the protein. Multiple phosphonate diester groups were available per molecule of gp120, allowing presentation of the electrophilic hapten in conjunction with diverse antigenic epitopes.

Robust polyclonal Ab responses in MRL/lpr and BALB/c mice immunized with **III** were observed by routine ELISA. Abs raised to **III** were bound at somewhat greater levels by immobilized **III** than control gp120 devoid of phosphonate diester groups (Fig. 2). Conversely, Abs raised to control gp120 recognized immobilized **III**, but the binding was 3–4-fold lower than by immobilized gp120 (e.g. at serum dilution of 1:1000, A_{490} 0.44 ± 0.03 for immobilized **III** and 1.40 ± 0.03 for immobilized

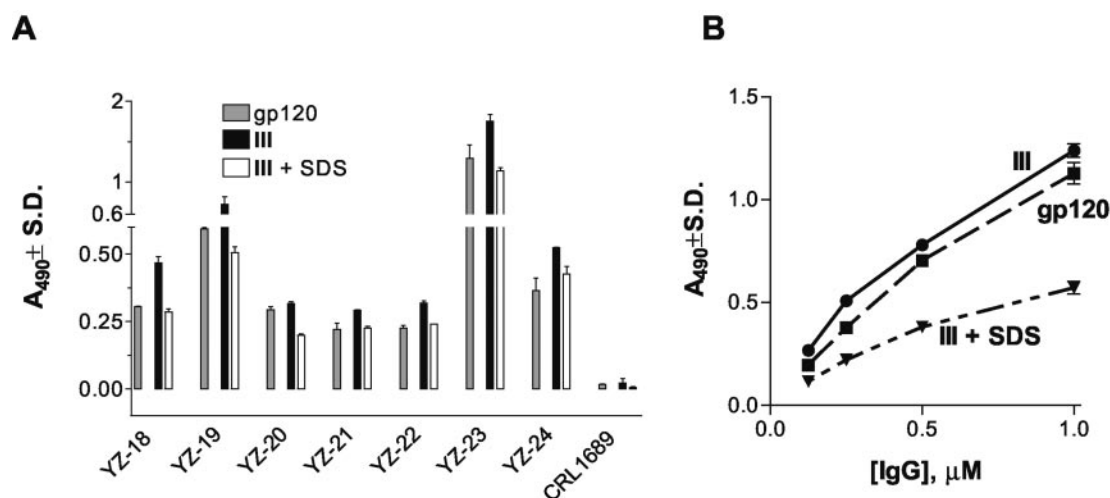


FIG. 3. **Irreversible III binding by monoclonal Abs.** ELISA showing SDS-resistant III binding by tissue culture supernatants containing mAbs (YZ series) (A) and monoclonal IgG purified from clone YZ18 (B) raised by immunization with gp120-CRA III. mAb CRL 1689 is an irrelevant monoclonal IgG with same isotype as mAbs YZ21 and YZ23. Immobilized antigens are gp120 and III. SDS-resistant III binding is indicated by bars and the curve labeled III + SDS.

gp120). III binding by nonimmune Abs was negligible, indicating that indiscriminate covalent binding at the hapten groups was not a problem. The observed differences in the antigenic reactivity of gp120 and III were held to be sufficiently small to proceed with further Ab studies. To facilitate high throughput screening, the feasibility of measuring irreversible III binding by Abs was studied by ELISA. After binding of polyclonal Abs anti-III Abs to the immobilized antigens, ELISA plates were treated with the denaturant SDS to remove reversibly bound Abs. SDS treatment allowed essentially complete removal of anti-III Abs bound by control gp120 devoid of hapten phosphonate groups. In comparison, 13–40% of the overall anti-III Ab binding activity consistently remained bound to immobilized III after SDS treatment in three repeat experiments. SDS-electrophoresis and immunoblotting with Abs to mouse IgG confirmed formation of irreversible Ab-III complexes in boiled reaction mixtures (Fig. 2B, inset, lane 3, estimated mass from extrapolated standard curve of molecular mass standards, ~400 kDa; large complexes can be formed by binding of multiple Abs to hapten groups in III).

Catalytic Activity—mAbs were prepared from MRL/lpr mice immunized with gp120-CRA III. This mouse strain develops lupus-like autoimmune disease attributable to the dysfunctional Fas-receptor gene. Spontaneous development of proteolytic Abs (18) and increased synthesis of esterase Abs in response to immunization with phosphonate monoester haptens (19, 20) have been reported in this mouse strain. Supernatants from 712 hybridoma wells (two splenocyte-myeloma cell fusions) were screened for SDS-resistant binding to III. IgG from seven wells was positive for this activity. After cloning of the cells by limiting dilution, monoclonal IgG from the supernatants of the seven cell lines was purified, and the binding assays were repeated (Fig. 3; clones YZ18, IgG2a, κ ; YZ19, IgG2b, κ ; YZ20, IgG2a, κ ; YZ21, IgG2a, κ ; YZ22, IgG2a, κ ; YZ23, IgG2a, κ ; and YZ24, IgG1, κ). Of total binding observed without SDS treatment of the ELISA plates, residual binding after the detergent treatment was 43–83% in 4 repeat assays. All seven mAbs were also bound by gp120 devoid of hapten CRA groups determined by routine ELISA without SDS treatment, indicating that they are not directed to neopeptides generated by chemical modification procedures used for III preparation. An irrelevant mAb (clone CRL 1689) displayed no detectable binding of III or gp120.

Of seven mAbs with irreversible III binding activity, slow

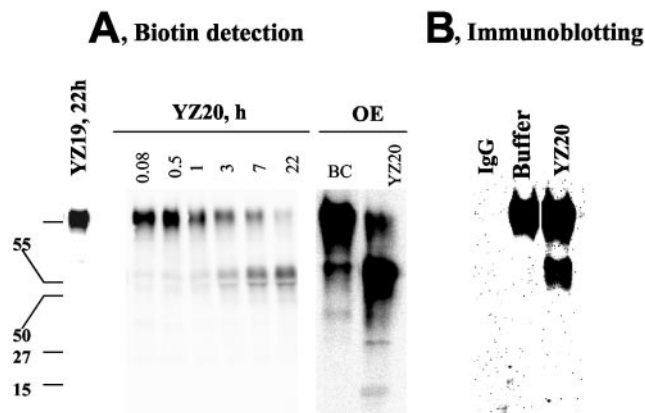


FIG. 4. **Cleavage of Bt-gp120 by mAb YZ20.** A, streptavidin peroxidase-stained blot of SDS-electrophoresis gels showing time-dependent Bt-gp120 cleavage by mAb YZ20 and lack of cleavage by mAb YZ19 (22 h incubation). IgG, 1 μ M; Bt-gp120, 0.2 μ M. OE, overexposed lanes showing Bt-gp120 incubated for 22 h in diluent and with YZ20 IgG (1 μ M). Product bands at 27 and 15 kDa are visible in addition to the major 50–55 kDa bands. B, anti-gp120-peroxidase stained blot of SDS-electrophoresis gel showing gp120 (1 μ M) incubated with diluent or YZ20 IgG (1 μ M, 24 h).

cleavage of Bt-gp120 by three mAbs was detected (YZ18, YZ20, YZ24), determined by the appearance of biotin-containing fragments of the protein in SDS-electrophoresis gels. The electrophoretic pattern of Bt-gp120 cleaved by mAbs YZ18 and YZ24 were similar to that shown for mAb YZ20 in Fig. 4. mAb YZ20 was further studied as it cleaved Bt-gp120 ~5-fold more rapidly than the other two mAbs. The consumption of gp120 was time-dependent (Fig. 4A). Major biotin-containing cleavage products with apparent mass 55 and 50 kDa were observed along with less intensely stained bands at 27 and 15 kDa. A band at 35 kDa was visible in overexposed gels, but this does not represent a product of mAb cleavage, as it was present at similar density in control incubations of Bt-gp120 in diluent. A control-irrelevant mAb (clone CRL 1689) did not cleave Bt-gp120. Immunoblotting using polyclonal anti-gp120 Abs confirmed that non-biotinylated gp120 is also susceptible to cleavage by the mAb (55-kDa cleavage product, Fig. 4B). Both detection methods allow quantification of gp120 cleavage by measuring depletion of intact gp120. Neither method provides guidance about the complete product profile or product concen-

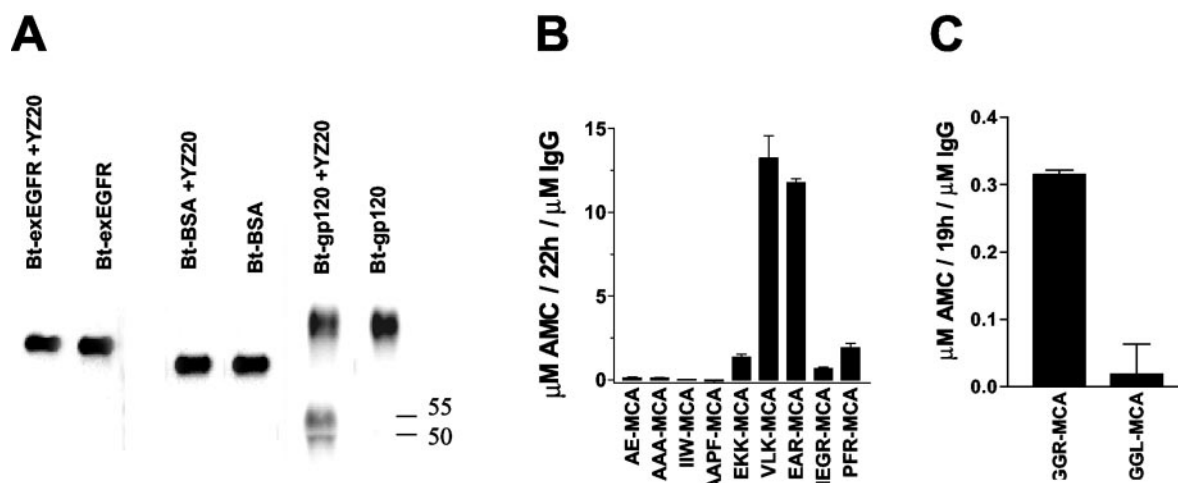


FIG. 5. **Lack of cleavage of Bt-bovine serum albumin (BSA) and Bt-exEGFR by mAb YZ20 (A) and preferential cleavage at basic residues (B and C).** A, streptavidin peroxidase-stained blots of biotinylated proteins (0.2 μM) incubated with mAb YZ20 (1 μM , 24 h). B, fluorimetric determination of mAb YZ20 (0.5 μM) catalyzed cleavage of peptide-MCA (AMC) substrates (200 μM , 22 h). C, cleavage of GGR-MCA and GGL-MCA by mAb YZ20 (0.5 μM). Concentration of both substrates was held at 12.5 μM because of limited solubility of Gly-Gly-Leu-MCA. Blocking groups at the N termini of the substrates were: succinyl, AE-MCA, AAA-MCA, AAPF-MCA, IIV-MCA; *t*-butyloxycarbonyl, EKK-MCA, VLK-MCA, IEGR-MCA, EAR-MCA; benzyloxycarbonyl, GGR-MCA, GGL-MCA. Values are the means of three replicates \pm S.D.

tration, because Bt-gp120 contains minimal amounts of biotin (~ 1 mol/molgp120), and the polyclonal Abs used for immunoblotting do not react equivalently with the cleavage products.

mAb YZ20 did not cleave biotinylated bovine serum albumin or the extracellular domain of the epidermal growth factor (exEGFR), indicating selectivity for gp120 (Fig. 5A). Attempts to identify the bonds cleaved by mAb YZ20 were unsuccessful. N-terminal sequencing of the 55- and 50-kDa bands yielded identical sequences (TEKLWVTVYY), corresponding to the N-terminal residues of gp120. Sequencing of the 15-kDa band from the YZ20 reaction mixture did not yield detectable phenylthiohydantoin derivatives of amino acids, possibly because of a blocked N terminus. Identification of the 27-kDa gp120 fragment is complicated because of its comigration with the Ab light chain in reducing gels. Because identification of the precise bonds in gp120 cleaved by the mAb was not central to the present study, we turned to the use of model peptide substrates for determination of scissile bond preferences. A fluorimetric assay was employed to determine mAb-catalyzed cleavage of the amide bond linking aminomethylcoumarin to the C-terminal amino acid in a panel of peptide-MCA substrates (Fig. 5B). The peptide-MCA substrates were used at excess concentration (200 μM), permitting detection of even weakly cross-reactive catalytic Abs. Selective cleavage at Arg-MCA and Lys-MCA was observed, with no evident cleavage on the C-terminal side of neutral or acidic residues. To confirm that the rate differences are because of recognition of the basic residue at the cleavage site (as opposed to remote residues), we studied two tripeptide substrates identical in sequence except for the N-terminal residue at the scissile bond, Gly-Gly-Arg-MCA and Gly-Gly-Leu-MCA. The former substrate was cleaved at detectable levels by Ab YZ20 (0.31 ± 0.01 (S.D.) μM aminomethylcoumarin/19 h/ μM IgG), whereas the fluorescence intensity in reaction mixtures of the latter substrate and the Ab was statistically indistinguishable from background values observed in assay diluent (0.02 ± 0.04 μM MCA/19 h/ μM IgG; $p > 0.05$; student's *t* test, unpaired; Fig. 5C). The basic residue preference is consistent with the presence of positively charged amidino groups neighboring the phosphonate groups in the immunogen (III) and selective cleavage on the C-terminal side of Arg/Lys residues by germ line-encoded proteolytic Abs observed previously (21, 22).

Attainment of the desired catalytic properties, *i.e.* the ability

TABLE I
Kinetic parameters for cleavage of Bt-gp120 and Boc-EAR-MCA by MAb YZ20

IgG (1 μM) was incubated with Bt-gp120 (0.14–2.2 μM ; 13 h) or Boc-EAR-MCA 31 (1000 μM , 6 h). Cleavage of Bt-gp120 was determined by measuring depletion of the 120-kDa intact protein band on SDS-gels run in duplicate and of EAR-MCA by fluorimetry in triplicate. Kinetic parameters for Bt-gp120 cleavage were computed using the general quadratic equation describing a one-site binding interaction and, for EAR-MCA, by fitting the data to the Michaelis-Menten equation (see "Materials and Methods").

Antigen	K_m	k_{cat}	k_{cat}/K_m
	M	min^{-1}	$M^{-1} \text{min}^{-1}$
Bt-gp120	2.0×10^{-6}	$3.4 \pm 0.1 \times 10^{-3}$	1.7×10^3
EAR-MCA	$4.0 \pm 1.2 \times 10^{-4}$	$3.3 \pm 0.4 \times 10^{-2}$	8.4×10^1

to combine high affinity for individual antigens with rapid turnover, can be judged from the K_m and k_{cat} parameters (mol of antigen cleaved/mol of Ab/unit time). The K_m of mAb YZ20 for Bt-gp120 was about 200-fold smaller than its preferred peptide-MCA substrate (EAR-MCA; Table I; single letter code for amino acids), consistent with development of specificity for gp120 by immunization with III. Twelve mol of EAR-MCA were cleaved per mol of mAb YZ20 over the course of the reaction (22 h), indicating that the mAb is capable of turnover, a defining feature of a catalyst. Turnover of Bt-gp120 was ~ 10 -fold lower than that of EAR-MCA. Previously, conventional non-Ab serine proteases were reported to cleave short peptide more rapidly than large proteins (23), presumably because the former substrates are more readily accessible to the catalytic site.

Nucleophilic Reactivity—gp120 hydrolysis by mAb YZ20 was inhibited by hapten CRA II (Fig. 6), confirming the serine protease-like character of the mAb. II inhibition of mAb YZ20 cleavage of gp120 was 90-fold more potent than inhibition of mAb c23.5 cleavage of VIP (IC_{50} , 0.4 and 36.0 μM , respectively). The latter mAb was obtained by immunization with VIP devoid of phosphonate diester groups (3). Superior reactivity of the hapten CRA with mAb YZ20 is consistent with the conclusion of strengthened Ab nucleophilicity in response to immunization with phosphonate groups present in the gp120-CRA immunogen.

To confirm induction of nucleophilicity, irreversible hapten CRA I binding by polyclonal IgG was measured. The hapten

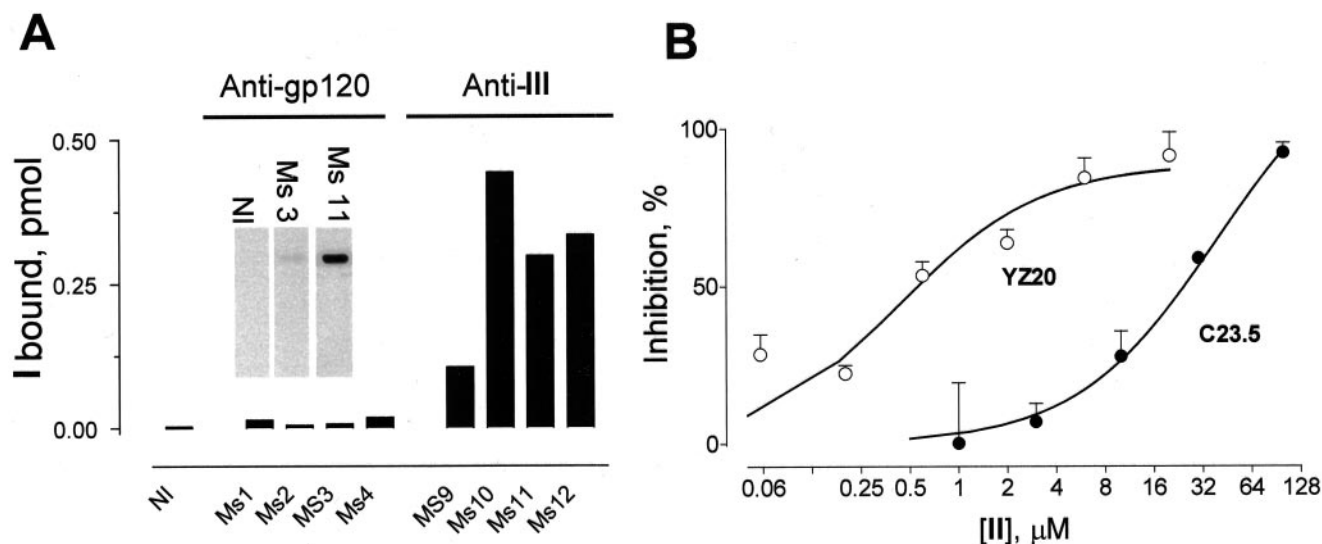


FIG. 6. Enhanced hapten CRA I covalent binding by polyclonal IgG from mice immunized with III (A) and potent inhibition of mAb YZ20 cleavage of Bt-gp120 by hapten CRA II (B). A, binding of hapten CRA I ($10 \mu\text{M}$), determined by incubation with IgG ($0.4 \mu\text{M}$) from BALB/c mice immunized with III (Ms9–12) or control gp120 (Ms1–4) for 60 min, SDS-electrophoresis, and quantification of the biotin-containing band at 150 kDa. NI, nonimmune IgG (pooled from 20 mice). Inset, representative SDS-electrophoresis lanes showing hapten CRA I binding by IgG from a mouse immunized with III (Ms11), a mouse immunized with gp120 (Ms3), and nonimmune IgG (NI). B, Bt-gp120 ($0.1 \mu\text{M}$) cleavage by mAb YZ20 ($1 \mu\text{M}$, 4 h) and [^{125}I]VIP ($\sim 100 \text{ pM}$, 45,000 cpm) cleavage by mAb c23.5 (20 nM , 18 h) were measured in the presence of increasing III concentrations. In the absence of III, 15 and 40% of available Bt-gp120 and VIP, respectively, were cleaved.

CRA does not contain antigenic epitopes belonging to gp120, and noncovalent binding interactions are not anticipated to contribute to its irreversible binding by Abs. IgG samples from all four mice immunized with III displayed superior I binding compared with IgG from mice immunized with control gp120 (mean values, 0.31 and 0.01 pmol I; $p < 0.02$, Student's t test, unpaired observations) as well as pooled nonimmune IgG (Fig. 6). BALB/c mice were studied in this immunization. It may be concluded that synthesis of nucleophilic Abs in response to immunization with III is not restricted to autoimmune hosts (mAbs to gp120-CRA III were prepared from MRL/lpr mice).

DISCUSSION

The goal of this study was to strengthen the intrinsic serine protease-like reactivity of Abs and direct the reactivity to cleavage of gp120. Improved irreversible binding of hapten CRA by Abs after immunization with gp120-CRA III was evident, and the hapten CRA was a potent inhibitor of gp120 cleavage by a mAb. These observations suggest adaptive improvement of Ab nucleophilicity induced by the phosphonate diester groups. Specificity of the Abs for gp120 was obtained by traditional noncovalent mechanisms, *i.e.* recognition of gp120 epitopes located in the proximity of the phosphonate diester groups. No cleavage of unrelated proteins by the gp120-cleaving mAb was observed, and the K_m value of cleavage of a model peptide was 200-fold greater than of gp120 cleavage, indicating the absence of indiscriminate proteolysis.

Proteolysis entails Ab attack on the backbone of gp120, whereas the phosphonate electrophiles are located in Lys side chains of the immunogen. Because mAbs raised to gp120-CRA displayed proteolytic activity, the nucleophile developed to recognize the side chain electrophiles must enjoy sufficient conformational freedom to attack the polypeptide backbone. Movements of individual amino acids in Ab combining sites after binding to antigen have been reported (24, 25). Epitope mapping and mutagenesis studies of certain proteolytic Abs indicate that the catalytic residues do not participate in stabilization of the Ab-antigen ground state complex (26, 27), suggesting that the mobility of the nucleophile may not be restricted by initial noncovalent Ab-antigen interactions. Nat-

urally occurring mAbs to VIP (17) and gp41 (4) cleave multiple peptide bonds in these antigens, which may be explained by hypothesizing formation of alternate transition states in which the nucleophile is free to initiate nucleophilic attack on spatially neighboring peptide bonds (for review, see Ref. 28). Understanding the extent of conformational freedom of Ab nucleophiles is important, because there is no viable alternative to locating the peptide bond mimetic in the side chains when large proteins must be used to induce the synthesis of catalytic Abs. In addition to direct structural analysis of nucleophile movements in available catalytic Abs, the length and flexibility of the linker utilized to attach the phosphonate groups at Lys side chains can be varied in future studies to assess the flexibility of the catalytic site. In the case of synthetic peptide immunogens, the phosphonate groups can be incorporated within the peptide backbone to better mimic the intended scissile bond (7). However, synthetic peptides often fail to assume conformations similar to their cognate determinants in full-length proteins, in which case anti-peptide Abs do not recognize the parent proteins.

The fully competent catalytic machinery found in modern non-Ab serine proteases has presumably evolved in response to selection pressures that optimized each of the rate-limiting steps in the catalytic cycle. In comparison, a CRA immunogen can at best select for Abs with the greatest covalent attack capability. No selection for hydrolysis of the acyl-Ab complex or the subsequent product release steps is anticipated, which may account for observations of limited Ab turnover. Two previous attempts to raise esterase Abs indicated the formation of irreversible substrate binding by Abs (29, 30), suggesting the need to optimize events occurring after nucleophilic attack by the Abs. Furthermore, structural refinements of the immunogen could be implemented to help guide the Ab-antigen complex toward the catalytic pathway, *e.g.* inclusion of a component that binds a water molecule and facilitates hydrolysis of the acyl-protein complexes. Notwithstanding this weakness, the results reported here represent a significant advance toward developing antigen-specific proteolytic Abs. Previously, several Abs with haptenic ester-hydrolyzing activity have been raised

based on the premise that catalytic sites capable of noncovalent stabilization of the oxyanionic transition states can be formed *de novo* over the course of adaptive sequence diversification of Ab variable domains (8, 9). This approach has not been successful for development of proteolytic Abs. Pollack *et al.* (31) describe the failure of a phosphonate monoester analog of Phe-Leu-Ala to induce proteolytic Ab synthesis. No attempt was made in this study to recruit the intrinsic properties of natural Abs for the purpose of protease synthesis, *i.e.* their nucleophilicity and selective recognition of basic residues adjacent to the cleavage site. Recently, phosphonate monoesters were discovered to form covalent bonds with nucleophiles in serine proteases, but their reactivity is weaker than the diester used in the present study, and no detectable reaction occurs unless an adjacent positive charge is present (6, 12).

Evidence for increased potency because of the catalytic function has recently been published in regard to Ab antagonism of the biological effects of VIP, a 28-amino acid neuropeptide (32, 33). Concerning gp120, a major hurdle has been to induce the synthesis of Abs that recognize the determinants involved in viral entry, *i.e.* the binding sites for host CD4 and chemokine receptors. Most Abs raised to monomer gp120 are directed to its variable region epitopes, and the Abs do not neutralize diverse HIV-1 strains found in different geographical locations (34). Reversibly binding Abs must bind at or near the receptor binding sites of gp120 to sterically hinder HIV entry into host cells. Proteolytic Abs offer the potential advantage of gp120 inactivation even if cleavage occurs at a site that does not itself participate in binding to host cells. Discussion of the immunotherapeutic potential of mAbs to gp120-CRA III is beyond the scope of the present study, but initial HIV-1 neutralization studies suggest that certain mAbs raised to gp120-CRA III neutralize the HIV-1 primary isolate ZA009 (peripheral blood mononuclear cell cultures; infection was measured by determining p24 antigen concentrations).² A potential pitfall is that proteolytic Abs to monomer gp120-CRA may not recognize trimeric gp120 on the surface of HIV-1, as observed for reversibly binding Abs to the protein (35). The CRA immunogen techniques described in the present study are readily applicable to recently developed recombinant mimetics of trimeric gp120 (36) as well as whole HIV-1 particles.

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REFERENCES

1. Tramontano, A., Golobov, G., and Paul, S. (2000) in *Chemical Immunology: Catalytic Antibodies* (Paul, S., ed) Vol. 77, pp. 1–17, S. Karger GmbH, Basel,

- Switzerland
2. Lacroix-Desmazes, S., Moreau, A., Sooryanarayana-Bonnemain, C., Stieltjes, N., Pashov, A., Sultan, Y., Hoebeke, J., Kazatchkine, M. D., and Kaveri, S. V. (1999) *Nat. Med.* **5**, 1044–1047
3. Paul, S., Sun, M., Mody, R., Tewary, H. K., Stemmer, P., Massey, R. J., Gianferrara, T., Mehrotra, S., Dreyer, T., Meldal, M., and Tramontano, A. (1992) *J. Biol. Chem.* **267**, 13142–13145
4. Hifumi, E., Okamoto, Y., and Uda, T. (1999) *J. Biosci. Bioengin.* **88**, 323–327
5. Nossal, G. J. (2002) *Immunol. Rev.* **185**, 15–23
6. Paul, S., Tramontano, A., Golobov, G., Zhou, Y. X., Taguchi, H., Karle, S., Nishiyama, Y., Planque, S., and George, S. (2001) *J. Biol. Chem.* **276**, 28314–28320
7. Taguchi, H., Burr, G., Karle, S., Planque, S., Zhou, Y. X., Paul, S., and Nishiyama, Y. (2002) *Bioorg. Med. Chem. Lett.* **12**, 3167–3170
8. Tramontano, A., Janda, K. D., and Lerner, R. A. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 6736–6740
9. Schultz, P. G., and Lerner, R. A. (1995) *Science* **269**, 1835–1842
10. Wagner, J., Lerner, R. A., and Barbas, C. F., III (1995) *Science* **270**, 1797–1800
11. Zhou, G. W., Guo, J., Huang, W., Fletterick, R. J., and Scanlan, T. S. (1994) *Science* **265**, 1059–1064
12. Nishiyama, Y., Taguchi, H., Luo, J. Q., Zhou, Y. X., Burr, G., Karle, S., and Paul, S. (2002) *Arch. Biochem. Biophys.* **402**, 281–288
13. Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., and Weigle, M. (1972) *Science* **178**, 871–872
14. Green, N. M. (1965) *Biochem. J.* **94**, 23–24
15. Brown, P. M., Debanne, M. T., Grothe, S., Bergsma, D., Caron, M., Kay, C., and O'Connor-McCourt, M. D. (1994) *Eur. J. Biochem.* **225**, 223–233
16. Karle, S., Nishiyama, Y., Zhou, Y. X., Luo, J., Planque, S., Hanson, C., and Paul, S. (2003) *Vaccine* **21**, 1213–1218
17. Sun, M., Gao, Q. S., Kirmarskiy, L., Rees, A., and Paul, S. (1997) *J. Mol. Biol.* **271**, 374–385
18. Bangale, Y., Karle, S., Zhou, Y. X., Lan, L., Kalaga, R., and Paul, S. (2003) *FASEB J.* **17**, 628–635
19. Tawfik, D. S., Chap, R., Green, B. S., Sela, M., and Eshhar, Z. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 2145–2149
20. Sun, J., Takahashi, N., Kakinuma, H., and Nishi, Y. (2001) *J. Immunol.* **167**, 5775–5785
21. Kalaga, R., Li, L., O'Dell, J. R., and Paul, S. (1995) *J. Immunol.* **155**, 2695–2702
22. Golobov, G., Sun, M., and Paul, S. (1999) *Mol. Immunol.* **36**, 1215–1222
23. Noda, Y., Jujiwara, K., Yamamoto, K., Fukuno, T., and Segawa, S. I. (1994) *Biopolymers* **34**, 217–226
24. Jimenez, R., Salazar, G., Baldrige, K. K., and Romesberg, F. E. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 92–97
25. Braden, B. C., and Poljak, R. J. (1995) *FASEB J.* **1**, 9–16
26. Gao, Q. S., Sun, M., Rees, A., and Paul, S. (1995) *J. Mol. Biol.* **253**, 658–664
27. Paul, S., Volle, D. J., Powell, M. J., and Massey, R. J. (1990) *J. Biol. Chem.* **265**, 11910–11913
28. Paul, S. (1996) *Mol. Biotechnol.* **5**, 197–207
29. Rao, G., and Philipp, M. (1991) *J. Protein Chem.* **10**, 117–122
30. Lefevre, S., Debat, H., Thomas, D., Friboulet, A., and Avalle, B. (2001) *FEBS Lett.* **489**, 25–28
31. Pollack, S. J., Hsiun, P., and Schultz, P. G. (1989) *J. Am. Chem. Soc.* **111**, 5961–5962
32. Berisha, H. I., Bratut, M., Bangale, Y., Colasurdo, G., Paul, S., and Said, S. I. (2002) *Pulm. Pharmacol. Ther.* **15**, 121–127
33. Voice, J. K., Grininger, C., Kong, Y., Bangale, Y., Paul, S., and Goetzl, E. J. (2003) *J. Immunol.* **170**, 308–314
34. Moore, J., and Trkola, A. (1997) *AIDS Res. Hum. Retroviruses* **13**, 733–736
35. Kwong, P. D., Doyle, M. L., Casper, D. J., Cicala, C., Leavitt, S. A., Majeed, S., Steenbeke, T. D., Venturi, M., Chaiken, I., Fung, M., Katinger, H., Parren, P. W., Robinson, J., Van Ryk, D., Wang, L., Burton, D. R., Freire, E., Wyatt, R., Sodroski, J., Hendrickson, W. A., and Arthos, J. (2002) *Nature* **420**, 678–682
36. Kwong, P. D., Wyatt, R., Sattentau, Q. J., Sodroski, J., and Hendrickson, W. A. (2000) *J. Virol.* **74**, 1961–1972

² S. Paul, S. Karle, and C. Hanson, unpublished information.