Exceptional Amyloid β Peptide Hydrolyzing Activity of Nonphysiological Immunoglobulin Variable Domain Scaffolds

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Nucleophilic sites in the paired variable domains of the light and heavy chains (V_L and V_H domains) of Ig can catalyze peptide bond hydrolysis. Amyloid β (Aβ)-binding Igs are under consideration for immunotherapy of Alzheimer disease. We searched for Aβ-hydrolyzing human IgV domains (IgVs) in a library containing a majority of single chain Fv clones mimicking physiological V_L-V_H-combining sites and minority IgV populations with nonphysiological structures generated by cloning errors. Random screening and covalent selection of phase-display IgVs with an electrophilic Aβ analog identified rare IgVs that hydrolyzed Aβ mainly at His^εL-Gln^εL^5. Inhibition of IgV catalysis and irreversible binding by an electrophilic hapten suggested a nucleophilic catalytic mechanism. Structural analysis indicated that the catalytic IgVs are nonphysiological structures, a two domain heterodimeric V_L (IgV_L-ε-t) and single domain V_L clones with aberrant polypeptide tags (IgV_L-ε'-t'). The IgVs hydrolyzed Aβ at rates superior to naturally occurring Igs by 3–4 orders of magnitude. Forced pairing of the single domain V_L with V_H or V_L domains resulted in reduced Aβ hydrolysis, suggesting catalysis by the unpaired V_L domain. Ångstrom level amino acid displacements evident in molecular models of the two domain and unpaired V_L domain clones explain alterations of catalytic activity. In view of their superior catalytic activity, the V_L domain IgVs may help attain clearance of medically important antigens more efficiently than natural Igs.

The antigen-combining sites of immunoglobulins found in higher organisms are composed of the variable domains of light and heavy chain subunits (V_L and V_H domains). The individual V_L and V_H domains can bind antigens independently of each other, but the paired V_L-V_H structure consistently expresses superior antigen binding affinity because of cooperative antigen-binding forces contributed by the two domains (1). High affinity Igs are generated by adaptive V domain sequence diversification over the course of B lymphocyte differentiation, a process in which antigen binding to mutated B cell receptors (surface Igs associated with signal-transducing proteins) drives the selective expansion of the cells. Adaptive Ig maturation entails the use of one each of ~50 inherited V_L and V_H genes, diversification at the junctions of the V_L-D_L gene segments and V_H-D_H-catalysis gene segments, and somatic mutation over the entire length of the V domains.

Following initial noncovalent binding of antigen, some Igs proceed to catalyze its chemical transformation. Examples of Ig-catalyzed reactions include hydrolysis of polypeptide antigens (2, 3), hydrolysis of nucleic acids (4, 5), and various acyl transfer reactions of other antigen classes (6). Proteolytic Igs that utilize serine protease-like covalent hydrolytic pathways have been described (7, 8). Serine protease-like catalytic triads have been identified in the V domains of Igs by site-directed mutagenesis and crystallography (9, 10). The catalytic mechanism involves nucleophilic attack on the electrophilic carbonyl of peptide bonds. Electrolytic phosphonate diesters originally developed as covalent probes for the nucleophilic site of serine proteases bind catalytic Igs irreversibly and inhibit their catalytic activity (7, 11, 12). The strength of Ig-antigen noncovalent binding often exceeds that of enzyme-substrate binding. An important limitation holding back the application of catalytic Igs for clearance of undesirable antigens is that their catalytic rate constants (turnover number; k_cat) are small compared with enzymes. Evidently, Ig adaptive selection is geared toward noncovalent immune complexation (the ground state stabilization step), and the ability of Igs to recognize the high energy transition state complex that must be stabilized to accelerate chemical reactions is limited. This is supported by observations that IgMs, the first and least diversified Ig class produced during B cell differentiation, express superior catalytic rate constants than Igs produced by the cells at later stages of their adaptive differentiation (12).

Accumulation of amyloid β peptide (Aβ)2 aggregates in the brain is thought to be a central contributor to neurodegenera-
tive changes underlying Alzheimer disease (AD). Administration of monoclonal IgGs that bind Aβ reversibly to transgenic mice overexpressing human Aβ clears brain Aβ deposits and improves cognitive function (13, 14). Suggested mechanisms explaining the favorable effect of peripherally administered IgG are as follows: (a) Aβ containing immune complexes formed by small amounts of IgGs that cross the blood-brain barrier are removed by Fc receptor-mediated uptake by resident macrophages in the brains, the microglia (14); (b) Aβ binding to the IgG constrains the peptide into a nonaggregable conformation (15); (c) IgG bound to FcRn receptors on the blood-brain barrier accelerates Aβ exit from the brain to periphery blood (16); and (d) binding of peripherally circulating Aβ by IgG disrupts equilibrium between the central and peripheral compartments, causing compensatory Aβ release from the brain (17). In principle, IgGs that catalyze the hydrolysis of Aβ can be applied to clear Aβ. We reported naturally occurring IgMs and isolated light chain subunits (IgLs) that hydrolyze Aβ impede Aβ aggregation and inhibit Aβ-induced neurotoxicity (18, 19). However, these IgGs hydrolyze Aβ slowly, and development of more efficient catalysts will help advance the use of catalytic IgGs for Aβ clearance.

We report here the search for efficient Aβ-hydrolyzing Ig fragments in a human IgV domain (IgV) library in which the majority of clones are single chain Fv constructs (scFv-t; a V_L domain attached via a linker peptide to a V_H domain). The scFv scaffold mimics the physiological structure of antigen-combining sites. A minority of clones in the library are nonphysiological V domain structures generated by repertoire cloning errors. Unexpectedly, the nonphysiological two domain and single domain IgV_L fragments expressed exceptional Aβ hydrolyzing efficiency. scFv-t derivatives obtained by repairing a high activity single domain IgV_L displayed reduced catalytic activity. The observations suggest that novel Ig structures freed of constraints imposed by the physiological organization of V domains can be the source of efficient catalysts to medically important antigens.

**MATERIALS AND METHODS**

**Electrophilic Compounds—Syntheses and Ig binding characteristics of these compounds are reported:** E-hapten 1 and 2 (20) and E-hapten 3 (12). Bt-E-Aβ40 was prepared by reacting biotinylated Aβ1–140 (Aβ40; 10 mg, 2.1 μmol) with diphenyl- N-[O-(3-sulfosuccinimidyl)sulfoethyl]-amino(4-amidophenyl)methane phosphonate (10.6 mg, 12.5 μmol) in DMSO. The reaction mixture was purified by RP-HPLC (Waters) and lyophilized. Its identity and purity were determined by mass spectrometry using a matrix of 99.9% C266H380N62O71P2S2, 1427.2, 1141.9, and 951.8. scFv-t derivatives of single domain IgV_L-t' 5D3 were prepared by inserting the deleted V_L residues 8–115 (Kabat numbering). For this, full-length V_L cDNA was amplified by PCR using as template the IgV-pHEN2 DNA library and back/forward primers containing ApaLI/NotI restriction sites (respectively, GGTAATGCACCTTACAGTGTCGCGGCGGAGCTGTCGAGTCCTATGTGCCGGCCGCGGGGAAAAGGTGTTGGGGGCGATGC, and the cdNA digested with ApaLI/NotI was ligated into similarly digested plasmid IgV_L-t' 5D3 DNA with T4 DNA ligase (Invitrogen). Full-length light chain L-t' 5D3 was prepared by Mutagenex by a chimeregism method (23) using as starting materials the V_L domain of IgV_L-t' 5D3 and human κ chain constant domain (obtained from pLC-huCk (24)) and cloned into pHEN2 vector as the Ncol/NotI-digested fragment. cDNAs for the homodimeric IgV_L-t' form of clone 5D3 were prepared by PCR with Mutagenex. Briefly, V_L cDNA was amplified by PCR from the IgV_L-t' 5D3 template using back/forward primers containing ApaLI/NotI restriction sites (respectively, AAAATGCACCTTACAAATGTGTTAGCAGCGTCTC/AAAGCGGCAGCGGGTGTGCTCCAGCTTGGTGTGCTTGGGTCGCGGTGGGTCGCGGGCAGGTGCGAGGTGGGTCGCGCGGGTCGCGAGGTGGGTCGCGCGGGTCG, and the cdNA digested with ApaLI/NotI was ligated into pHEN2 vector. The nucleotide sequence of all constructs determined by dideoxy nucleotide sequencing in the 5' to 3' and 3' to 5' directions was identical (Applied Biosystems, ABI PRISM® 3100 Genetic Analyzer). Following electroporation of IgV phagemid DNA into HB2151 cells, soluble IgVs were purified from periplasmic extracts as before. Total protein was determined by the microBCA kit (Pierce). For mass spectrometry (25), the IgV band was excised from the SDS-electrophoresis gel stained with GelCode Blue (Pierce), subjected to dehydration in 50% acetonitrile and SpeedVac drying, reduced (dithiothreitol) and alkylated (iodoacetamide), and digested with sequencing grade trypsin (Promega) and Lys-C (Wako) for 20 h at 37 °C. Following extraction of gel fragments with acetonitrile/formic acid, digested peptides obtained by ZipTip C18 (Millipore) fractionation using 5 μl of aqueous 50% acetonitrile containing 2% formic acid were analyzed by mass spectrometry using a matrix of α-cyano-4-hydroxycinnamic acid (ABI 4700 MALDI-TOF/TOF mass spectrometer). Predicted monoisotopic peptide sequence and a c-myc epitope are located at the IgV C terminus.

Expression levels were 1–3 mg of IgV/liter of bacterial culture, determined by anti-c-myc immunoblotting. Soluble IgVs were purified from periplasmic extracts of HB2151 cells by metal affinity chromatography. Further purification was by anion exchange FPLC (MonoQ HR 5/5 column; 0–1 mM NaCl in 50 mM Tris buffer, pH 7.4, containing 0.1 mM CHAPS). Purity was determined by SDS-gel electrophoresis and immunoblotting. IgV phages (1012 colony-forming units) were packaged using the hyperphage method (22) and incubated (2 h, 37 °C) with Bt-E-Aβ40 in 0.07 ml of 10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS). Phages with bound Bt-E-Aβ40 were captured using anti-biotin antibody coupled to agarose gel (0.22 ml settled gel; Sigma) and washed with 100 ml of PBS containing 0.1% bovine serum albumin. Reversibly bound phages were eluted by incubation of the gel in 0.2 ml of 100 μM Aβ40 for 1 h with slow mixing, and the residual phages covalently complexed to Bt-E-Aβ40 were eluted with 0.4 ml of 0.1 mM glycine, pH 2.7. scFv-t derivatives of single domain IgV_L-t' 5D3 were prepared by inserting the deleted V_L residues 8–115 (Kabat numbering). For this, full-length V_L cDNA was amplified by PCR using as template the IgV-pHEN2 DNA library and back/forward primers containing ApaLI/NotI restriction sites (respectively, GGTAATGCACCTTACAGTGTCGCGGCGGAGCTGTCGAGTCCTATGTGCCGGCCGCGGGGAAAAGGTGTTGGGGGCGATGC, and the cdNA digested with ApaLI/NotI was ligated into similarly digested plasmid IgV_L-t' 5D3 DNA with T4 DNA ligase (Invitrogen). Full-length light chain L-t' 5D3 was prepared by Mutagenex by a chimeregism method (23) using as starting materials the V_L domain of IgV_L-t' 5D3 and human κ chain constant domain (obtained from pLC-huCk (24)) and cloned into pHEN2 vector. The nucleotide sequence of all constructs determined by dideoxy nucleotide sequencing in the 5' to 3' and 3' to 5' directions was identical (Applied Biosystems, ABI PRISM® 3100 Genetic Analyzer). Following electroporation of IgV phagemid DNA into HB2151 cells, soluble IgVs were purified from periplasmic extracts as before. Total protein was determined by the microBCA kit (Pierce). For mass spectrometry (25), the IgV band was excised from the SDS-electrophoresis gel stained with GelCode Blue (Pierce), subjected to dehydration in 50% acetonitrile and SpeedVac drying, reduced (dithiothreitol) and alkylated (iodoacetamide), and digested with sequencing grade trypsin (Promega) and Lys-C (Wako) for 20 h at 37 °C. Following extraction of gel fragments with acetonitrile/formic acid, digested peptides obtained by ZipTip C18 (Millipore) fractionation using 5 μl of aqueous 50% acetonitrile containing 2% formic acid were analyzed by mass spectrometry using a matrix of α-cyano-4-hydroxycinnamic acid (ABI 4700 MALDI-TOF/TOF mass spectrometer). Predicted monoisotopic peptide sequence and a c-myc epitope are located at the IgV C terminus.
mass values were obtained using MS-Fit for protein data base searches (Protein Prospector, University of California, San Francisco). IgM was purified from human sera as described (18).

Hydrolysis and Binding Assays—Hydrolysis of 125I-Aβ40 was determined as described (18). Briefly, 125I-Aβ40 prepared by the chloramine-T method was purified by RP-HPLC (2.2 Ci/μmol). The 125I-Aβ40 (~0.1 nm, ~30,000 cpm/tube) was treated with IgVs in PBS containing 0.1 mM CHAPS and 0.1% (w/v) bovine serum albumin; intact peptide was separated from fragments by precipitation with trichloroacetic acid, and acid-soluble radioactivity was counted and corrected for background values in control assay tubes incubated in diluent without Ig (mean ± S.D., 18 ± 6%; n = 7 assays). This procedure affords estimates of hydrolysis concordant with RP-HPLC separation of the reaction mixtures. Apparent kinetic parameters were estimated by fitting hydrolysis rates observed at varying 125I-Aβ40 concentrations mixed with a constant amount of 125I-Aβ40 to the following equation: \( v = \frac{V_{\text{max}}[A\beta40]}{K_m + [A\beta40]} \), where \( V_{\text{max}} \) is the maximum velocity at saturating Aβ40 concentrations, and \( K_m \) is the concentration at which half-maximal velocity was observed. To identify the reaction products, reaction mixtures of nonradiolabeled synthetic Aβ40 or Aβ42 (100 μM; American Peptide Co.) incubated with IgVs in PBS/CHAPS were desalted by gel filtration (Bio-Rad micro Bio-spin 6 columns), lyophilized, and subjected to MALDI-TOF MS with positive ion mode, 20,000 V. RP-HPLC of Aβ40-Ig reaction mixtures and ESI-MS identification of the product have been described previously (18). Hydrolysis of the amide bond linking 7-amino-4-methylcoumarin (AMC) to the C-terminal amino acid of peptide-AMC substrates (Peptides International) was measured in PBS/CHAPS buffer by fluorimetry with authentic AMC as reference (λem 470 nm; λex 360 nm (12)). Hydrolysis of biotinylated proteins was determined by SDS-electrophoresis using peroxidase-conjugated streptavidin to stain blots of the gels (18). IgV covalent binding to biotinylated E-hapten 

RESULTS

Aβ40 Hydrolyzing IgVs—We reported previously the hydrolysis of Aβ40 by polyclonal IgM purified from humans without dementia (18). Here we searched for Aβ40-hydrolyzing human IgVs in a library composed of ~10^7 clones. A majority of the clones in the library are scFv-t constructs with the domain organization Vt-L-Vh-1-t, where Li denotes the 16-residue peptide SS(GGGGS)5-GGSA joining the VL domain C terminus to the VH domain N terminus, and t denotes the 26-residue C-terminal peptide containing the c-myec peptide and His6 tags (7). A minority of clones possess unusual IgV structures generated by cloning errors (see below). Sixty three IgVs purified from the periplasmic extracts of randomly picked clones by His6 binding to nickel affinity columns were tested for 125I-Aβ40 hydrolyzing activity. Two IgVs with activity markedly superior to the remaining clones were identified (Fig. 1A). The activity of the empty vector control extract (pHEN2 devoid of an IgV insert) was within the assay error range (50 cpm/μl, corresponding to mean background acid soluble radioactivity + 3 S.D.). The phagamid DNA of the high activity IgV clone 2E6 was re-expressed in 15 individual bacterial colonies. All recloned colonies secreted IgV with robust 125I-Aβ40 hydrolyzing activity (Fig. 1B), ruling out trivial sample preparation variations as the cause of proteolytic activity. As before, the purified extract of the control empty vector clone did not hydrolyze 125I-Aβ40.

In previous studies, electrophilic phosphonate groups incorporated within polypeptides were bound covalently by catalytic Ig nucleophilic sites, with noncovalent binding at the peptide epitopes conferring specificity to the reaction (21). We employed the biotinylated Aβ40 analog containing phospho-
nates at Lys\textsuperscript{16} and Lys\textsuperscript{28} side chains to isolate $\alpha$B40 catalysts displayed on phage surface (Bt-E-$\alpha$B40; Fig. 2A). Phage IgVs treated with Bt-E-$\alpha$B40 were captured using immobilized antibody, and noncovalently bound phage IgVs were eluted by treatment with excess $\alpha$B40 (designated noncovalently selected IgVs), and the irreversible phage IgV immune complexes were eluted by acid disruption of the biotin-antibody complexes (designated covalently selected IgVs). The frequency of IgVs with robust $\alpha$B40 hydrolyzing activity was increased by covalent selection (Fig. 2B). Four of 7 IgVs obtained by covalent selection at 2 $\mu$M $\alpha$B40 hydrolyzed $^{125}$I- $\alpha$B40 at rates >400 cpm/h, compared with 2 of 63 IgVs with this level of activity identified by random screening. Phage selections conducted at increased $\alpha$B40 concentration (10 $\mu$m) yielded less active IgVs (Fig. 2B), consistent with the prediction of more efficient selection of catalysts at the lower ligand concentration. Eighteen IgVs recovered by noncovalent selection displayed no or little hydrolytic activity.

IgV Primary Structure and Activity Validation—The cDNAs for IgV clone 2E6 obtained without phage selection and three covalently selected IgVs with the greatest $\alpha$B40 hydrolyzing activity (clones 5D3, 1E4, and 5H3) were sequenced. Identical nucleotide sequences were obtained for each clone sequenced from the 5' to 3' direction and the 3' to 5' direction. The cDNA sequences indicated that IgV 2E6 is a dimer of two different $\nu$L domains with the intervening linker peptide and the expected C-terminal tag (designated heterodimeric IgV$_{1,2}$-$t$). Sequences in supplemental Fig. S1). IgVs 5D3, 1E4, and 5H3 are single domain $\nu$L clones with unexpected C-terminal polypeptide segments, designated IgV$_{1,2}$-$t$' clones, where $t'$ denotes the following: (a) the expected linker peptide, (b) a 15–28-residue aberrant peptide sequence in place of the $\nu$V$_{1}$ domain composed of ~115 residues, and (c) the expected 26 residue peptide sequence containing the c-myc and His$_8$ sequences (Fig. 3A and supplemental Fig. S1).

Two clones were studied further, IgV$_{1,2}$-$t$ 2E6 and IgV$_{1,2}$-$t'$ 5D3. Their deduced protein masses predicted from the cDNA sequences are, respectively, 27 and 17 kDa. Denaturing electrophoresis of the IgVs purified by 2 cycles of nickel-affinity chromatography and anion exchange FPLC (IgV$_{1,2}$-$t$ 2E6 and IgV$_{1,2}$-$t'$ 5D3) fractions corresponding, respectively, to retention times 10–11 and 23–23.5 min; supplemental Fig. S2) revealed silver stained and anti-c-myc stainable protein bands close to the predicted mass of the monomer proteins (IgV$_{1,2}$-$t$ 2E6, 29 kDa; IgV$_{1,2}$-$t'$ 5D3, 18 kDa; Fig. 3B). The presence of the c-myc peptide epitope confirms that these are IgV bands. In view of its unusual structure, the identity of IgV$_{1,2}$-$t'$ 5D3 monomer band was confirmed further by tryptic digestion and mass spectroscopy (supplemental Table S1). All observed spectroscopic signals originated from peptides within the predicted IgV$_{1,2}$-$t'$ structure deduced from the cDNA sequence, and the peptide signals were consistent with deletion of $\nu$V$_{1}$ residues 8–115 predicted from the cDNA sequence. Additional IgV bands were detected prior to anion exchange chromatography (low mass IgV$_{1,2}$-$t$ 2E6 band at 18 kDa; high mass IgV$_{1,2}$-$t'$ 5D3 bands at 36, 50, 58, 67, and 74 kDa). All of these bands were stained by anti-c-myc antibody (Fig. 3B). As irrelevant proteins are not stained by the anti-c-myc antibody, there is no evidence of non-IgV contaminants. We concluded that the anomalous low mass band is an IgV$_{1,2}$-$t'$ self-degradation product, and the high mass bands are IgV$_{1,2}$-$t'$ aggregates. $^{125}$I- $\alpha$B40 hydrolyzing activities of both IgVs remained constant after one and two cycles of nickel-affinity chro-
matography but were increased following further FPLC purification that removed the degradation product and aggregates (Table 1; by 3.1- and 31.2-fold, respectively, for the IgVL2-t and IgVL2-t'). These observations are consistent with Aβ hydrolysis by the unaggregated IgVs.

Sequence of 24 randomly picked clones indicated that most IgVs in the library are scFv-t constructs (83.3% clones), with only rare representation of IgV1-2-t and IgV1-2-t' constructs (respectively, 12.5 and 4.2% clones; supplemental Table S2). The cumulative probability that all four Aβ40-hydrolyzing IgVs identified in the present study are IgV1-2-t or IgV1-2-t' clones by random chance is very small \( p = 0.9 \times 10^{-3}; \) computed as 0.125 × 0.042). It may be concluded that the rare IgV structures favor expression of Aβ hydrolysis compared with the physiological VL-VH paired structure of scFv-t clones. This is supported by comparisons of IgV catalytic activities with the previously reported polyclonal human IgM preparations and a mononclonal IgM from a patient with Waldenstrom's macroglobulinemia (18). IgV1-2-t 2E6 and IgV1-2-t' 5D3 hydrolyzed

![Figure 3](image-url)

**FIGURE 3. Structure and hydrolytic activity of IgVL2-t 2E6 and IgVL2-t' 5D3.** A, schematic representation of IgV structures. scFv-t, VL, and VH domains connected by a peptide linker with t, the His\(^6\)-c-myc tag located at the C terminus; IgVL2-t 2E6, a heterodimer of two VL domains with the linker and t; IgV1-2-t' 5D3, a single domain VL with t at the C terminus, corresponding to the peptide linker, a short peptide region in place of the VH domain and the t tag. B, SDS-electrophoresis of IgVs. Lanes 1 and 2, respectively, IgVL2-t 2E6 purified by metal affinity chromatography followed by anion exchange FPLC and stained with silver and anti-c-myc antibody. Lanes 3 and 4, respectively, IgVL2-t 2E6 purified by two cycles of metal affinity chromatography and stained with silver and anti-c-myc antibody. Lanes 5 and 6, respectively, IgVL2-t' 5D3 purified by metal affinity chromatography followed by anion exchange FPLC and stained with silver and anti-c-myc antibody. Lanes 7 and 8, respectively, IgVL2-t' 5D3 purified by two cycles of metal affinity chromatography and stained with silver and anti-c-myc antibody. C, \(^{125}\)I-Aβ40 hydrolysis (cpm/μg Ig: means ± S.D.) by monoclonal IgM Yvo, a pooled human polyclonal IgM preparation, IgVL2-t 2E6, and IgVL2-t' 5D3. \(^{125}\)I-Aβ40, -30,000 cpm (0.1 mU). The monoclonal and polyclonal IgM preparations are described previously (18). Data are from assays at 90–405 μg/ml IgMs and 0.075–0.55 μg/ml IgVs.

**TABLE 1**

| Catalyst | Specific activity, cpm/μg of protein (mean ± S.D.) |
|----------|--------------------------------------------------|
| IgVL2-t | 1318 ± 375 1220 ± 134 4495 ± 1035 |
| IgVL2-t' | 2660 ± 114 2273 ± 308 61401 ± 5274 |

**FIGURE 4.** 125I-Aβ40 hydrolysis by single domain IgVL2-t' 5D3 and its paired V domain derivatives. A, hydrolytic activity of the IgVL2-t' (C) and its scFv-t derivatives (B). 125I-Aβ40, -30,000 cpm, (-0.1 mU). Values are means ± S.D. Incubation, 18 h. B, SDS-electrophoresis gels of the IgVL2-t' stained with silver and anti-c-myc antibody. Lanes 3 and 4, respectively, an example scFv-t derivative of S3D (clone S3D-E6) stained with silver and anti-c-myc antibody. The bands at 18 and 30 kDa are, respectively, the IgVL2-t' and scFv-t. B, hydrolytic activity of full-length L-t 5D3 and homodimeric IgVL2-t' 5D3. 125I-Aβ40 hydrolysis assayed as in A. Inset, lanes 1 and 2, respectively, SDS-electrophoresis gels of the L-t stained with silver and anti-c-myc antibody. Lanes 3 and 4, respectively, the IgVL2-t' stained with silver and anti-c-myc antibody. The bands at 30 and 27 kDa are, respectively, the L-t and IgVL2-t'.

125I-Aβ40 with potencies superior to the IgMs by 3–4 orders of magnitude (Fig. 3C).

**Repaired IgV 5D3 Versions**—The aberrant t' region of IgVL1-t' 5D3 contains a deletion of VH domain residues 8–115 (Kabat numbering). Four scFv-t constructs were generated from the IgVL1-t' by inserting the deleted residues derived from full-length VH domains represented in the library (supplemental Fig. S3). The repaired scFv-t 5D3 derivatives migrated at the expected mass in electrophoresis gels (30 kDa, example in Fig. 4A, inset). Their 125I-Aβ40 hydrolyzing activity was consistently lower than the parent IgVL1-t' (by ~82–167-fold, computed by rate comparisons in the linear region of the hydrolysis curves; Fig. 4A). This suggests that Aβ40 hydrolysis occurs at an autonomous catalytic site in the IgV1-t' VH domain that is suppressed by pairing with VH domains.

Intermolecular noncovalent bonding between isolated light chains can generate dimeric light chain structures (29). To assess whether noncovalently associated IgV-t' dimers containing paired VL-VL structures might account for the hydrolytic activity, we prepared the homodimeric IgV1-2-t 5D3 hydrolyzed 125I-Aβ40 poorly...
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The hydrolytic activity of both IgV clones was saturable with increasing Aβ40 concentrations (1–100 μM) nonradioactive Aβ40 mixed with 0.1 nM [125I]Aβ40. Kinetic parameters are reported in Table 2. MALDI-MS of nonradioactive Aβ40 treated with IgVL2-t 2E6 or IgVL-t’ 5D3 indicated similar product profiles (Fig. 5, A and B). The prominent products were Aβ-(1–14) and Aβ-(15–40), suggesting hydrolysis of the His14–Gln15 peptide bond (Fig. 5E; observed and calculated m/z values in Fig. 5 legend). Smaller signals for the following peptide products were also detected as follows: Aβ-(1–15), Aβ-(1–20), Aβ-(16–40), and Aβ-(21–40). The corresponding scissile bonds in Aβ40 are Gln14–Lys16 and Phe20–Ala21. Similar product profiles were evident in reaction mixtures of the longer Aβ42 peptide treated with the IgVL2-t 2E6 (Fig. 5, C and D), except that additional minor products suggesting cleavage at the Lys28–Gly29 and Gly29–Ala30 bonds were evident. As accurate product quantification by MALDI-MS is difficult, we also conducted RP-HPLC of Aβ40 treated with IgVL2-t 2E6. This indicated depletion of the intact Aβ40 peak, accompanied by appearance of a major peptide product absent in control chromatograms of the IgV alone or Aβ40 alone (supplemental Fig. S4A). The product peak was identified as Aβ-(1–14) by ESI-MS, confirming the His14–Gln15 bond as the major cleavage site.

The [125I]Aβ40 hydrolysis measurements were conducted using a small amount of the Aβ40 substrate (0.1 nM) mixed with excess albumin (1 mg/ml; 15 μM), which can serve as an alternate substrate for promiscuous catalysts. As hydrolysis of [125I]Aβ40 was detected readily, the IgVs do not appear to be non-specific catalysts. In addition, there was no evidence for hydrolysis of several irrelevant biotinylated polypeptides (ovalbumin, soluble extracellular domain of the epidermal growth factor receptor, human immunodeficiency virus gp120, protein A; supplemental Fig S4B).

Previous reports have identified promiscuous IgGs present in human blood using model fluorogenic peptide substrates (12). IgVL2-t 2E6 and IgVL-t’ 5D3 failed to hydrolyze the model peptide substrate appreciably (Table 3), whereas a representative human polyclonal IgM preparation 9010 hydrolyzed large amounts of Arg/Lys-containing peptide substrates. The data indicate specific Aβ hydrolysis by the IgVs.

Previous reports have indicated that proteolytic IgGs utilize a serine protease-like catalytic mechanism entailing nucleophilic attack on the electrophilic carbonyl of peptide bonds (7, 9). This was the basis for the covalent phage IgV selection in this study. To confirm the mechanism, we studied the reactivity of IgVL2-t 2E6 and IgVL-t’ 5D3 with the electrophilic phosphate diester E-hapten-1 (Fig. 6A), a compound originally developed as a covalent inhibitor of serine proteases (30). E-hapten-1 inhibited [125I]Aβ40 hydrolysis by both clones (Fig. 6B). The biotin-containing version of the phosphate diester,
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E-hapten-2, formed 30-kDa covalent adducts with IgV\textsubscript{L2}-t 2E6 that were stable to boiling and SDS treatment (Fig. 6B, inset). Control hapten-3, a poorly electrophilic analog of E-hapten-2, did not form detectable adducts with the IgV\textsubscript{L2}-t. The results support a nucleophilic catalytic mechanism.

IgV\textsubscript{L2}-t 2E6 and IgV\textsubscript{L1}-t’ 5D3 (20 μg/ml) did not bind detectably to immobilized Bt-A\textsubscript{40} in enzyme-linked immunosorbent assay tests (A\textsubscript{490} < 0.06). Under similar conditions, the reference anti-\(\alpha\)B monoclonal IgG (6E10, 0.1 μg/ml) afforded readily detected binding activity (A\textsubscript{490} 0.7 ± 0.01).

**Molecular Models**—Intramolecular H-bonding between triads and dyads of amino acids enhances the nucleophilicity of certain side chains responsible for enzymatic catalysis. Examples are the hydroxyl side chains of Ser, Thr, and Tyr residues activated by spatially neighboring general bases contributed by His, Lys, Arg, Tyr, Glu, and Asp residues (31–34). We screened molecular models of IgV\textsubscript{L2}-t 2E6 and IgV\textsubscript{L1}-t’ 5D3 for side chain hydroxyls located within 4 Å of atoms that can serve as general bases as described in Ref. 31. One triad and several dyads fulfilling this requirement were found in each of the catalysts (supplemental Fig. S5 and supplemental Table S3). The presence of the potential nucleophiles is consistent with the mechanism of IgV catalysis suggested by electrophilic inhibitor studies. Several complexes containing the candidate nucleophilic residues of IgV\textsubscript{L2}-t 2E6 asp to Gln\textsuperscript{105} of the major A\textalpha B40 scissile bond were evaluated by molecular modeling. Among these, the complex containing VL1 domain Thr\textsuperscript{105} asp to the scissile bond was the energetically most favored structure. This complex also contained various noncovalent stabilizing interactions between IgV\textsubscript{L2}-t 2E6 and A\textalpha B40, including VL1 domain Gly\textsuperscript{21} and Ala\textsuperscript{23} backbone atoms hydrogen bonded with, respectively, A\textalpha B40 Asp\textsuperscript{23} backbone and side chain atoms.

The V\textsubscript{L} domain of clone 5D3 is highly catalytic in the unpaired IgV\textsubscript{L1}-t’ state and poorly catalytic when paired with a second V domain in scFv-t or homodimeric IgVL\textsubscript{2}-t states. Substrate movements of electronegative atoms can weaken or strengthen H-bonds and thereby modulate the nucleophilic and proteolytic activities (35, 36). Frequent backbone displacements on the order of 0.5–1.7 Å were evident by energy minimization of the V\textsubscript{L} domain modeled in the unpaired state versus the paired scFv-t or IgVL\textsubscript{2}-t states (Fig. 7, A and B). Spatial displacement of amino acid side chains that influence H-bonding strength and increase or decrease the nucleophilic reactivity could also occur by virtue of rotation around single bonds (see supplemental Table S3 for changes of inter-residue distances within the potential nucleophilic sites in the unpaired and paired V\textsubscript{L} domain states). The modeling results therefore suggest the feasibility of altered nucleophilic reactivity and provide a rational explanation for unequal catalysis by various V\textsubscript{L} domain-containing molecules.

**DISCUSSION**

Our observations indicate the superior A\textalpha B hydrolyzing activity of V\textsubscript{L} domains expressed in the IgV\textsubscript{L2}-t and IgV\textsubscript{L1}-t’ scaffolds compared with scFv-t constructs mimicking physiological antigen-combining sites. The catalytic activity is also strikingly superior to previously reported catalytic IgMs that contain fully natural A\textalpha B-combining sites (18). Several IgV\textsubscript{L2}-t and IgV\textsubscript{L1}-t’ clones with exceptional A\textalpha B hydrolyzing activity were identified from the library. Inclusion of full-length V\textsubscript{H} domains in these clones uniformly suppressed the catalytic activity. Certain previous studies have presented evidence for hydrolysis of polypeptides (37) and nucleic acid (38) by isolated Ig light chain subunits. In contrast to catalysis, high affinity antigen binding by noncovalent means depends on cooperative V\textsubscript{L}-V\textsubscript{H} domain interactions, and heterodimeric V\textsubscript{L}-V\textsubscript{H} domain complexes invariably bind antigens noncovalently at levels superior to the binding activity of either V domain alone. It may be concluded that the catalytic sites are frequently present in IgV\textsubscript{L} domains, but the physiological Ig scaffold does not favor expression of catalytic activity.

**TABLE 3**

| Substrate | Hydrolysis, μM AMC/h/μM IgVL2-t 2E6 | Hydrolysis, μM AMC/h/μM IgVL1-t’ 5D3 | Hydrolysis, μM AMC/h/μM IgM 9010 |
|-----------|----------------------------------|-----------------------------------|----------------------------------|
| AE-AMC    | ND                               | ND                                | ND                               |
| AAA-AMC   | ND                               | ND                                | ND                               |
| IWW-AMC   | ND                               | ND                                | ND                               |
| AAPF-AMC  | ND                               | ND                                | ND                               |
| EKK-AMC   | 0.030 ± 0.001                    | 0.104 ± 0.001                     | 13.1 ± 1.4                       |
| VLK-AMC   | 0.005 ± 0.001                    | 0.023 ± 0.001                     | 6.9 ± 0.4                        |
| EAR-AMC   | 0.001 ± 0.001                    | 0.022 ± 0.001                     | 1.4 ± 0.6                        |
| IEGR-AMC  | 0.010 ± 0.001                    | 0.022 ± 0.001                     | 2.0 ± 0.7                        |
| PFR-AMC   | ND                               | ND                                | 37.4 ± 0.7                       |

**FIGURE 6. Inhibition of IgV catalysis by E-hapten-1.** A, E-hapten-1 is an active site-directed inhibitor of serine proteases. E-hapten-2, a biotinylated analog of E-hapten-1, was used to detect covalent IgV adducts. E-hapten-3, the unesterified phosphonic acid analog of E-hapten-1, is the poorly electrophilic control compound. B, inhibition of IgV\textsubscript{L2}-t 2E6 and IgV\textsubscript{L1}-t’ 5D3 hydrolysis by E-hapten-1. IgV\textsubscript{L2}-t 2E6 (0.55 μg/ml) or IgV\textsubscript{L1}-t’ 5D3 (0.075 μg/ml) was treated with E-hapten-1 for 2 h followed by determination of 125I-A\textalpha B hydrolytic activity. Inset, streptavidin-peroxidase-stained blots of SDS gels showing IgV\textsubscript{L2}-t 2E6 (20 μg/ml) treated for 18 h with 0.1 mM E-hapten-2 (lane 2) or E-hapten-3 (lane 3). Lane 1, IgV\textsubscript{L2}-t 2E6 stained with silver.
Ig light chain homodimers overproduced by cancerous B cells in multiple myeloma patients can bind certain antigens (39, 40). There is no naturally occurring Ig homolog of IgVL1-2'-t 2E6, a heterodimer of two V_L domains. Homodimeric IgVL1-2'-t constructs containing the individual V_L domains of IgVL1-2'-t 2E6 were without appreciable Aβ hydrolyzing activity, suggesting that both V_L domains in the heterodimer are important in maintaining the integrity of the catalytic site. Three Aβ-hydrolyzing clones with the IgVL1-2' scaffold were also identified. In energy-minimized molecular models of one such clone, the small V_L domain peptide in the C-terminal segment was revealed as a disordered region without the β sheet structure typical of the Ig fold. Moreover, the proximity of the V_L peptide region to the V_H domain was insufficient to anticipate that it contributes to Aβ recognition by the V_H domain catalytic site. Noncovalent intermolecular association of the single domain IgVL1-2'-t can be hypothesized to generate homodimeric V_L-V_L-combining sites. However, the stable homodimeric IgVL1-2'-t derivative containing its two V_L domains was devoid of catalytic activity, supporting attribution of catalysis to the unpaired V_L domain. No natural Igs with an unpaired, functionally active V_L domain are known. In extant organisms, the closest functional homolog of the unpaired catalytic V_L domain are certain jawed fish and cameldid Igs containing a single V_H domain, which is thought to bind antigen in its unpaired state (41, 42). The V_L and V_H domains express appreciable sequence identity with each other, and modern Igs have likely evolved by duplication and sequence diversification of a common primordial gene encoding the Ig fold (43). The phylogenetic origin of Ig catalysis and deterioration or improvement of the catalytic function over the course of evolution of the immune system remains to be examined.

Electrophilic compounds that react irreversibly with the active site of serine proteases inhibited the Aβ hydrolyzing activity and formed irreversible complexes with the catalytic IgVs. This suggests a nucleophilic catalytic mechanism as deduced for other proteolytic Igs from inhibitor, mutagenesis, and crystallography studies (7, 9, 10). Protein nucleophilic reactivity is generated by intramolecular activation reactions within dyads and triads formed by precisely positioned amino acids, e.g. by hydrogen bonding between the Ser haloxy side chain and an imidazole nitrogen. Even small, sub-Å side chain movements can weaken the bonding and induce loss of active site nucleophilic reactivity. Noncovalent antigen binding, on the other hand, is mediated by weak and more numerous interactions at several contact residues in Ig-combining sites (1). Loss of any single contact because of a minor conformational change may weaken noncovalent antigen binding, but an abrupt transition from the binding state to a nonbinding state is less likely.

Molecular modeling of the single domain IgVL1-2'-t 3D3 suggested the likelihood of minor structural perturbations upon pairing the catalytic V_L domain with another V domain, helping explain the poor catalytic activity of the scFv-t and homodimeric IgVL1-2'-t versions of the molecule. Compared with the suppressive effect of V_L-V_H and V_H-V_H pairing, the catalytic activity of the single domain V_L was tolerant to inclusion of the C-terminal κ constant domain. This is significant, because it opens the route to inclusion of C-terminal moieties that reduce IgV clearance, e.g. polyethylene glycol or the Ig Fc fragment (44–46). Engineering stable catalyst versions with sufficient longevity in vivo is an important goal for clinical applications. scFv-t constructs have short half-lives in peripheral blood (47), in view of their small size, the IgVs may also be subject to rapid clearance in vivo. Another route to prolonging the lifetime of IgVs is their recloning within the physiological IgG scaffold (48). The two domain IgVL1-2'-t 2E6 is a heterodimeric structure that should allow development of a IgG-like structure with a combining site formed by the two V_L domains.

Drugs currently employed to treat AD do not arrest the underlying pathology and progressive cognitive decline. Aβ oligomer accumulation is thought to be a major cause of neuronal death and dysfunction in the AD brain (49, 50). Small amounts of peripherally infused Aβ binding monoclonal IgGs traverse the blood-brain barrier, and IgG-facilitated Aβ clearance has emerged as a novel therapeutic strategy with the potential to halt cognitive decline in AD patients (51). Reversibly binding IgGs can at best bind two antigen molecules. Large quantities of stoichiometrically binding monoclonal IgGs are usually required for immunotherapy. Catalytic Igs hold the potential of clearing Aβ efficiently by virtue of the specific Aβ degrading activity. For example, from its k_cat value in Table 1, a single IgVL1-2'-t 5D3 molecule is predicted to digest 4320 Aβ molecules in 3 days at excess Aβ concentration. The enzyme neprilysin has received attention as a potential Aβ-clearing AD drug (52). The k_cat of neprilysin for Aβ is comparable with the IgVs reported here (53). Neprilysin, however, also hydrolyzes irrelevant polypeptides (54), whereas the IgVs did not degrade.

FIGURE 7. Models of the V_L domain of IgVL1-2'-t 3D3 (black) superimposed on the VL domains of its heterodimeric scFv-t derivative clone 3D3-E6 (red, A) and its homodimeric IgVL1-2'-t 3D3 derivative (red, B). Cα atoms belonging to amino acids with r.m.s. deviation >1.5 Å are identified, and overall r.m.s. deviations the superimposed models are indicated.
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non-Aβ polypeptides detectably. Aβ degradation by an IgM at a substantially lower rate than the IgGs was previously reported to inhibit Aβ aggregation and Aβ-induced neurotoxicity (18). The IgVs hydrolyze the His14–Gln15 bond and, at lower levels, other peptide bonds located in the central Aβ region. The aggregability of various synthetic Aβ fragments is generally weaker than full-length Aβ (55–57), but the precise functional effects of IgV-catalyzed Aβ hydrolysis remain to be examined. Concerns have been raised that Aβ binding IgGs can induce inflammation (58) and vascular microhemorrhages (59, 60) caused, respectively, by immune complex-stimulated release of microglial inflammatory mediators and IgG-stimulated Aβ deposition in cerebral blood vessels. The IgVs reported here do not form immune complexes detectably. They degrade Aβ permanently, minimizing the risks of inflammatory mediator release and Aβ re-deposition in the vascular wall. The recently reported phase II clinical trial of a reversibly binding anti-Aβ monoclonal IgG in patients with mild-to-moderate AD patients highlights the importance of searching for safer and more effective immunotherapeutic reagents (61). A dose-limiting incidence of vasogenic edema in magnetic resonance images was evident in this trial. At lower doses of the IgG, cognitive performance tended to improve, but the effect did not reach statistical significance in the intent-to-treat population. However, upon exclusion of patients homozygous for the apolipoprotein E4 allele, post-hoc analysis suggested significantly improved cognitive functions in the remaining patient subgroup. The apolipoprotein E4 allele is known to predispose AD patients to increased amyloid accumulation (62).

In summary, the specific and efficient Aβ degrading activity of the IgVs supports evaluation of their efficacy and safety in attaining Aβ clearance. The potential medical utility of catalytic IgGs to microbial antigens and cancer-associated antigens has been discussed previously (28), but the low catalytic rate constants of physiological IgGs have been a barrier to their clinical application. Our observations suggest that enhanced catalysis can be achieved by placing the V_{L} domains within non-physiological two domain and single domain scaffolds. If this finding proves generally applicable, development of efficient catalysts specific for other clinically important antigens should be feasible.

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