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Antigen Recognition by an Antibody Light Chain*

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A monoclonal antibody to vasoactive intestinal polypeptide (VIP) was reduced and alkylated and its light and heavy chains were purified by denaturing gel filtration. Following renaturation, the light chain displayed sequence-specific binding of VIP. The specific VIP binding activity of several fractions spanning the light chain peak recovered from the gel filtration column was constant, the light chain was electrophoretically homogeneous, the VIP binding activity was precipitated by anti-light chain antibody but not anti-heavy chain antibody and the activity remained associated with a light chain fraction recovered by resolutive chromatography on a hydroxylapatite column. N-terminal amino acid sequencing of the light and heavy chain fractions confirmed the purity of these proteins and suggested that the V_L and V_H regions belonged to κ-family II and γ-family III, respectively. The VIP-binding affinity of the light chain was only 5-fold lower than that of the parent antibody and the light chain did not bind unrelated peptides. These observations suggest that light chains display structural characteristics necessary for high affinity antigen binding.

Antigen binding sites in antibodies are formed by the variable regions of light and heavy chains. Delineation of antigen interactions with the individual subunits of antibodies is of interest for several reasons, including: (i) the presence of free light chains in B-lymphocytes (1) and in the circulation of patients with myelomas (B-lymphocyte tumors) (2), and (ii) derivation of high affinity antibodies *in vitro* from randomly combined libraries of light and heavy chains is dependent on pairing of subunits that display appropriate interactions with each other and the antigen (3). Purified heavy chains (4–8) and heavy chain variable regions can bind antigens (9) with affinities approaching those of native antibodies. Light chains purified from polyclonal antibodies, in contrast, display little (6–8) or no antigen binding activity (4, 5). Dimers of light chain secreted by myeloma cells are known to bind haptens. However, their hapten-binding affinities are several orders of magnitude smaller than native antibodies (10–12) and the binding is usually interpreted to be fortuitous and unrelated to the activity of light chains *in vivo*. Study of crystals of antigen-antibody complexes has shown that the number and area of antigen contacts at light and heavy chain residues is comparable (13), but it has been suggested that the subset of residues that contribute to the free energy of binding are located primarily in the heavy chain (14). These observations have lead to suggestions that the heavy chain makes the major contribution in antigen binding, with the light chain serving as a relatively nonspecific partner.

The variable regions of several light chains possess sequences notably similar to active site sequences found in some serine proteases (15). The demonstration of VIP[1] hydrolysis by light chains purified from human autoantibodies (16) suggests the potential utility of these light chains as proteases, either alone or in combination with noncatalytic heavy chains of a different specificity. These reagents can be predicted to display substrate specificities dependent at least in part on the strength and specificity with which the light chains bind VIP. We undertook, therefore, a detailed study of the binding properties of a light chain purified from a monoclonal antibody to VIP. We observed that this light chain displays sequence-specific and high affinity binding of VIP. This observation suggests that it should be possible to develop novel light chain reagents with antigen-specific activity, including a catalytic activity, and permits conception of an antigen-specific biological function for free light chains *in vivo*.

EXPERIMENTAL PROCEDURES

Antibody Subunits—Monoclonal antibody c23.5 was raised by immunization of mice with VIP conjugated via NH_2 groups to keyhole limpet hemocyanin and purified from ascites fluid by ammonium sulfate precipitation and affinity chromatography on protein G-Sepharose chromatography (17). Nonimmune IgG_{2a}, κ secreted by a myeloma served as the control protein (UPC10, Sigma; the isotype of the control antibody and the anti-VIP antibody are identical). The two types of antibodies were reduced and alkylated by a method similar to that described in Ref. 4. Briefly, 5 mg of antibody was reduced with 2-mercaptoethanol (0.2 M, 3 h) followed by iodoacetamide (0.3 M, 15 min) in 0.05 M Tris-HCl, 0.15 M NaCl with the pH maintained at 8.0 using Tris base. The reaction mixture was concentrated (Centriprep-10, Amicon) and immediately separated by high performance gel filtration in 6 M guanidinium chloride, pH 6.5, on two Superose-12 columns (Pharmacia) connected in series (flow rate, 0.4 ml/min). The fractions were dialyzed against 50 mM Tris-HCl, 0.02% sodium azide, pH 7.3 (12–14 kDa cut-off), for 4 days (4 °C) with four buffer changes. Assuming complete equilibration across the dialysis membrane, the final guanidinium concentration in the renatured protein solutions was 1 μM. Chromatography of renatured light chains on hydroxylapatite (Bio-Gel HPHT 100 × 7.8 mm, Bio-Rad) was done at pH 6.8 using a gradient of sodium phosphate (10–300 mM) dissolved in 10 μM calcium chloride, 0.02% sodium azide, pH 6.8. Electrophoresis was on gradient polyacrylamide gels (8–25%) using a Phast system (Pharmacia LKB Biotechnology Inc.). Immunoblotting of the gels was with rabbit anti-mouse heavy (Fc) chain (Axell) and light (κ) chain antibodies (Cappel) followed by staining with a goat anti-rabbit IgG-peroxidase conjugate (Cappel) (18).

Protein Sequencing—The purified antibody subunits (10 μg each) were adsorbed on polyvinylidine difluoride membranes (ProBlott cartridges; Applied Biosystems) according to instructions supplied by the manufacturer and their N-terminal amino acid sequence was determined using a pulsed liquid phase sequenator with on-line phenylthiohydantoin-derivative detection (Applied Biosystems, model 477A). With β-lactalbumin as standard, the initial yield for sequencing was 53%,

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[1] The abbreviations used are: VIP, vasoactive intestinal peptide; CDRs, complementarity determining regions.

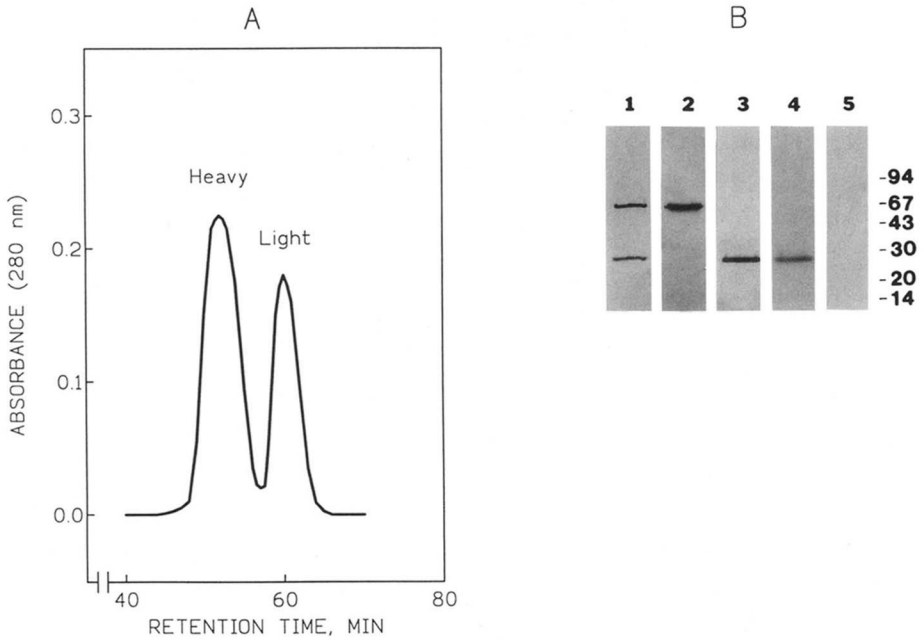

FIG. 1. **Separation of the heavy and light chains from anti-VIP antibody by gel filtration.** Monoclonal antibody c23.5 (5 mg) was reduced and alkylated and chromatographed in 6 M guanidinium chloride, pH 6.5, on two Superose-12 gel filtration columns (Pharmacia) connected in series (*A*). Pooled fractions corresponding to the heavy chain peak (retention time: 54–56 min) and the light chain peak (retention time: 60–66 min) were electrophoresed (8–25% polyacrylamide gels) under reducing conditions and stained with silver (*lanes 2* and *3*, respectively) (*B*). *Lane 1* shows a silver-stained gel of the parent antibody electrophoresed under reducing conditions. *Lanes 4* and *5* are immunoblots of the light chain fraction stained with antibody to mouse light chain and heavy chain, respectively.

and the repetitive yield was >90%. The sequence data reported here are based on amino acid yields >20 pmol in individual sequencing cycles.

[^{125}I-Tyr^{10}]VIP Binding—Synthetic VIP (HSDAVFTDNYTRLRKQ-MAVKKYLNSILN-NH_2; peptide content 81%, Bachem) was radioiodinated using chloramine-T and [^{125}I-Tyr^{10}]VIP was purified and identified as described (19). Binding of [^{125}I-Tyr^{10}]VIP (approximately 0.05 nM) by intact antibody or renatured antibody subunit fractions was measured in duplicate according to Ref. 20 with the following modifications: (i) after incubation with radiolabeled peptide, 25 μl of human γ-globulins (100 μg; Sigma) was added as carrier protein, and (ii) protein-bound VIP was precipitated by addition of polyethylene glycol (M_r 8,000; Sigma) to 20% (w/v). In some assays, bound [^{125}I-Tyr^{10}]VIP was precipitated with rabbit antibodies to mouse light chains (κ) (Cappel) or Fc (Axell) (50 μl of a 40 μg/ml dilution) (20). The binding data were corrected for nonspecific binding (<5% of available radioactivity) determined by incubation in the presence of 1 μM unlabeled VIP.

RESULTS

VIP Binding by Purified Antibody Subunits—A monoclonal antibody to VIP (clone c23.5) was reduced and acetylated in a nondenaturing buffer, solvent conditions described to favor reduction of intersubunit S-S bonds and minimize reduction of intrasubunit S-S bonds (4). Since heavy and light chains form dimers and higher order aggregates by noncovalent interactions (21), they were separated by gel filtration in a denaturing solution (6 M guanidinium chloride) (Fig. 1). SDS-electrophoresis showed a single silver-stained 60-kDa band in the heavy chain fraction and a 25-kDa band in the light chain fraction. After a second round of gel filtration, guanidinium chloride was removed by dialysis and saturable binding of [^{125}I-Tyr^{10}]VIP (binding displaced by excess unlabeled VIP) by the refolded light and heavy chains was measured. Both proteins displayed binding activity. The specific binding activity was essentially constant across the width of the light chain peak recovered from the gel filtration column (fractions 58–61; 4964, 4830, 4210, and 4967 cpm/μg of L-chains, respectively) (Fig. 2), suggesting that the activity is attributable to the light chain. Similarly, fractions spanning the heavy chain peak displayed comparable specific activities (fractions 52–55; 1504, 2026, 1800,

Since strong antigen binding by light chains has not been described previously, additional control experiments were performed. Immunoblotting of SDS gels revealed staining of the 25-kDa light chain band with anti-light chain antibody. Staining with anti-heavy chain antibody was undetectable. An antibody to mouse light chain (κ) precipitated the [^{125}I-Tyr^{10}]VIP binding activity (2,325 ± 149 cpm) of the light chain (1.2 μg/assay), but essentially no binding activity was detected by precipitation with an equivalent concentration of antibody to the heavy chain. [^{125}I-Tyr^{10}]VIP complexed with the parent antibody (0.15 nM) was precipitated effectively by both types of antibodies (anti-light chain antibody, 4782 ± 580 cpm; anti-heavy chain antibody, 3056 ± 660 cpm). We concluded that the binding was not due to trace contamination with heavy chains or heavy-light chain complexes.

A single protein sequence was detected in the antibody subunit preparations by N-terminal amino acid sequencing. The sequence of the N-terminal 27 residues of the light chain was: Asp-Val-Val-Met-Thr-Gln-Thr-Pro-Leu-Thr-Leu-Ser-Val-Thr-Ile-Gly-Gln-Pro-Ala-Ser-Ile-Ser-*X*-Lys-Ser-Ser-Gln, and of the N-terminal 29 residues of the heavy chain, Glu-Val-Lys-Leu-Val-Glu-Ser-Gly-Gly-Gly-Leu-Val-Lys-Pro-Gly-Gly-Ser-Leu-Lys-Leu-Ser-*X*-Ala-Ala-Ser-Gly-Phe-Thr-Phe (*X*, unidentified residues). The amino acids on the C-terminal side of light chain residue 27 (*Gln*) and heavy chain residue 29 (*Phe*) could not be identified with certainty due to diminished amino acid yields (<10 pmol). A comparison of the N-terminal sequences with sequence data in Ref. 22 suggested that the light chain was a member of κ-chain family II. The anti-VIP light chain contains all 10 invariant residues found at corresponding positions in the N-terminal region of this family and its remaining 16 residues are also found among other members of this family. Similar analysis suggested that the heavy chain variable region belonged to the γ-chain family IIIA. As in the case of the light chain, all of the N-terminal amino acids of the anti-VIP heavy chain are found at corresponding positions among members of

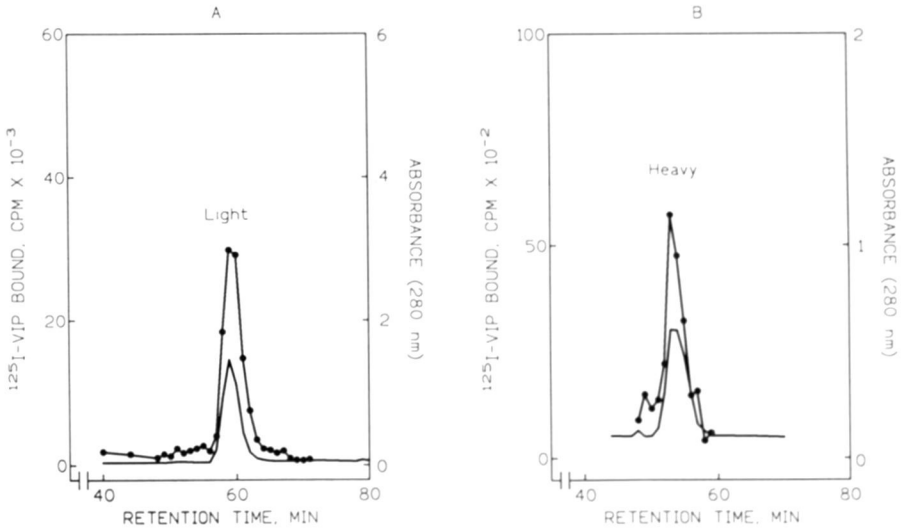

FIG. 2. **Saturable binding of [^{125}I-Tyr^{10}]VIP by purified light chains (*A*) and heavy chains (*B*).** Pooled light chains (500 µg) and heavy chains (135 µg) from Fig. 1 were rechromatographed in 6 M guanidinium chloride on a gel filtration column (Superose-12). The fractions were renatured by dialysis and binding of [^{125}I-Tyr^{10}]VIP (0.1 nM) by duplicate aliquots (light chains, 20 µl; heavy chains, 50 µl) of the column fractions was measured (see "Experimental Procedures").

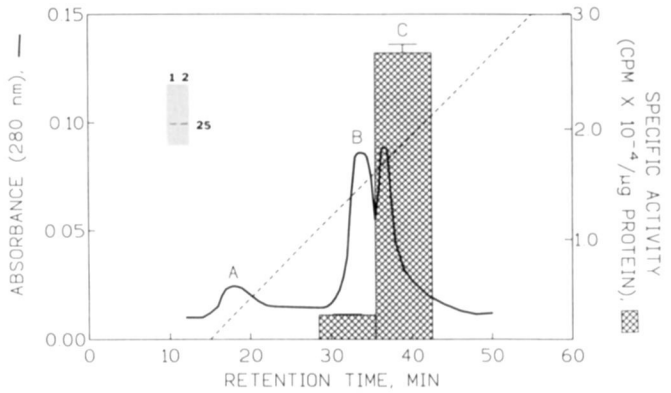

FIG. 3. **Hydroxylapatite chromatography of light chains: separation of a high activity subpopulation.** Light chains (325 µg) purified by gel filtration (see Fig. 1) were chromatographed on a high performance hydroxylapatite column using a gradient of sodium phosphate, pH 6.8 (10–300 mM, 45 min; *dotted line*). Aliquots of pooled fractions corresponding to the two major A_{280} peaks (*solid line*) were assayed for [^{125}I-Tyr^{10}]VIP binding (*hatched bar*). Data for the binding are normalized for protein content to permit direct comparison of binding activity. *Inset* shows a silver-stained SDS-polyacrylamide gel (8–25%) of the pooled fractions corresponding to peaks *B* and *C* (*lanes 2* and *1*, respectively).

and 22 in the heavy chain may correspond to acetylated Cys, since Cys is invariant at these positions.

Chromatography of the light chain fraction on hydroxylapatite revealed two poorly resolved major components (labeled *B* and *C* in Fig. 3) and at least one well-resolved minor protein component (labeled *A*). Identical amounts (3 µg of protein) of the three peaks were subjected to N-terminal sequencing (11 cycles). The deduced sequence was identical in all three peaks (Asp-Val-Val-Met-Thr-Gln-Thr-Pro-Leu-Thr-Leu), corresponding to the N-terminal sequence of the light chain loaded on the column. The major light chain peaks (*B* and *C*) were analyzed further. As expected, a single 25-kDa light band was observed in both peaks by nonreducing SDS-electrophoresis and silver staining. Determination of VIP binding by pooled fractions corresponding to the two peaks showed that the specific [^{125}I-Tyr^{10}]VIP binding activity of the light chain in peak *C* (26,451 cpm/µg protein) was approximately 12-fold greater than that of peak *B* (2,231 cpm/µg protein). Since peaks *B* and *C* were not base-line resolved, contamination with high activity *C* could be responsible for the low-level activity of *B*.

Affinity, Specificity, and Binding Capacity—The binding affinities (*K_d* above) were estimated from Scatchard plots of binding of [^{125}I-Tyr^{10}]VIP mixed with increasing concentrations of unlabeled VIP (Fig. 4). Linear plots were evident for the light chain (r = 0.98) and the parent intact antibody (r = 0.97), with apparent *K_d* values 10.1 and 1.9 nM, respectively. The heavy chain data suggested two binding components with *K_d* 6.8 and 58.3 nM (r > 0.9 for each component). VIP binding capacities (per µg of protein) deduced from the x-intercepts of Scatchard plots were, light chain, 0.8 pmol; heavy chain, 0.05 and 0.13 pmol; and intact antibody, 6.4 pmol. In each case, the slope of a Hill plot of the data was close to unity, suggesting an absence of cooperativity. These data show that the light and heavy chains can bind VIP independently with affinities only 3–5-fold lower than that of intact antibody.

The VIP binding was observed in the presence of excess albumin (0.5%, w/v). Peptides unrelated in sequence to VIP (α-human atrial natriuretic peptide, neurotensin, bombesin, and eledoisin, 1 µM) were without detectable effect on the VIP binding activity of the light or heavy chain. In comparison, >90% of [^{125}I-Tyr^{10}]VIP bound by these proteins was displaced competitively by 1 µM unlabeled VIP. Since unfolding and folding of light chains could expose nonspecific binding sites, the light chain of a control nonimmune antibody (myeloma IgG_{2a}, κ) was purified and assayed for VIP binding under conditions identical to those used for the anti-VIP light chain. The control light chain did not display detectable VIP binding.

DISCUSSION

Several observations indicated that the VIP binding activity of the light chain fraction is not due to contamination with heavy chains or complexes of heavy and light chains, including demonstration of the electrophoretic purity of the light chains, detection of a single protein sequence by N-terminal sequencing, a constant specific activity across the width of the light chain peak recovered by gel filtration, precipitation of the VIP binding activity by antibody to light chains but not antibody to heavy chains, and retention of the VIP binding activity in a light chain fraction purified by hydroxylapatite chromatography. We concluded that the light chain is capable of independent recognition of VIP.

Excess albumin as well as several short and mid-sized peptides unrelated to VIP did not inhibit the binding of radioiodinated VIP by the light chains. A nonimmune light chain did not bind VIP. The VIP-binding affinity of the light chain was nearly equivalent to that of the high affinity component in the heavy chain preparation and only 5-fold lower than that of the

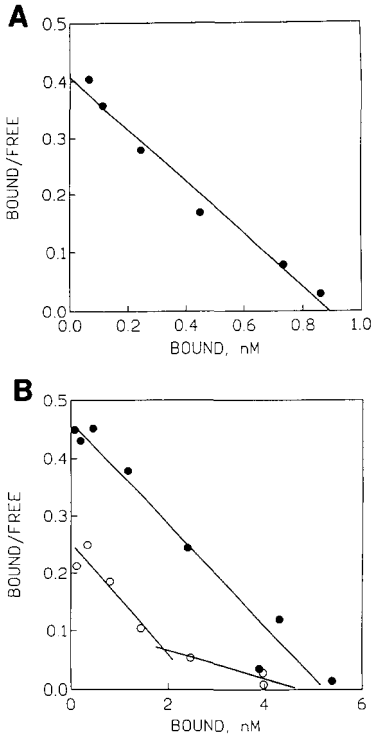

FIG. 4. **VIP-binding affinity of intact c23.5 antibody (*A*) and its purified heavy (○) and light chain subunits (●) (*B*).** Binding of [^{125}I-Tyr^{10}]VIP (0.16 nM) by antibody (1.7 nM), light chain (221 nM), or heavy chain (417 nM) in the presence of increasing concentrations of unlabeled VIP (0.2 nM to 1 μM) was determined. Antibody subunits were purified as described in the legend to Fig 2. Data (means of duplicates) were analyzed using LIGAND (Elsevier Biosoft).

is sequence-specific and can be attributed to the variable regions of the light chain.

The results of hydroxylapatite chromatography of light chains suggested that proper refolding of the protein was a major factor governing antigen binding. This column resolved the light chains into two major species (labeled *B* and *C* in Fig. 3), one with a high specific binding activity and another with little or no activity. N-terminal sequencing confirmed that both peaks were composed of the same light chain. The two peaks must represent, therefore, differently refolded light chains. Possible explanations for this observation are: (i) the two light chain states may be chemically different because of incomplete reduction of disulfide bonds, or (ii) chemically homogenous light chains may adopt alternative conformations. The possibility that *B* and *C* represent different aggregation states of the protein is unlikely, since establishment of equilibrium between the two states following chromatographic separation can be predicted to produce similar levels of binding activity. In view of the presence of two forms of the light chain, the binding capacity value deduced from Fig. 4 must underestimate the stoichiometry of VIP binding by the high activity species. On the other hand, computation of the binding affinity of the high activity species is not compromised, since VIP binding by the second species is apparently too low to produce a deviation of the Scatchard plot from linearity.

Improper refolding could help explain reports describing minimal antigen binding by light chains purified from polyclonal antibodies (4–8). For example, recovery of antigen binding activity in light chains denatured with SDS (5) or propionic acid (4, 6, 8) may be difficult compared to guanidinium chloride used in the present study. A second factor is the possibility of inactive aggregate-formation in concentrated solutions of antibody subunits (21). In the case of concentrated solutions of light chains derived from polyclonal antisera, formation of *heterologous* dimers is likely to be the predominant reaction, which could account for low antigen binding activity. In the present study, the VIP binding activity was associated with the light chain monomer peak (25 kDa) identified by gel filtration (Superose-12 column) in 6 M guanidinium chloride (Fig. 2) and in nondenaturing solvent (50 mM Tris, pH 7.7, 0.15 M NaCl, 0.025% Tween-20; not shown). These data do not permit evaluation of the relative levels of activity in the monomer and dimer forms of the light chain, since noncovalent dimerization could occur following column separation and exposure to VIP in the binding assay could also promote dimerization (23). In the case of the heavy chain of the anti-VIP antibody, overt aggregation was observed in the nondenaturing solvent; approximately 90% of the protein was recovered at the void volume of the gel filtration column (exclusion limit 2×10^6 kDa) and only 10% as the 50–60 kDa monomer (not shown). This type of aggregation may explain the apparent heterogeneity of VIP binding by the heavy chain preparation (Fig. 4).

Antigen binding entails contact with amino acid residues of both antibody subunits. The lengths of heavy and light chain CDRs in different antibodies are variable (22, 24), as are the relative extents of antigen contacts with heavy and light chains in different antigen-antibody complexes (13). Although the number and aggregate surface area of heavy chain contacts are generally somewhat greater, contacts at the light chain can approach 50% of the total interactions. Heavy chain preparations consistently show lower binding activity than the parent antibodies (1–6) and interactions at heavy chain residues alone are unlikely to account fully for high affinity antigen-antibody binding. Light chain contributions in antigen binding may be particularly important when favorable contacts with the heavy chain CDRs are limited, for example, in antibodies expressing short heavy chains CDRs.[2] In view of these considerations, we consider it unlikely that the anti-VIP light chain is unique in its high affinity antigen binding activity.

B-lymphocytes synthesize light chains in excess over heavy chains and secretion of free light chains by these cells has been demonstrated (1). Large amounts of light chains accumulate in the extracellular fluids and tissues of patients with light chain secreting tumors (2). There is compelling evidence that light chains can mediate peptide-bond cleavage (16), activate complement components (25), and suppress antibody synthesis (26). Observation of high affinity antigen binding by a light chain warrants study of the hypothesis that free light chains can simulate the antigen-specific functions of antibodies.

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[2] cDNA for the light and heavy chain variable regions of antibody c23.5 were obtained by the reverse transcriptase-polymerase chain reaction method, cloned into a phagemid vector, and sequenced by the dideoxynucleotide chain termination method (27). The deduced amino acid sequences suggest an aggregate of 26 residues in heavy chain CDRs and 32 residues in light chain CDRs (Q. S. Gao and S. Paul, unpublished). CDR3 in this heavy chain is short (4 amino acids) compared to its length in most mouse antibodies (mean, 8.7 residues; >95% of known heavy CDR3s are composed of 5 or more residues. Ref. 24)

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