Degradation of β-Amyloid by Proteolytic Antibody Light Chains†

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ABSTRACT: Deposition of beta-amyloid (Aβ) is considered an important early event in the pathogenesis of Alzheimer's disease (AD). Clearance of Aβ thus represents a potential therapeutic approach. Antibody-mediated clearance of Aβ by vaccination inhibited and cleared Aβ deposition in animal models; however, inflammatory side effects were observed in humans. An alternative potentially noninflammatory approach to facilitate clearance is to proteolytically cleave Aβ. We screened 12 proteolytic recombinant antibody fragments for potential α-secretase activity, a naturally occurring enzyme that cleaves between the Lys16 and Leu17 residues of the amyloid precursor protein (APP). We utilized the synthetic α-secretase substrate, benzyloxycarbonyl-L-lysine *o*-nitrophenyl ester (Z-lys-o-Np) as a preliminary screen for α-secretase activity. Two antibody light chain fragments that hydrolyzed Z-lys-o-Np were identified. Aβ hydrolysis was studied using mass spectrometry to identify the cleavage patterns of the antibodies. The recombinant antibody light chain antibody fragment, c23.5, showed α-secretase-like activity, producing the 1−16 and 17−40 amino acid fragments of Aβ. The second light chain antibody fragment, hk14, demonstrated carboxypeptidase-like activity, cleaving sequentially from the carboxyl terminal of Aβ. These antibody light chains provide a novel route toward engineering efficient therapeutic antibodies capable of cleaving Aβ in vivo.

Alzheimer's disease (AD), a neurodegenerative disorder characterized by progressive dementia, judgmental impairment, delusions and irritability, affects over 4 million U.S. elderly. While dementia has long been affiliated with aging, around 70% of all cases of dementia are associated with AD, afflicting greater than 10% of those over 65 years of age (*1*). AD-associated dementia appears to be caused in part by disruption of nerve cell function, associated with the accumulation of senile plaques and neurofibrillary tangles in the brain. The principal component of the extracellular plaques is the β-amyloid protein (Aβ), an approximately 4 kDa fragment proteolytically derived from a larger amyloid precursor protein (APP). The neurofibrillary tangles are composed of tau, a phosphorylated microtubule-associated protein (*2*, *3*).

Because of the important role of Aβ in AD pathology and the correlation of APP mutations with AD (*4*−*6*), intense efforts have been directed at studying both the processing and biological function of APP (see for examples refs *7*−*10*). Proteolytic cleavage of APP by either α- or β-secretase releases the soluble (100−120 kDa) APP fragment (APPs) and generates a 10−12 kDa membrane-bound fragment. Further proteolytic processing of the β-secretase cleaved membrane bound fragment by γ-secretase will release the 4 kDa Aβ fragment. Three proteases control the processing of APP into Aβ: α-secretase cleaves APP between what would be residues 16(Lys) and 17(Leu) of Aβ, releasing APPs and leaving a nonamyloidogenic membrane bound protein; alternatively β-secretase can cleave APP generating the amino terminal of Aβ in the fragment retained in the membrane and releasing a slightly shorter soluble APP molecule; γ-secretase can then cleave the membrane-bound fragment at the carboxyl terminal of Aβ to release the amyloidogenic Aβ protein.

Since increased accumulation of Aβ, particularly Aβ42, appears to be a critical factor in AD pathology, decreasing Aβ concentration represents a potentially viable therapeutic approach. One approach to decrease Aβ accumulation and toxicity is to cleave the protein at the α-secretase site using proteolytic antibodies. Antibodies are well-known for the specificity with which they recognize the target antigen. Less recognized is the ability of certain antibodies to cleave polypeptide antigens. Several examples of cleavage by proteolytic antibodies and antibody light chain subunits found in certain disease states have been published (*11*−*13*), but it is not known whether this type of activity can be directed to other polypeptide antigens. Antibodies with esterase activity have been raised by immunization with oxy-anionic mimics of the transition state of transacylase reactions (*14*, *15*), but this strategy has not been successful in raising proteolytic antibodies (*16*), presumably because peptide bond hydrolysis is a more energetically demanding reaction. Here we report the feasibility of in vivo Aβ hydrolysis as a

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therapeutic for AD by demonstrating proteolysis of Aβ mediated by two different antibody light chains. We identified two recombinant proteolytic antibody fragments that hydrolyzed a synthetic substrate that mimics the cleavage site of α-secretase. We utilized mass spectrometry (MS) to identify the degradation products generated by hydrolysis of Aβ by the proteolytic antibody fragments. One antibody light chain showed primary cleavage at the α-secretase site, generating the amino terminal 1−16 and carboxyl terminal 17−40 fragments. The second antibody light chain demonstrated carboxypeptidase-like activity, cleaving sequentially from the carboxyl terminal.

EXPERIMENTAL PROCEDURES

Antibody Samples. Lyophilized samples of 12 different recombinant antibody samples were a generous gift of Dr. Sudhir Paul (University of Texas Health Science Center, Houston, Texas). Ten antibody light chains and two single-chain Fv constructs were analyzed. Of the light chains, seven were monoclonal Bence Jones protein purified from the urine of multiple myeloma patients (LAY, JOH, XOC, LEN, LOC, RHY, B-6) to electrophoretic homogeneity and described to cleave various model peptide-methylcoumarinamide substrates on the C-terminal side of basic residues with varying levels of catalytic activity (*17*). Three recombinant light chains (c23.5, hk14, GG63) were purified from bacterial periplasmic extract (*18*). These light chains have previously been reported to cleave vasoactive intestinal polypeptide (light chains c23.5 and hk14). L chain GG63 cleaves model peptides with specificity similar to the Bence Jones protein. The single chain Fv constructs (MRT-3, YZ17) were derived from murine phage display libraries by selection based on binding to phosphonate monoester haptens (*18*, *19*). Each of the purified protein preparations contained a major ∼29 kDa band corresponding to intact scFv or light chains. Certain preparations contained minor degradation products stainable with anti-*c-myc* antibody (the recombinant proteins contain a c-myc peptide tag close to their C-terminus) or anti-human light chain antibody (Bence Jones proteins) (*17*, *18*). The antibody fragments were lyophilized after purification from water, stored at 4 °C, and reconstituted in 1× PBS (0.137 M NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) to a concentration of 20 μM. The antibody samples were further diluted to working concentrations of 10 μM in 1× PBS before being used for kinetic analyses.

Purity of c23.5 Light Chain Antibody. To verify the purity of the c23.5 and hk14 samples, we analyzed the samples by SDS−PAGE. A major protein band at 28 kDa, corresponding to the c23.5 or hk14 light chain antibody fragment, was observed in each case (data not shown). The c23.5 sample also showed two much fainter protein bands corresponding to degradation products at approximately 15 and 13 kDa as reported previously (*20*).

Catalytic Assays. To test for antibody hydrolysis following lysine residues, we utilized the synthetic lysine substrate benzyloxycarbonyl-L-lysine *o*-nitrophenyl ester (Z-lys-o-Np) (Sigma-Aldrich, St. Louis, MO). A total of 10 μL of 10 μM antibody in 1× PBS was reacted with 10 μL of 10 mM Z-lys-o-Np dissolved in 100% methanol and diluted to the final concentration in 1× PBS (pH 7.4, $T = 25$ C). The extent of reaction was determined by following release of *o*-nitrophenol after 5 min by measuring OD_{400} using a Beckman DU 520 UV/Vis spectrophotometer. Proteolytic activity was calculated by subtracting the sample reading from a control aliquot containing only 10 mM Z-lys-o-Np in 1× PBS.

MS was used to identify degradation products of the hydrolytic reactions. We initially defined the MS assay conditions using the control protease, trypsin, and Aβ1−28 (Aβ28) since this model substrate is more readily soluble than Aβ1−40 or 42. A 1-mg vial of lyophilized Aβ28 (Sigma-Aldrich, St. Louis, Mo.) was dissolved in 100 μL of hexafluoro-2-propanol (HFIP) (Sigma-Aldrich), sonicated, and filtered to remove any undissolved particles, and then diluted with sample buffer (1× PBS, pH 7.4) to working concentrations of 0.1, 1, 5, and 10 mM. A 160 IU/mL concentration of trypsin (porcine pancreas containing 16 000 units/mg, Sigma-Aldrich, lot T-0134) was prepared (0.01 mg/mL of trypsin in 1 mM HCl). Aβ28 was reacted with trypsin for 5 min at 25 °C after which the products were identified by MS analysis. For MS analyses, 4 μL of the reaction mixture matrix containing each of the different concentrations of beta amyloid in trypsin (50:1 volume ratio) was added to 5 μL of α-cyano-4-hydroxy cinnamic acid in 50% acetonitrile containing 0.5% trifluoroacetic acid. A 2 μL aliquot of the above mixture was taken and spotted onto a stainless steel MS sample pin. MS analysis was performed using a Voyager-DE STR Biospectrometry Workstation operated in the positive ion mode and using the reflectron. The accelerating voltage was 20 000 V and data were acquired over a mass range of 1−5000 Da. Each spectrum was typically the average of 100 laser shots. MS data were obtained at different laser intensities and the spectrum showing the most product peaks was chosen.

Once suitable conditions were developed for observing proteolytic fragments resulting from digestion of Aβ28 by trypsin, we utilized similar conditions to test for antibody-mediated proteolysis of Aβ40. Since the principal objective of the MS assay is to identify the products of proteolysis, high substrate concentrations of Aβ were utilized to enhance the probability of observing minor product peaks.

For hydrolysis by antibody samples, 2.5 and 0.25 μM antibodies (PBS, pH 7.4) were reacted with 1 mM Aβ40 in 1× PBS (pH 7.4, 25 C). Five microliter aliquots were removed at time intervals of 0, 6 h, 1 day, 2 days, and 3 days and analyzed by MS as described above. Control samples were taken with 1 mM Aβ40 without any antibody, to rule out the possibility of Aβ40 self-degradation after 3 days in 1× PBS solution.

Zymogram Gel Protocol. A total of 15 μL of the antibody sample (2.5 μM in 1× PBS pH7.4) was mixed with 30 μL of the Laemmeli sample buffer (Bio-Rad, Hercules, CA), and a 20 μL aliquot was loaded onto precast 12% polyacrylamide zymogram gel (Bio-Rad). Proteins were separated by electrophoresis for 60 min at room temperature at 100 V. After electrophoresis, the gel was incubated in 100 mL of 1× Zymogram Renaturing buffer (Bio-Rad) with gentle agitation for 30 min at room temperature. The renaturing buffer was decanted and equilibrated in 10% Zymogram Development buffer (Bio-Rad) with gentle agitation. The renaturing buffer was then replaced and the gel was incubated overnight with gentle agitation. The gel was stained using 100 mL of 0.5% (w/v) Coomassie Blue R-250 stain (Phast Gel Blue, Sigma-Aldrich) and destained with a 100 mL

Table 1: Hydrolysis of 5 mM Z-lys-o-Np Substrate in 1× PBS, pH 7.4 at 25 °C by 5 μM Concentrations of Different Antibody Samples[a]

sample	OD_{400}
blank control	0.122
MRT − 3	nd
hK-14	0.143
GG 63	nd
YZ 17	nd
LAY	nd
JOH	nd
XOC	nd
LEN	nd
LOC	nd
RHY	nd
c23.5	0.328
BB-6	nd

[a] OD_{400} readings represent sample readings minus the blank control value without antibody. nd denotes samples in which activity was not detected.

solution containing 50% methanol and 10% acetic acid. Light bands against a dark background correspond to protein bands having proteolytic activity.

Evaluation of Kinetic Parameters. To determine the kinetic parameters of the c23.5 light chain antibody, we used the chromogenic substrate representing the α-secretase site, Z-lys-o-Np. Different concentrations of Z-lys-o-Np (0.25, 0.2, 0.16, 0.08, 0.04, 0.02 mM in pH 6.8 1× PBS) were reacted with 0.08 μM c23.5 light chain at 25 °C, and release of *o*-nitrophenol was followed as described above by following the OD_{400} at different time points. Product concentrations of *o*-nitrophenol were calculated by subtracting the OD_{400} value obtained from a sample of Z-lys-o-Np without added antibody from the samples containing antibody. Reaction rates at each substrate concentration were calculated, and the kinetic parameters, k_{cat} and K_M, were determined by plotting the substrate concentration versus the reaction rate and fitting the data by nonlinear regression using Michaelis−Menten kinetics.

RESULTS

Screen for α-Secretase Activity Using Z-lys-o-Np. Our preliminary screen for α-secretase like activity, which cleaves Aβ between lysine16 and leucine17, utilized the synthetic lysine substrate, Z-lys-o-Np. The sensitivity of the screening assay for antibody catalytic activity was limited by spontaneous release of *o*-nitrophenol from Z-lys-o-Np. The reactions were followed by measuring the release of the *o*-nitrophenol group after 5 min of incubation with the antibody. Under these conditions, we identified two proteolytic antibody light chains that hydrolyzed Z-lys-o-Np, c23.5 and hk14 (Table 1).

Mass Spectrometry (MS) Assay. The two proteolytic antibody light chains, c23.5 and hk14, were incubated with Aβ40 and production of proteolytic peptide fragments was analyzed using MS. In preliminary studies using trypsin as a catalyst and Aβ28 as substrate, we found that the minimal concentration of substrate needed to reproducibly detect product peptides was 1 mM (data not shown). This concentration of Aβ40 was utilized in all subsequent assays. Additional studies were conducted to establish the stability of Aβ40 in the absence of antibodies over prolonged

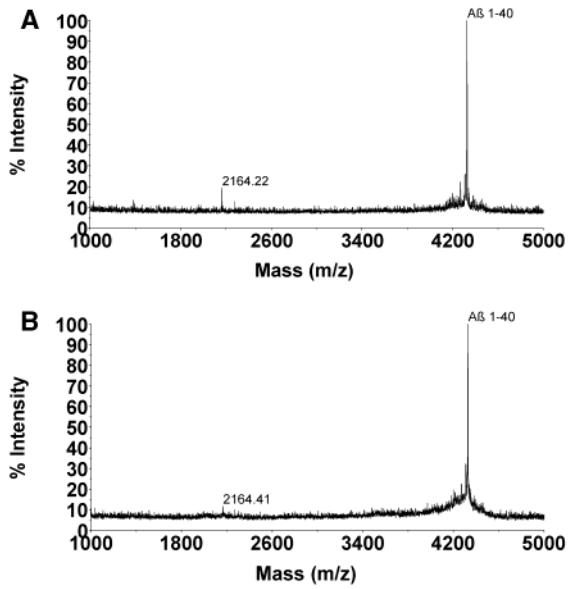

FIGURE 1: Assay for self-degradation products of Aβ. 1 mM Aβ40 in PBS (pH 7.4) was incubated at 25 °C over a three-day period to test for hydrolytic products. Mass spectrometry was performed on aliquots taken after (A) 0 h and (B) 3 days. The major peak at m/z = 4327 corresponds to full-length Aβ40, and the small peak at m/z = 2160 represents a minor contaminant in the original Aβ40 sample.

incubation times at 25 °C. No uncatalyzed degradation of Aβ40 was observed over 3 days of incubation. The only peptide species observed by MS were the full-length Aβ40 peptide (m/z 4327) and a minor contaminant present in the original peptide preparation (m/z 2164) (Figure 1).

Incubation of Aβ40 with antibody light chain c23.5 (Figure 2) and light chain hk14 (Figure 3) resulted in degradation of Aβ40 detectable by MS. Incubation of Aβ40 with 0.1μM c23.5 light chain showed significant hydrolysis after 1 day (Figure 2B), with essentially complete disappearance of the original Aβ40 peak at m/z 4327 by day 3 (Figure 2B). If Aβ40 is partially degraded by hydrolysis of the peptide bonds on the C-terminal side of its three lysine residues (positions 5, 16, and 28), the following peptide fragments could be generated (numbers refer to positions in the Aβ40 sequence with corresponding mass (in Da) indicated in parentheses): 1−5 (637); 29−40 (1062); 6−16 (1316); 17−28 (1331); 1−16 (1953); 17−40 (2385); 6−28 (2649); 1−28 (3286); 6−40 (3711); and 1−40 (4327). The two major proteolytic fragments observed following hydrolysis of Aβ40 by light chain c23.5 display m/z at 1953, corresponding to the amino terminal 1−16 fragment of Aβ40 (Figure 2A,B), and m/z at 2385, corresponding to the 17−40 carboxyl terminal fragment (Figure 2B). The concentration of the 17−40 fragment may decrease with time (Figure 2B) due to either the additional proteolytic cleavage following Lys28, or due to the aggregation with the 1−40 protein. Deletions at the more soluble amino terminal region of Aβ have been shown to facilitate aggregation of the subsequent peptides (*21*).

The second antibody light chain, hk14, also hydrolyzed Aβ40 (Figure 3) but with a distinctly different cleavage pattern than that observed with the c23.5 light chain. After 1 day of incubation with light chain hk14, two major product species were observed at m/z 2463 and m/z 1562, corresponding to the 1−20 and 1−13 amino acid Aβ fragments,

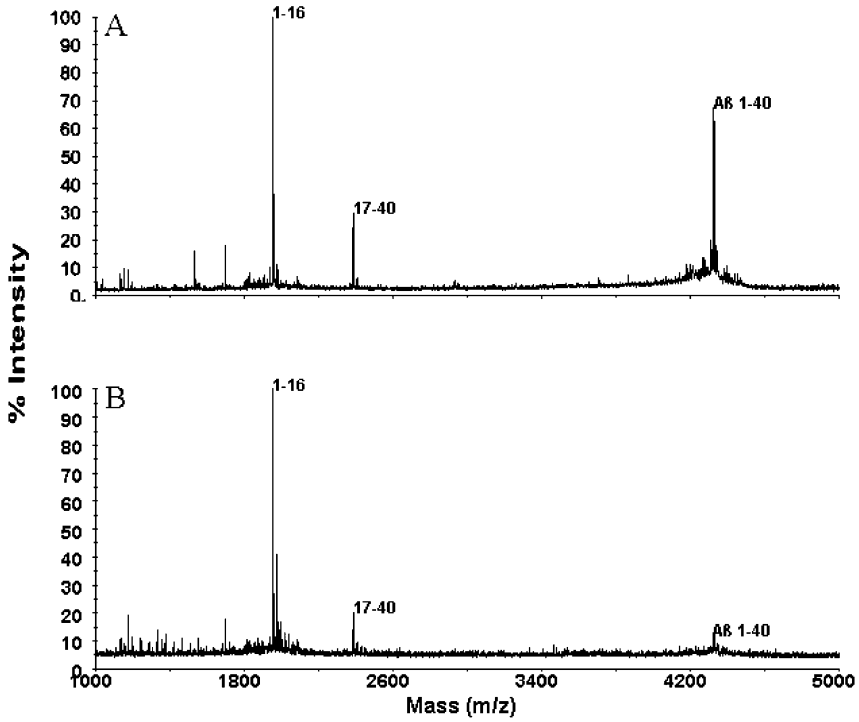

FIGURE 2: Proteolytic degradation of Aβ40 with antibody fragment c23.5 light chain. 1 mM Aβ40 was incubated with 2.5 μM c23.5 light chain in PBS (pH 7.4), at 25 °C over a three-day period to test for proteolytic activity. Mass spectrometry was performed on aliquots taken after (A) 0 day and (B) 3 days. Peaks corresponding to Aβ 1−16 (m/z = 1953), 17−40 (m/z = 2385), and full-length 1−40 (m/z = 4327) are indicated.

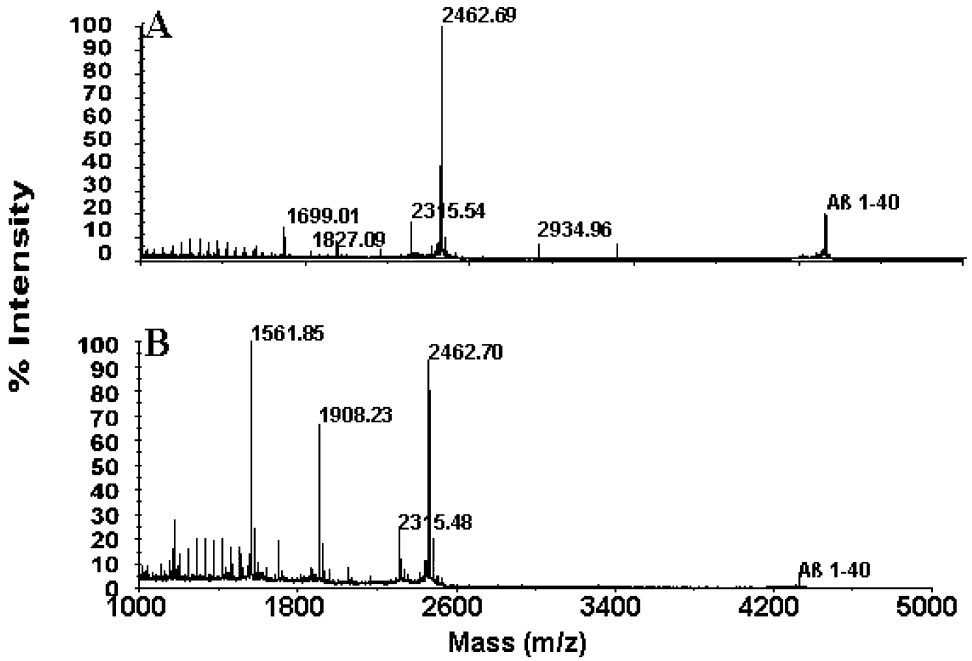

FIGURE 3: Proteolytic degradation of Aβ40 with antibody fragment hk14 light chain. 1 mM Aβ40 was incubated with 2.5 μM hk14 light chain in PBS (pH 7.4), at 25 °C over a three-day period to test for proteolytic activity. Mass spectrometry was performed on aliquots taken after (A) 1 day and (B) 3 days. The peak corresponding to full-length 1−40 (m/z = 4327) is indicated.

respectively (Figure 3A). We were able to identify MS peaks corresponding to nearly all of the amino Aβ fragments ranging from 1−5 to 1−40 amino acids in length differing only by a single residue at the C-terminus (Table 2 and Figure 3B). MS peaks corresponding to amino acid fragments 1−5 to 1−30 are visible in Figure 3B, while peaks corresponding to fragments 1−31 to 1−39 can be discerned by changing the z-scale (data not shown). Peptides smaller than the 1−5 fragment could not be observed using the MS protocol described due to the presence of solvent peaks. This hydrolytic fragment pattern representing sequential cleavage of single amino acids from the carboxyl terminal region of Aβ40 indicates that the hk14 light chain antibody fragment has carboxypeptidase-like activity toward Aβ.

Determination of c23.5 Light Chain Kinetic Constants. The kinetic parameters of light chain antibody C23.5 were determined using the synthetic substrate Z-lys-o-Np. Typical saturation kinetics expected for enzymatic hydrolysis were

Table 2: Molecular Mass of Expected and Observed Carboxyl Terminal Hydrolysis Products of Aβ40[a]

Aβ fragment (amino acid numbers)	expected fragment weight in kDa	observed fragment weight in kDa
1−40	4331	4329.79
1−39	4232	4232.02
1−38	4133	4133.08
1−37	4074	nd
1−36	4018	4018.07
1−35	3919	3918.44
1−34	3788	3787.66
1−33	3674	3676.48
1−32	3618	nd
1−31	3505	nd
1−30	3391	3391.30
1−29	3320	3320.30
1−28	3263	3261.98
1−27	3135	3132.99
1−26	3020	3023.10
1−25	2933	2933.97
1−24	2876	2875.70
1−23	2778	2777.79
1−22	2663	2663.81
1−21	2533	2532.79
1−20	2462	2461.75
1−19	2315	2315.51
1−18	2168	2168.40
1−17	2068	2067.75
1−16	1956	1956.19
1−15	1827	1827.06
1−14	1698	1698.98
1−13	1562	1561.90
1−12	1425	1425.94
1−11	1325	1325.56
1−10	1196	1198.46
1−9	1033	1032.67
1−8	976	976.39
1−7	889	891.64
1−6	774	773.43
1−5	637	637.39
1−4	481	nd
1−3	333	nd
1−2	204	nd
1−1	133	nd

[a] The expected fragment weights were calculated from the peptide property calculator located online at http://www.basic.nwu.edu/biotools/ProteinCalc.html. Observed peaks were identified by MS analysis of Aβ40 digestion by hk14 after 3 days as described in Figure 3 (See Figure 3B).

observed (Figure 4). The k_{cat} value is 2.52 ± 0.29/min and the K_M value is 346 ± 126 μM.

Detection of Proteolytic Activity in Zymograms. To verify that the α-secretase-like activity observed with c23.5 is attributable to the antibody light chain, we separated the components of the c23.5 light chain sample on a zymogram gel containing casein. For this, 24 and 12 μM samples of the light chain c23.5 and 0.01 μM trypsin were run on a 12% casein precast Zymogram gel. In this system, a proteolytic protein band is predicted to digest the casein contained within the gel resulting in an unstained band on a stained background. The light chain c23.5 showed a single proteolytic band having a molecular weight of approximately the same mass as the 23.8 kDa control proteolytic protein trypsin (Figure 5), indicating that the observed proteolytic activity was due to the antibody light chain protein and not trace amounts of a contaminating protease. Furthermore, the c23.5 light chain and hk14 light chain antibody samples were expressed and purified from *Escherichia coli* in a similar manner as three other antibody fragments studied that did not display proteolytic activity (YZ17, MRT-3, and GG63). We also tested these three light chain antibody fragments samples for activity toward Aβ40 using the MS assays, and none of them showed any hydrolysis of Aβ40 (data not shown).

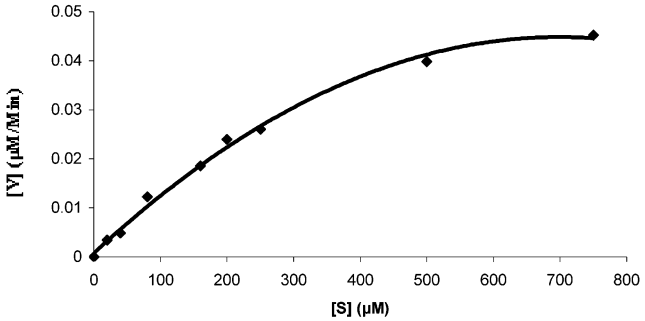

FIGURE 4: Saturation kinetic behavior of antibody fragment c23.5 light chain. Plot of reaction rate [*V*] vs substrate concentration [S] for the hydrolysis of 10 mM Z-lys-o-Np using 0.08 μM c23.5 light chain antibody fragment in 1× PBS, pH 6.8, T = 25 °C. Kinetic constants were determined by plotting the substrate concentration versus the reaction rate and fitting the data by nonlinear regression using Michaelis−Menten kinetics giving k_{cat} = 2.52 ± 0.29/min, and K_M = 346 ± 126 μM.

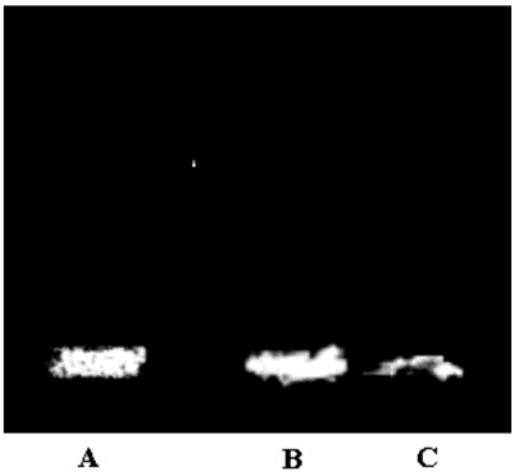

FIGURE 5: Zymogram gel indicating proteolytic activity of the c23.5 antibody. Lane (A) 0.01 μM trypsin; (B) 24 μM C23.5 light chain antibody; (C) 12 μM c23.5 light chain antibody. Lanes B and C indicate c23.5 samples (28 kDa) contain a single concentration-dependent proteolytic band of approximately the same size as the 23.8 kDa trypsin control.

DISCUSSION

Increased accumulation of Aβ, particularly Aβ42, appears to be a critical factor in AD pathology; decreasing Aβ concentration represents a potentially viable therapeutic or preventative approach. Immunization of mouse models for Aβ deposition with aggregated Aβ was shown to delay deposition of Aβ and to clear Aβ deposits already present in the brain (*22*). Passive immunization of these same mice by periodic injection of antibodies generated against Aβ also delayed the deposition of Aβ and reduced Aβ deposits that were already present (*23*). The clearance of Aβ deposits from brain tissue in an ex vivo assay was correlated with Fc receptor mediated phagocytosis (*23*), an inflammatory cell function. Behavior studies of mice that were immunized against Aβ also showed reduced memory loss and behavioral

impairment supporting the therapeutic value of this approach (*24*, *25*). However, there is considerable evidence that AD is an inflammatory disease (reviewed in ref *26*), and antibody-mediated clearance by phagocytosis could potentially exacerbate brain inflammation and damage. Recent Phase IIA studies testing whether Aβ vaccination could provide a similar benefit in humans were canceled due to potential inflammation in the central nervous system of selected patients, demonstrating the potential drawback of this approach (*27*). Further, passive immunization with antibodies against Aβ was also shown to lead to an increased risk of cerebral hemorrhage in mouse models of AD (*28*). Therefore, conventional antibody-mediated mechanisms for clearance of Aβ, while having very encouraging results, can lead to prohibitively dangerous side effects.

The present study indicates the feasibility of an alternative approach that utilizes the catalytic capabilities of antibodies to facilitate clearance of amyloid deposits and reduce the toxicity of Aβ. Since these recombinant antibody fragments lack the heavy chain constant region, they do not initiate an inflammatory response, reducing the risk of toxic side effects. Two antibody light chains were identified that cleave Aβ40. One antibody fragment, the hk14 light chain, has exoproteolytic activity cleaving from the carboxyl terminal residues from Aβ. Since the hydrophobic carboxyl terminal residues of the longer Aβ42 form have been shown to facilitate aggregation in vitro compared to the Aβ40 form (*29*, *30*), sequential cleavage of the carboxyl terminal residues could increase the solubility of Aβ and decrease formation of toxic aggregates.

The second antibody fragment, the recombinant light chain antibody, c23.5, has α-secretase like activity, degrading Aβ40 on the C-terminal side of lysine residues. This light chain antibody has previously been shown to possess serine protease-like activity, based on its covalent reactivity with phosphonate diesters that bind to the active site of serine proteases (*18*). While additional studies are needed to make accurate quantitative assessments of the concentrations of the different degradation products, clearly the most prominent peaks detected by MS resulting from hydrolysis of Aβ40 by light chain c23.5 are the 1−16 and the 17−40 fragments. Additional minor peaks corresponding to peptide fragments resulting from hydrolysis at the other lysine residues of Aβ40 are also evident. These data indicate that the primary cleavage site of Aβ40 by c23.5 light chain is identical to the α-secretase cleavage site, i.e., between Lys16 and Leu17. MS analysis may not identify all the carboxyl terminal fragments that are produced since the amino terminal region of Aβ40 is more soluble than the carboxyl terminal (*29*, *31*), and amino terminal deletions of Aβ40 have been shown to result in increased aggregation rates compared to the intact peptide (*21*).

Antibody light chain c23.5 demonstrates typical saturation kinetics observed for enzymes. The turnover of c23.5 light chain is 2.5/min using the ester substrate. This turnover rate compares favorably with the turnover of previously published esterase antibodies (reviewed in refs *14* and *15*)). The light chain has previously been observed to cleave vasoactive intestinal polypeptide (VIP) with $K_M \sim 0.2$ micromolar and $k_{cat} \sim 0.01$/min (*20*). The lower K_M for VIP is consistent with its derivation from an antibody raised by immunization with VIP. The present study suggests that the light chain can also recognize other peptide substrates, albeit with lower affinity for the peptide ground state as 1 mM substrate concentrations were used in the present experiments. Similarly, antibody light chain *hk*14 can also recognize peptide substrates other than its original target antigen, and can sequentially cleave amino acids from the carboxyl-terminus similar to carboxypeptidases.

Other potential approaches to supplement proteolytic cleavage of the Aβ peptide have been studied, in particular insulin-degrading enzyme (IDE). IDE besides cleaving insulin, regulates extracellular levels of Aβ peptide by degradation in vitro (*32*) and in vivo (*33*), and also degrades other polypeptide hormones including insulin growth factor, atrial natriuretic peptide, glucagon, and beta-endorphin. The sequences around these different IDE cleavage sites show little or no similarity to each other (*34*), suggesting a broad substrate specificity for IDE. Cleavage of Aβ by IDE can enhance oligomerization of Aβ at physiological concentrations in vitro due to the formation of more hydrophobic, truncated N-terminal fragments (*35*). We have observed this same effect with the c23.5 antibody in preliminary studies where cleavage after Lys16 should result in formation of a more hydrophobic 17−40 fragment, enhancing oligomerization (data not shown). Because IDE has activity toward a number of substrates that may also influence cellular function, its use as a therapeutic may have unwanted side effects.

The proteolytic antibody light chains identified here also should have broad substrate specificity; however, the real value of these antibody fragments as potential therapeutics for degrading Aβ will depend on the success of future antibody engineering studies to increase the specificity of the parent light chains and to control oligomerization. Recombinant antibody affinities can be increased substantially by various in vitro affinity maturation protocols (*36*−*38*). The specificity of recombinant proteolytic antibody light chains for a given substrate can be increased without significantly decreasing k_{cat} by pairing the light chain with heavy chains that bind the target substrate (*39*). Turnover rates for recombinant antibodies can also be increased by affinity maturation techniques using appropriate phage display technology coupled with selection for catalytic activity as previously demonstrated (*18*). Finally, engineering bispecific antibody fragments can also increase proteolytic targeting: a construct where one scFv site contains the catalytic activity, and the second scFv targets a particular protein or even morphology. While the rate of proteolytic degradation of Aβ need not be extremely robust to achieve a therapeutic effect since Aβ burdens accumulate slowly over a period of years and antibodies can have long half-lives in vivo allowing for prolonged degradation of Aβ, the proteolytic activity needs to be targeted specifically to Aβ. Since affinity maturation procedures can increase the affinity of antibodies into the femtomolar range (*38*), the light chains described here are promising reagents from which specific antibodies to Aβ can be prepared. Since both Aβ40 and Aβ42 form similar supramolecular and fibrillar forms although at different rates and with different intermediate morphologies (*40*−*42*), future studies will need to address whether it is desirable to engineer antibodies that have specificity for a particular form or morphology of Aβ. Additionally, given the small size of the recombinant antibody fragments,

transport across the restrictive blood-brain barrier can be facilitated.

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