Antibody (Ab) nucleophilic reactivity was studied using hapten and polypeptide antigens containing biotinylated phosphate diester groups (covalently reactive antigen analogs, CRAs). Polyclonal IgG from healthy donors formed covalent adducts with a positively charged hapten CRA at levels superior to trypsin. Each of the 16 single chain Fv clones studied expressed a similar reactivity, indicating the V domain location of the nucleophiles and their broad distribution in diverse Abs. The formation of hapten CRA-Fv adducts was correlated with Fv proteolytic activity determined by cleavage of a model peptide substrate. Despite excellent nucleophilicity, proteolysis by IgG proceeded at lower rates than trypsin, suggesting that events occurring after nucleophilic attack on the substrate limit the rate of Ab proteolysis. The extracellular domain of the epidermal growth factor receptor with phosphate diester groups at Lys side chains and a synthetic peptide corresponding to residues 421–431 of human immunodeficiency virus glycoprotein (gp) 120 with the phosphate diester at the C terminus formed covalent adducts with specific polyclonal and monoclonal Abs raised by immunization with epidermal growth factor receptor and synthetic gp120-(421–436) devoid of phosphate diester groups, respectively. Adduct formation was inhibited by extracellular domain of the epidermal growth factor receptor (exEGFRβ) and synthetic gp120-(421–436) devoid of phosphate groups, suggesting that the nucleophiles are located within the antigen binding sites. These results suggest the innate character of the Ab nucleophilic reactivity, its functional coordination with non-covalent adaptive binding interactions developing over the course of B cell maturation, and novel routes toward permanent inhibition of Abs.

Many enzymes exploit covalent interactions with substrates to catalyze chemical transformations. On the other hand, most studies on Ab catalysis have focused on non-covalent binding forces as the mechanism by which the energy barrier between reactants and products is lowered, e.g., the electrostatic forces that stabilize the negatively charged oxyanionic transition state of ester hydrolysis (reviewed in Refs. 1 and 2). The underlying assumption has been that Abs interact with their ligands exclusively by non-covalent means. Initial indications that natural Abs express chemical reactivity indistinguishable from enzymes came from reports of proteolytic and nuclease activity of autoantibodies (3, 4). Similar activities were later found in Ab light chains from multiple myeloma patients (5), alloantibodies from patients with transfusion-induced hemophilia (6), Abs raised by routine immunization with polypeptides (7, 8), and anti-idiotypic Abs to anti-enzyme Abs (9). From mutagenesis and inhibitor studies, it appears that the proteolytic activity of natural Abs originates from nucleophilic mechanisms similar to those utilized by conventional serine proteases (10, 11). The catalytic activity of natural Abs could be construed to violate the principles of B cell clonal selection theory. Antigen-specific Abs develop over the course of the immune response by sequence diversification of germ line genes encoding the V domains followed by selective antigen binding by B cell receptors with the greatest affinity, which stimulates clonal proliferation. Catalysis entails chemical transformation of the antigen and release of products (surface immunoglobulins associated with Igα and Igβ subunits), which is predicted to result in the cessation of B cell selection. Therefore, adaptive selection of Ab catalytic activity may be a disfavored event. For this reason, catalysis by naturally occurring Abs is often assumed to be a molecular accident arising from sequence variability of the V domains as opposed to a general phenomenon with functional implications.

The foregoing restrictions do not apply to the initial step in the catalytic cycle of serine proteases. In analogy with conventional enzymes, a nucleophile belonging to a proteolytic Ab (Fig 1A, Nu) is conceived to initiate nucleophilic attack on the antigen following the formation of the non-covalent ground state complex. Adaptive selection of Ab catalytic activity is fully compatible with B cell clonal selection if the outcome is the formation of a covalent acyl-Ab complex as occupancy of the B cell receptor will be maintained. Whether the catalytic cycle is completed depends on the efficiency of hydrolysis of the acyl-Ab complex and release of the product. Recently, hapten phosphonate esters have been developed as probes for covalent binding to the active site nucleophiles in Abs displaying serine
protease and serine esterase activity (11, 12) (designated CRAs). These compounds can be applied for direct study of Ab chemical activity independent of additional activities needed for accomplishment of catalysis. In addition, the phosphonates can be placed within peptides and proteins (Fig. 1, B and C) for studying the interplay between Ab nucleophilic reactivity and non-covalent forces permitting specific recognition of individual polypeptides.

We describe here observations suggesting the broad distribution of nucleophilic reactivity in IgG and recombinant Fv preparations at levels exceeding that of the conventional serine protease trypsin. Originally prepared as probes for catalytic Abs, CRA analogs of EGFFR and an HIV gp120-synthetic peptide were observed to form covalent adducts with ordinary Abs raised by immunization with antigens devoid of the phosphonate groups, suggesting that adaptive maturation processes favor the expression of nucleophilic reactivity. These observations argue for Ab nucleophilicity as a force responsible for shaping the expressed Ab repertoire and suggest novel routes toward permanent inactivation of Abs.

MATERIALS AND METHODS

Abs—Human polyclonal IgG was prepared by affinity chromatography on protein G-Sepharose (Amersham Biosciences) from the sera of six healthy human subjects (laboratory codes 1086, 1087, 1088, 1091, 1092, and 1518). IgG from pooled serum from eight BALB/c mice (4–6 weeks) was obtained similarly. Preparation of polyclonal Abs by hyperimmunization with synthetic Cys-gp120 (421–436) (KQINMQVQVKAMYA, residues 421–436 of gp120 HIV SF2 strain) conjugated to keyhole limpet hemocyanin is described by Karle et al. (13). Polyclonal Abs to exEGFR were raised by immunizing female BALB/c mice (5–6 weeks) intraperitoneally with exEGFR (10 µg/injection) on days 0, 27, and 49 in RIBI™ adjuvant and with AS151 tumor cells (105 cells in saline) on day 14. Monoclonal Abs to exEGFR (clones C225, H11, and C111.6) were purchased from Labvision (Fremont, CA). A control monoclonal anti-BSA IgG (clone BGN/H8) was from Biogenesis (Dr. O'Connor-McCourt) (15) was reacted with 6-biotinamidohexanoic acid N-hydroxysuccinimide ester (50 nmol, Sigma) in 0.53 ml of 10 mM HEPES, 150 mM NaCl, 0.1 mM CHAPS, pH 7.5, buffer (50 min, 25 °C). Unreacted biotinylation reagent was removed by gel filtration (Micro Gel-Superdex 75; Bio-Rad) followed by exEGFR (0.33 mg/ml) then reacted with compound b (136 nmol) in 3.3-mM buffer for 2 h. Following the removal of excess compound b by gel filtration in 50 mM Tris-HCl, 100 mM glycine, 0.1 mM CHAPS, pH 7.8, the concentration of free amines in the initial and CRA-dervitized proteins was measured using fluorescamine (16). Biotin content determined using 2-4′-hydroxyazobenzene-benzoic acid (17) was 1.1 mol/mol exEGFR. The density of phosphonate diester labeling was 19 mol/mol exEGFR. Total protein was measured using BCA (Pierce). Some experiments were done using exEGFR CRA IVa. This compound is identical to CRA IV but for the presence of a disulfide bond in linker. To prepare CRA IVa, the precursor diphenyl-N-(3-sulfosuccinimidyl)-3,3′-dithiodiisopropylphosphinylaminomethane phosphonate (compound c) was synthesized as described for compound b using 3,3′-dithiobis(sulfosuccinimidyl-propionate) (Pierce) (yield 6.0 mg, 21.4%; tR 24.49 min, >98 purity; 20–50% acetonitrile in 0.1% trifluoroacetic acid, 60 min; m/z 751 (MH + )). Labeling with biotin and compound c was as described for CRA IV (biotin and phosphonate diester content of CRA IVa, respectively, 2.3 mol and 18.3 mol/mol exEGFR). Synthesis of peptidyl-CRAs V and Va and their chemical characterization are described by Taguchi et al. (18). CRA V was conjugated with BSA using γ-maleimidobutryric acid N-hydroxysuccinimide ester as described previously (13). BSA was pre-treated with diphenyl-N-(benzoyloxycarbonyl)aminomethane phosphonate (BSA, 21.3 µmol; phosphonate, 0.5 mM; solv; 10 mM phosphate (pH 7.4), containing 5% acetonitrile; 15.5 h) to biotin binding sites. CRA VBSA molar ratio was 3.9 determined from consumption of ~SH groups using Ellman’s reagent. The storages of CRAS I–III was at 70 °C as 10 mM solutions in N,N-dimethylformamide. CRA IV and IVa were stored at ~70 °C in 50 mM Tris-HCl, pH 8.0, 0.1 mM glycine, 0.1 mM CHAPS. CRAs V and Va were stored at ~70 °C as 10 mM solutions in N,N-dimethylformamide.

ELISA—Maxisorp 96-well microtiter plates (Nunc) were coated with gp120-(421–436) conjugated to BSA (20-ng peptide equivalent/well for details regarding peptide conjugation method see Ref. 13). CRA V was conjugated to BSA (20 ng of peptide-CRA equivalent/well), exEGFR (200 ng/well), or exEGFR-CRA V (200 ng of protein equivalent/well) in 100 mM sodium bicarbonate buffer (pH 8.6) for 2 h. Plates were essentially as described previously (13). Bound murine IgG was detected with goat anti-mouse IgG-horseradish peroxidase conjugate (1:1000, specific for the constant domain of the heavy chain, Sigma).

Irreversible CRA Binding—Following incubation of biotinylated CRAs with Abs or trypsin (porcine, type IX, Sigma) in 50 mM Tris-HCl, 100 mM glycine, 0.1 mM CHAPS, pH 7.7, at 37 °C, the reaction mixtures were boiled (5 min) in 2% SDS and subjected to SDS-PAGE (4–20%, Bio-Rad, or 8–25% Phast gels, Amersham Biosciences). Electrobloot blotting and biotin detection procedures using streptavidin-horseradish peroxidase and a chemiluminescent substrate (Supersignal, Pierce) have been described previously (11). Imaging and quantification was on x-ray film (XAR-5; Kodak Co.) using a radiographic light box (UVP, UT) or Fluoro-STM Multimager (Bio-Rad). Band intensities are expressed in arbitrary unit areas (AAU). Valid comparisons of band intensities from different experiments is not possible because exposure and development times were not held constant. Disopropyl fluorophosphate (DFP) (Sigma) was kept at 4 °C until used. In some experiments, purified and biotinylated BSA (8 mol of peptide-CRA equivalent/mol protein, Pierce) was electrophore- sed at several concentrations in parallel with the samples and the biotin content of the CRA adducts was determined. Pseudo-first order rate constants (kobs) were computed from reaction progress curves by fitting to the equation B − Bmax(1 − exp(−kobs t)), where B represents adduct concentration at various times and Bmax represents the initial Ab concentration.

Immunoblotting with goat anti-exEGFR IgA Abs was as described previously (10).

Proteolysis Assay—Catalytic activity was measured by fluorometric determination (λex 360 nm, λem 470 nm; Varian Cary Eclipse) of the cleavage of amide bond linking amidinomethylcoumarin to the C-termi-
nal amino acid in short peptide-MCA substrates (Pro-Phe-Arg-MCA, Boc-Glu-Ala-Arg-MCA, and Boc-Ile-Glu-Ala-Arg-MCA (200 μM), Peptide International) (10). Catalysts were incubated with peptide-MCA substrates in 50 mM Tris HCl, 0.1 M glycine, 0.025% Tween 20, pH 8.0, at 37 °C in 96-well plates. In some assays, a comparison of IgG and trypsin proteolytic activity was done in 10 mM sodium phosphate, pH 7.4, 0.137 M NaCl, 2.7 mM KCl, 0.1 mM CHAPS. Authentic aminometh-ylcoumarin (Peptide International) was used to construct a standard curve from which product release was computed in molar values.

RESULTS

Ab Nucleophilicity Identified with Hapten CRAs—Phosphonate hapten CRAs I-III (Fig. 1) are analogs of known active site-directed inhibitors of serine proteases (19). Similar to the serine protease trypsin, IgG from a healthy human subject formed adducts with CRA I that were resistant to boiling and the denaturant SDS (Fig. 2, IgG, 150-kDa adducts; trypsin, 21-kDa adducts). Pooled IgG from immunologically unmanipulated BALB/c mice formed similar CRA I adducts. The positively charged amidino group in CRA I was originally incorporated in this compound to allow selective recognition of trypsin, which displays preference for basic residues at the P1 site (the residue immediately adjacent to the cleavage site in peptide substrates) (for review see Ref. 20). CRA II lacks the positively charged amidino group adjacent to the covalently reactive phosphorus atom. IgG was 240-fold less reactive with CRA II than with CRA I, suggesting the trypsin-like P1 specificity of Abs. CRA III, which contains the weaker methoxy leaving group. CRAs IV and V are intended, respectively, to permit detection of nucleophiles in specific Abs to exEGFR and residues 421–431 of gp120. The biotin and phosphate diester groups were incorporated in CRA IV at Lys side chains. CRA Va contains biotin at the N terminus and the phosphate diester at the C terminus.
function of reaction time (Fig. 2B). The velocity of the reaction for IgG was 14.5-fold greater than for trypsin measured under identical conditions (172.7 ± 14.2 and 11.9 ± 0.6 AAU/min, respectively, from linear regression of Fig. 2B data). Assuming that hydrolysis of the phosphorylated-protein complex is equivalent (see reaction schemes in Fig. 1), it may be concluded that the nucleophilic efficiency of IgG is superior to that of trypsin.

IgG preparations from healthy humans and immunologically unmanipulated mice have been documented to cleave small model peptide substrates on the C-terminal side of basic residues. The cleavage activity was observed in each of several IgG preparations examined. The activity co-migrated with intact 150-kDa IgG in denaturing gel filtration studies, and it was expressed by Fab (fragment antigen binding) preparations made by papain digestion (22). In this study, we compared the proteolytic activity of trypsin and IgG from a healthy human subject (the same preparation as in nucleophilicity studies illustrated in Fig. 2). With Glu-Ala-Arg-MCA and Pro-Phe-Arg-MCA substrates, initial rates of proteolysis by IgG were, respectively, $1.8 \times 10^7$ and $6.8 \times 10^6$-fold smaller than by trypsin (Fig. 3, A and B, determined from the slopes of the progress curves). Glu-Ala-Arg-MCA is the preferred substrate for trypsin. Glu-Ala-Arg-MCA and Pro-Phe-Arg-MCA are the preferred substrates for human IgG determined from previous screening of a panel of peptide-MCA substrates (22). The magnitude of proteolysis by this IgG preparation falls within the range reported previously for other human IgG preparations. Despite its superior nucleophilic reactivity, the IgG is evidently a poor catalyst compared with trypsin.

CRA I and DFP (another active site-directed inhibitor of serine proteases) inhibited the catalytic activity of IgG-catalyzed peptide-MCA cleavage (Fig. 3C), and DFP inhibited the irreversible binding of CRA I by the IgG (by 95%). These results provide assurance that CRA I binds the catalytic sites of IgG. As DFP binds the active site of serine proteases, its inhibitory effect confirms the serine protease character of the CRA I binding sites of IgG. Electrophoresis of CRA I-IgG adducts under reducing conditions revealed labeling of both subunits by the hapten CRA, evident as biotin-containing bands at 50-kDa heavy chain bands and 25-kDa light chain bands (Fig. 3D). Irreversible CRA I binding activity of IgG was lost by preheating the protein at 60 °C for 10 min, indicating the dependence of the nucleophilic reactivity on the native protein conformation.

Each of the five polyclonal IgG preparations from healthy humans displayed irreversible binding to CRA I (Table I). Each of 16 randomly picked scFv clones from a human library formed CRA I-adducts (see example in Fig. 4A), indicating the V domain location of the binding site and suggesting that the nucleophilic reactivity is a shared property of diverse Abs. 91% of the total protein available in Fv MM-F4 shown in Fig 4A (GenBank accession number AF522073) displayed nucleophilic reactivity (computed as mol biotin/mol Fv protein in the 27-kDa CRA I adduct band; Fv valency 1; reaction conditions as shown in Fig. 4). Analyzed by electrophoresis under non-reducing conditions, some scFv reaction mixtures contained CRA I adducts at 55–90 kDa in addition to the monomer scFv adducts at 27 kDa. All of the CRA-adduct bands were also stainable with Ab to c-Myc, confirming the presence of scFv in the adducts (the recombinant proteins contain a 10-residue c-Myc peptide) (10). The tendency of scFv to form aggregates has been reported previously (23). Diminished levels of CRA I-adducts were detected when an scFv clone was treated with DFP prior to CRA I treatment (by 72%). The rate of covalent adduct formation by different Fv clones was variable over a 34-fold range (Table I), indicating distinct levels of nucleophilic reactivity of different Abs. The reactivity of the five polyclonal IgG samples, which represent mixtures of different Abs, was less variable (by 5.4). A comparison of the peptide-MCA cleaving activity (Glu-Ala-Arg-MCA substrate) and irreversible CRA I binding by the scFv clones indicated a strong correlation ($p < 0.005, r^2 = 0.77$) (Fig. 4B), confirming the functional importance of superior nucleophilic reactivity.

**Specific Covalent Binding of Peptidyl and Protein CRA**—
Protein CRA IV and peptide CRA Va were analyzed to assess whether antigen-specific Abs can express nucleophilic reactivity coordinated with non-covalent recognition of the antigen. CRA IV is the extracellular domain of a tumor-associated protein, exEGFR, presenting diverse antigenic epitopes derivitized at Lys side chains with the phosphonate diester group, 714 Da). CRA Va corresponds to residues 421–431 of the HIV coat protein gp120 along with the amidino surrogate of this peptidyl CRA has been reported previously (18). Abs raised by routine immunization with exEGFR and the synthetic peptide corresponding to residues 421–436 of gp120 were initially employed to assay the antigenic integrity of these CRAs. ELISA studies indicated that the binding of CRAs IV and Va (conjugated to BSA) by polyclonal Abs to exEGFR and synthetic gp120-(421–436), respectively, was only marginally lower than of the control antigens devoid of phosphate diester groups, i.e. exEGFR and gp120-(421–436), respectively (Fig. 5). Evidently, the epitope structure of the two antigens is
preserved despite the introduction of the phosphonate diester in Lys side chains (CRA IV) and at the C terminus (CRA Va).

No binding of anti-exEGFR or anti-gp120-(421–436) Abs to immobilized calmodulin and albumin was detected (A490/H11021 0.05 at antisera dilution 1:1000), confirming the absence of nonspecific protein binding effects. Immobilized CRA IV and CRA Va (conjugated to BSA) did not display unusual binding to non-immune Abs used as controls for ELISA, indicating that the phosphonate diester group does not result in indiscriminate covalent binding effects.

Covalent binding by the Abs was studied using denaturing electrophoresis as described for the hapten CRAs. Saturable formation of biotin-containing IV adducts with Abs to exEGFR was evident (nominal mass of 250 kDa). CRA IV adducts of

FIG. 3. Catalytic and hapten CRA I binding characteristics of human IgG (1518) and trypsin. A, time course of cleavage of PFA-MCA (Pro-Phe-Arg-MCA, 200 μM) (IgG, 500 nM; trypsin, 0.1 nM). B, time course of cleavage of EAR-MCA (Glu-Ala-Arg-MCA, 200 μM) (IgG, 500 nM; trypsin, 0.1 nM). C, DFP (5 mM) and CRA I (0.1 mM) inhibition of peptide-MCA (mixture of Glu-Ala-Arg-MCA, Pro-Phe-Arg-MCA, and Ile-Glu-Gly-Arg-MCA, 67 μM each) cleavage by IgG (375 nM) and trypsin (1 nM), respectively, 21- and 1.5-h reaction. D, representative streptavidin-peroxidase-stained blots of reducing SDS-polyacrylamide gels showing inhibition of CRA I (10 μM) adduct formation by DFP (5 mM) and preheating of the proteins for a 10-min (IgG, 1 μM; trypsin, 1 μM), 1-h reaction. Treatment with DFP for 30 min prior to incubation with CRA I. Values in A–C are means of three replicates ± S.D.

TABLE I

| Antibody       | Hapten I-irreversible binding, AAU × 10^5 | N   |
|----------------|------------------------------------------|-----|
| Human serum    | 33.7 ± 20.4                              | 5   |
| IgG            | 928 ± 688                                | 16  |
| scFv           | 1050                                     | 16  |
|                | 55–1900                                  |     |

FIG. 4. Single chain Fv reactivity with hapten CRA I (A and B) and correlation with proteolysis (B). A, reducing SDS-electrophoresis gels showing Fv (clone MM-F4) adducts with CRA I stained with streptavidin-peroxidase (lane 2), anti-c-Myc antibody (lane 3), and silver (lane 4). Lane 1, standard proteins used for gel calibration. For the reaction in lane 2, Fv (0.45 μM) was treated with CRA I (200 μM; 60-min reaction). The minor c-Myc-containing band in lane 2 is a degradation product that co-purifies with full-length Fv on the nickel column as it contains the His6 tag. B, values for cleavage of EAR-MCA (Glu-Ala-Arg-MCA, γ axis; 200 μM; 17-h reaction time) and binding of CRA I (200 μM; 60 min) by purified Fv from eight clones (MM 18, 20, 24, F4, F5, F6, F11, and F14). Correlation was assessed by linear regression (183 FU, 1 μM aminomethylcoumarin). Dotted lines, 95% confidence limits; FU, fluorescence units.

non-immune IgG were not detectable (Fig. 6). Because CRA IV concentration is small (0.2 μM in Fig. 6), the formation of adducts similar to those observed using hapten CRA I is not predicted (Fig. 2, 100 μM CRA I). Few or no adducts were formed in the presence of exEGFR (1 μM), but adduct formation
was not impeded by an equivalent concentration of calmodulin, indicating that the covalent binding reaction is at or near the antigen binding site of the Abs. The 250-kDa CRA IV adducts were stainable with anti-IgG (data not shown). Each of three commercially available monoclonal Abs to exEGFR formed covalent adducts with CRA IVa (according to the suppliers, Ab C225 binds residues 351–364 in the extracellular domain of EGFR; the linear peptide determinant recognized by Abs H11 and C111.6 is not known, but both Abs bind the extracellular domain of the protein), an irrelevant monoclonal Ab did not, and the formation of the adducts by the monoclonal Abs was inhibited by exEGFR devoid of phosphonate diester groups but not by the unrelated protein calmodulin. Essentially, similar results were obtained using CRA Va (Fig. 7). The formation of biotin-containing 152-kDa adducts was saturable as a function of time (mass of CRA Va, 2.2 kDa), adduct formation was inhibited by the gp120-(421–436)-BSA conjugate (3 μM) but not an equivalent concentration of BSA, and the reaction with non-immune IgG proceeded slowly compared with the specific Abs.

The pseudo-first order rate constant \( k_{obs} \) for accumulation of CRA IV adducts of polyclonal IgG to exEGFR was \( 1.0 \pm 0.1 \) h\(^{-1}\). Because no reaction was detected with non-immune IgG, a precise estimate of \( k_{obs} \) is not possible. Using the detection sensitivity of the imaging system as the upper limit for accumulation of adducts over the period of observation in Fig. 6 (133 AU), the upper limit for \( k_{obs} \) is \( 7.2 \times 10^{-3} \) h\(^{-1}\). Similarly, \( k_{obs} \) for accumulation of anti-peptide IgG adducts of CRA Va was 496-fold greater than that of non-immune IgG adducts (17.8 ± 3.3 h\(^{-1}\) and 0.4 × 10\(^{-1} \) ± 0.1 × 10\(^{-1} \) h\(^{-1}\), respectively) (see data in Fig. 7).

**DISCUSSION**

Activated nucleophilic residues in conventional serine proteases react covalently with phosphonate diester probes, e.g. the Ser residue activated by hydrogen bonding in the catalytic Ser-His-Asp triad of serine proteases. The presence of such nucleophiles in proteolytic and esterolytic Abs has been deduced from mutagenesis and covalent phosphate binding studies (10–12). Nucleophilic attack on the substrate is the rate-limiting step in catalysis by certain enzymes (24). As the reported catalytic rate constants (\( k_{cat} \)) of Abs are generally

**Fig. 5.** Antibody binding by phosphonate diester containing protein CRA IV and peptide CRA V determined by conventional ELISA procedures. A, comparison of binding of immobilized CRA IV by antiserum to exEGFR (■) and control non-immune serum (□). B, comparison of binding of immobilized CRA Va by antiserum to a gp120-(421–436)-keyhole limpet hemocyanin conjugate (■) and control non-immune serum (□). C, comparison of binding of immobilized exEGFR by anti-exEGFR antiserum. The pseudo-first order rate constant \( k_{obs} \) for accumulation of 250-kDa adducts of CRA IV was 1.0 ± 0.1 h\(^{-1}\). No adducts were formed by an equivalently treated control monoclonal Ab (BGN/H8). D, biotin-containing 250-kDa adducts formed by treatment of CRA IV (0.2 μM) for 2 h with polyclonal IgG to exEGFR (0.5 μM) in the absence (lane 1) and presence of exEGFR (1 μM; lane 2) or calmodulin (1 μM; lane 3). In control reactions, CRA IV (0.2 μM) was treated for 2 h with non-immune IgG (0.5 μM; lane 4) or boiled polyclonal IgG to exEGFR (0.5 μM, 10 min at 100 °C; lane 5). CRA IVa (0.2 μM) treated with monoclonal IgG c225 (0.5 μM; clones C225, H11, and 111.6) as a function of time. No adducts were formed by an equivalently treated control monoclonal Ab (BGN/H8). E, streptavidin-peroxidase-stained blot showing 250-kDa adducts formed by treating CRA IVa with polyclonal anti-EGFR IgG (0.7 μM) for 8 h. F, intensities of the 250-kDa band from panel A (in arbitrary area units). G, accumulation of 250-kDa biotin-containing adducts of CRA IV (0.2 μM) with monoclonal Abs to EGFR (0.5 μM; clones C225, H11, and 111.6) as a function of time. H, intensities of the 250-kDa band from panel G (in arbitrary area units). I, streptavidin-peroxidase-stained 152-kDa adducts in non-reducing SDS-electrophoresis gels formed with anti-gp120-(421–436) IgG (1 μM) and presence of albumin (3 μM peptide equivalents). J, streptavidin-peroxidase-stained 152-kDa adducts in non-reducing SDS-electrophoresis gels formed with anti-gp120-(421–436) IgG (1 μM; 1 h) in the absence of competitor proteins (lane 1) and presence of exEGFR (1 μM; lane 2) or calmodulin (1 μM; lane 3) and calmodulin (1 μM; lane 4) in the absence of competitor proteins (lane 1) and presence of exEGFR (1 μM; lane 2) or calmodulin (1 μM; lane 3) and calmodulin (1 μM; lane 4).

**Fig. 6.** Specific irreversible binding of exEGFR protein CRA IV by polyclonal and monoclonal Abs to EGRF. A, streptavidin-peroxidase-stained blot showing 250-kDa adducts formed by treating CRA IV (0.2 μM) with polyclonal anti-EGFR IgG (0.7 μM) for increasing lengths of time (0.05, 1, 2, 4, 6, and 8 h as shown in lanes 1–8, respectively). Lane 9, the reaction mixture of CRA IV (0.2 μM) incubated with control non-immune IgG (0.7 μM) for 8 h. B, intensities of the 250-kDa band from panel A (in arbitrary area units). C, accumulation of 250-kDa biotin-containing adducts of CRA IV (0.2 μM) with polyclonal Abs to EGFR (0.5 μM; clones C225, H11, and 111.6) as a function of time. No adducts were formed by an equivalently treated control monoclonal Ab (BGN/H8). D, biotin-containing 250-kDa adducts formed by treatment of CRA IV (0.2 μM) for 2 h with polyclonal IgG to exEGFR (0.5 μM) in the absence (lane 1) and presence of exEGFR (1 μM; lane 2) or calmodulin (1 μM; lane 3). In control reactions, CRA IV (0.2 μM) was treated for 2 h with non-immune IgG (0.5 μM; lane 4) or boiled polyclonal IgG to exEGFR (0.5 μM, 10 min at 100 °C; lane 5). CRA IVa (0.2 μM) treated with monoclonal IgG c225 (0.5 μM; clones C225, H11, and 111.6) as a function of time. No adducts were formed by an equivalently treated control monoclonal Ab (BGN/H8). E, streptavidin-peroxidase-stained 250-kDa adducts formed by treating CRA IVa with polyclonal anti-EGFR IgG (0.7 μM) for 8 h. F, intensities of the 250-kDa band from panel A (in arbitrary area units). G, accumulation of 250-kDa biotin-containing adducts of CRA IVa (0.2 μM) with polyclonal Abs to EGFR (0.5 μM; clones C225, H11, and 111.6) as a function of time. No adducts were formed by an equivalently treated control monoclonal Ab (BGN/H8). H, biotin-containing 250-kDa adducts formed by treatment of CRA IVa (0.2 μM) for 2 h with polyclonal IgG to exEGFR (0.5 μM) in the absence (lane 1) and presence of exEGFR (1 μM; lane 2) or calmodulin (1 μM; lane 3). In control reactions, CRA IVa (0.2 μM) was treated for 2 h with non-immune IgG (0.5 μM; lane 4) or boiled polyclonal IgG to exEGFR (0.5 μM, 10 min at 100 °C; lane 5). I, CRA IVa (0.2 μM) treated with monoclonal IgG c225 (0.5 μM; clones C225, H11, and 111.6) as a function of time. No adducts were formed by an equivalently treated control monoclonal Ab (BGN/H8). J, biotin-containing 250-kDa adducts formed by treatment of CRA IVa (0.2 μM) for 2 h with polyclonal IgG to exEGFR (0.5 μM) in the absence (lane 1) and presence of exEGFR (1 μM; lane 2) or calmodulin (1 μM; lane 3). In control reactions, CRA IVa (0.2 μM) was treated for 2 h with non-immune IgG (0.5 μM; lane 4) or boiled polyclonal IgG to exEGFR (0.5 μM, 10 min at 100 °C; lane 5).
orders of magnitude lower than those of enzymes, it has generally been assumed that the deficiency resides in the nucleophilic reactivity of Abs. Studies reported here indicate otherwise. Despite their low proteolytic activity, IgG preparations displayed stronger nucleophilic reactivity than trypsin determined from rates of the formation of covalent adducts with hapten phosphonate diesters. The study of polyclonal IgG and individual scFv clones indicated an apparently universal nucleophilic reactivity. In control experiments, the reactivity was lost upon thermal denaturation, consistent with expectations that activation of the nucleophile is dependent on the native structure of the protein. Covalent Ab binding to the phosphonate diester was inhibited by the established serine protease- and kinase (34) activities. Conceivably, nucleophilic Ab reactivity is unclear. In addition to protease and phosphonate diester hapten (32), but its relationship with in-

sider Abs to protein CRA IV and peptidyl CRA Va. We did not examine the presence of nucleophilic sites in the constant domains, as the present study was conducted in the context of catalytic activity attributed to the V domains. As the genes encoding the V and constant domains express certain sequence identities (27), the existence of constant domain nucleophiles can not be excluded. Notwithstanding their impressive nucleophilic reactivity, the rate of catalysis by Abs is limited. Presumably, this is because of energetic barriers associated with the deacylation and product release steps (Fig. 1). This statement does not conflict with observations of correlated proteolysis and nucleophilicity of the scFv clones, because increased accumulation of the acylated reaction intermediate will accelerate proteolysis according to the laws of mass action regardless of limitations at subsequent steps in the reaction cycle. In addition to proteases, diverse enzymes involved in chemical transformation of lipids, carbohydrates, and nucleic acids owe their catalytic power to covalent mechanisms (28–30). Some of these enzymes are reported to react with phosphonate probes (31). An aldolase Ab has been raised by immunization with a phosphonate diester hapten (32), but its relationship with innate Ab nucleophilicity is unclear. In addition to protease and esterase activities, Abs express nuclease (4), peroxidase (33), and kinase (34) activities. Conceivably, nucleophilic Ab reactivity described here may play a role in these reactions.

Specific polyclonal and monoclonal Abs to EGFR and synthetic gp120-(421–436) peptide displayed covalent binding to the CRA analogs of these antigens (CRAs IV and Va, respectively) at levels substantially greater than non-immune IgG, indicating that the nucleophiles express their reactivity in coordination with non-covalent antigen binding interactions. Therefore, non-covalent Ab-antigen binding may be interpreted as a mechanism that permits more efficient delivery of the electrophiles (phosphonate groups) to the Ab nucleophiles. The cognate antigens devoid of phosphonate diester groups inhibited the covalent reaction, suggesting spatial proximity be-

between the nucleophile and residues at which non-covalent bind-
taking takes place. The following conditions must be met to explain the experimentally observed antigen-specific formation of the CRA adducts. (a) The germ line-encoded nucleophiles must be retained in the Ab combining sites, or novel nucleophiles must generated over the course of adaptive Ab specialization. (b) A mechanism must be available to allow an improved approach of the Ab nucleophile within covalent binding distance of the phosphate probe. Precise spatial alignment of Ab nucleophiles in register with the phosphonate groups in CRAs IV and Va is unlikely because the Abs were raised by immunization with polypeptides that do not contain these groups. Conversely, the phosphonate electrophiles were placed at the side chain Lys residues of protein CRA IV and the C terminus of peptide CRA Va without foreknowledge of the spatial relationship between the non-covalent and nucleophilic binding sites in the Abs. These considerations suggest that the nucleophiles enjoy sufficient conformational freedom to make contact with imprecisely located phosphonate electrophiles in the antigenic epitope. The mobility of individual amino acids in Ab-combining sites following binding to antigen has been reported by other groups (35, 36). Previous epitope mapping and mutagenesis studies (11, 37) indicate that the catalytic residues of proteolytic Abs participate minimally in stabilizing the Ab-antigen ground state complex, suggesting that the mobility of the nucleophile is not restricted by non-covalent binding interactions. Further support for this model is available from observations that monoclonal Abs to vasoactive intestinal peptide (38) and gp41 (8) can cleave multiple peptide bonds in these antigens, presumably by the formation of alternate transition states in which the nucleophile is free to initiate attack on spatially neighboring peptide bonds.

Adaptive improvement in the rate of catalysis by Abs is limited by the mechanisms responsible for clonal selection of B cells. If product release exceeds the rate of transmembrane signaling by the B cell receptor, which is necessary to stimulate cell division, cellular proliferation will cease. On the other hand, there is no bar to adaptive improvement of Ab nucleophilicity as suggested by the results of this study. The improved nucleophilic reactivities of antigen-specific Abs described here result from routine immunization with polypeptides. It is difficult to ascribe the reactivity to a fortuitous immunological phenomenon, because it was observed in polyclonal Abs directed to two different antigens and three distinct monoclonal Abs. Nucleophilic attack on the natural counterparts of the phosphonate groups in CRAs IV and Va, i.e. the electrophilic carbonyl groups in the peptide backbone and side chain amides, is predicted to result in the formation of covalent acyl-Ab complexes (Fig. 1), allowing prolonged occupancy of the B cell receptor and favoring emergence of Abs with improved reactivity. Admittedly, the phosphonate diester group in CRAs is more electrophilic than the carbonyl group in proteins antigens, but Ab nucleophilicity is comparable or superior to that of trypsin, suggesting the feasibility of nucleophilic Ab attack on protein antigens. Two examples of Abs with the ability to form irreversible covalent complexes with hapten antigens have been reported previously (39, 40), and certain Abs display SDS-resistant binding to albumin.2 Ab-nucleophilic reactivity could conceivably contribute to Ab antigen binding without formation of stable covalent bonds. For instance, the nucleophilic reaction may lead to a structure with partial covalent character that does not progress to the acyl-Ab complex because no mechanism is available to donate a proton to the nitrogen atom of the leaving group (C-terminal peptide

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2 S. Paul and R. Dannenbring, unpublished data.
Chemically Reactive Antibodies

10. Gao, Q.-S., Sun, M., Rees, A., and Paul, S. (1995) selective blockade of the catalytic activity. Moreover, to the proteinic CRAs may be hypothesized to permit permanent and cilitated by non-covalent binding interactions, peptidyl and proteinic CRAs may be hypothesized to permit permanent and selective blockade of the catalytic activity. Moreover, to the extent that expression of nucleopolyhedrin coordinated with non-

covalent antigen binding is a universal Ab characteristic, CRA inhibition may be a generally useful means to inhibit biological effects regardless of catalytic activity. CRAs IV and Va, for example, may be used to study the functional roles of Abs from patients with systemic sclerosis and lupus, which are reported to bind EGFR (44) and synthetic gp120-421-436 (45), respectively.

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Broadly Distributed Chemical Reactivity of Natural Antibodies Expressed in Coordination with Specific Antigen Binding Activity
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