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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 14 (2004) 4529–4532

Antibody light chain-catalyzed hydrolysis of a hepatitis C virus peptide

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Received 28 April 2004; revised 12 June 2004; accepted 12 June 2004
Available online 3 July 2004

Abstract—A panel of human monoclonal and recombinant antibody light chains was screened for cleavage of the synthetic peptide corresponding to a neutralizing epitope of hepatitis C virus (residues 192–205 of envelope glycoprotein E1). One of the 39 light chains studied hydrolyzed the Val197–Ser198 bond of the peptide with K_m and k_{cat} values of $223 \pm 7\,\mu M$ and $0.087 \pm 0.001\,min^{-1}$.

Antibodies (Abs) with peptidase activity specific for microbial proteins represent a promising means for inactivation of pathogenic microbes.[1] A number of Abs that catalyze the hydrolysis of low-molecular-weight esters have been successfully obtained by immunization with transition state analogs.[2] This strategy, however, has not yielded peptidase Abs until now,[3] presumably because peptide bond hydrolysis is a more complex and energetically demanding reaction. In contrast, several examples of peptide/protein cleavage by naturally occurring Abs and Ab light chain subunits found in certain disease states have been reported.[4] Most proteolytic Abs and Ab fragments hydrolyze small amide substrates promiscuously by a serine peptidase-like mechanism.[4,5] Selective hydrolysis of larger peptides by Abs is more rare, and when observed, evidently entails noncovalent epitope binding in concert with nucleophilic attack on peptide bonds.[4,6] Light chain subunits capable of cleaving the targeted peptide are appropriate starting materials for molecular engineering of therapy-grade catalytic Abs, as the technologies have recently become available for improving the catalytic properties if needed. For example, improved catalytic selectivity can be obtained by mutagenesis at the appropriate residues in the complementarity determining regions of light chains, which can enhance the affinity of peptide ground state recognition.[7] Alternatively, the catalytic selectivity of light chains can be directed to the targeted antigenic epitope by pairing with heavy chains known to bind the epitope.[8]

The goal of this study was to identify a candidate Ab light chain suitable for molecular engineering of catalysts selective for the envelope glycoprotein E1 of hepatitis C virus (HCV). HCV infection is one of the leading causes of liver disease, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma.[9] The development of immunotherapeutics for HCV is an active area of research.[10] We recently reported a human monoclonal HCV neutralizing Ab that binds a defined determinant located in the E1 protein (residues 192–205).[11] In principle, the heavy chain of this Ab could serve as the donor directing the selectivity of a peptidase light chain toward E1, provided the light chain expresses an active site capable of recognizing this E1 peptide determinant as a catalytic target. In this study, we screened a panel of well-characterized Ab light chains for the capability of hydrolyzing the synthetic peptide corresponding to E1 residues 192–205. One light chain displayed the desired activity.

The panel of light chain screened was a random collection composed of 27 Bence Jones proteins isolated from

Keywords: Antibody; Peptidase; Hepatitis C virus.
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doi:10.1016/j.bmcl.2004.06.044

the urine of multiple myeloma patients,[12] 11 recombinant human light chains,[13] and one recombinant mouse light chain.[14] The light chains were purified to electrophoretic homogeneity as described previously.[4b,5d,14,15] By silver staining and sodium dodecylsulfate-polyacrylamide gel electrophoresis, a predominant monomer light chain band with mass 25–27 kD was observed. Some light chain preparations contained a dimmer band reducible with 2-mercaptoethonol.[4b] Both bands were stainable with anti-human light chain antibody by immunoblotting experiments.[4b] The substrate peptide, HCV E1 192–205 (**1**; Tyr-Glu-Val-Arg-Asn-Val-Ser-Gly-Val-Tyr-His-Val-Thr-Asn), was synthesized with the conventional solid-phase method using Fmoc-protection chemistry.[16] The peptide **1** (100 μM) was incubated in the presence or absence of light chains (0.5 μM) in phosphate buffered saline (pH 7.4) containing 0.01 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.05% Tween-20, and 2.5% CH_3CN at 37 °C for 48 h. The reaction was terminated by adding 1% TFA and analyzed by HPLC. Of 39 light chains screened, one light chain from a multiple myeloma patient (light chain LAY) showed **1** cleaving activity, evident from the appearance of two new HPLC peaks that were absent in the peptide reaction mixtures containing other light chains or no light chain. The cleavage profile of the E1 peptide **1** by light chain LAY is shown in Figure 1A. Molecular mass of products P1 and P2 determined by electro-spray ionization mass spectrometry were in good agreement with calculated values for 198–205 and 192–197 of E1, respectively (Fig. 1B), indicating that the Val197–Ser198 bond was cleaved.

Cleavage of **1** by LAY light chain was further studied using ^{125}I-labeled **1**,[17] which permits quantitative measurement of the cleavage product. HPLC analysis of ^{125}I-**1** ($(841 \pm 7) \times 10^3$ cpm) incubated with LAY light chain (0.5 μM) revealed a single radioactive product (Fig. 1C). As expected, no appreciable cleavage of the peptide was detected in samples incubated with a control myeloma light chain LEN or without light chain. The detection of a single radiolabeled **1** fragment is in agreement with the results of studies using nonradioactive **1** as substrate, if the radioactive peptide is assumed to carry ^{125}I at a single site. In this case, only one radiolabeled product is anticipated if the peptide is cleaved at a single site, as suggested by the data shown in Figure 1A and B. Kinetic parameters were determined from the substrate concentration dependency of the reaction. The light chain LAY (0.25 μM) was incubated with varying concentrations (0.1, 1.0, 10, 100, 300 μM) of **1** containing a constant concentration of ^{125}I-**1** (~1 nM) at 37 °C for 45 h. Substrate consumption was estimated from the amount of radioactivity in the product fractions with the assumption that the radiolabeled and nonradiolabeled peptides are cleaved at the same rate ([product] = [**1**] × (% radioactivity in the product fraction)/100). K_m and k_{cat} values obtained from the best-fit curve to the Mechaelis–Menten equation ($V/V_{max} = [S]/(K_m + [S])$; $k_{cat} = V_{max}/[\text{light chain}]$) were 223 ± 7 μM and 0.087 ± 0.001 min^{-1} ($r^2 > 0.99$). The submillimolar K_m value is in the same range as for cleavage of peptide substrates by conventional peptidase enzymes,[18] suggesting modest recognition of the **1** ground state. In comparison, K_m values for proteolysis by Abs specialized for recognition of individual peptide ground states are 2–5 orders of magnitude smaller.[4,6,15]

Previous studies indicate that certain light chains utilize a nucleophilic mechanism for catalysis.[4h,5b,d,6b,c] An active site-directed serine peptidase inhibitor, diphenyl *N*-[6-(biotinamido)hexanoyl]amino(4-amidinophenyl)methanephosphonate[19,20] (**2**; Fig. 1D), was assessed for capability of inhibiting ^{125}I-**1** cleaving activity of LAY light chain. Phosphonates including **2** have previously been validated as irreversible inhibitors and covalent active site modifiers of serine peptidases[19,21] and certain peptidase Abs by kinetic and structural studies.[5d,20,22] Phosphonate **2** inhibited hydrolysis of ^{125}I-**1** by the light chain in a concentration dependent manner (Fig. 1D). Analysis of **2**-treated LAY light chain subjected to heating (100 °C, 5 min) and denaturing gel electrophoresis revealed a single **2**-containing band at 27 kD corresponding to the **2**-light chain adduct (Fig. 1D, inset). The apparent irreversible reaction suggests that inhibition of the peptidase activity by **2** is due to covalent modification of the active site nucleophile in the light chain.

Several lines of evidence suggest that the peptidase activity identified in the present study belongs to the light chain as opposed to a trace contaminant. Notably, **2** inhibited the peptidase activity, but the electrophoresis analysis failed to detect a **2**-reactive species other than the light chain itself. Other light chains purified in the manner identical to the peptidase light chain did not display the activity. *N*-Terminal sequence analysis of the peptidase light chain preparation has previously yielded a single peptide sequence corresponding to the κII subgroup light chain, with no evidence for the presence of contaminating proteins.[4g] Gel filtration studies of the light chain in a denaturing solvent (6 M guanidine hydrochloride) failed to remove the peptidase activity, suggesting the absence of noncovalently associated adventitious peptidases.[4b]

The peptidase light chain identified in this study was previously described to hydrolyze model peptide substrates on the C-terminal side of basic amino acid residues.[4b] While hydrolysis of **1** at the Val197–Ser198 bond was readily detected, there was no observable cleavage of the only bond containing a basic amino acid residue, Arg195–Asn196. In addition, no cleavage at other Val–X bonds in peptide **1** was evident (Val194–Arg195, Val200–Tyr201, and Val203–Thr204). It may be concluded that the presence of a basic amino acid is not an absolute requirement for the hydrolysis reaction, and recognition of the Val residue alone is not sufficient for the reaction to proceed. Several light chains screened in the present study were reported to hydrolyze small peptide methylcoumarylamides,[4b] but only one displayed the desired **1** cleaving activity. This light chain is distinguished, therefore, by its favorable interactions with peptide **1**. The **1**-hydrolyzing light chain offers novel routes to immunotherapy of HCV infection, but further improvements in efficiency and specificity are

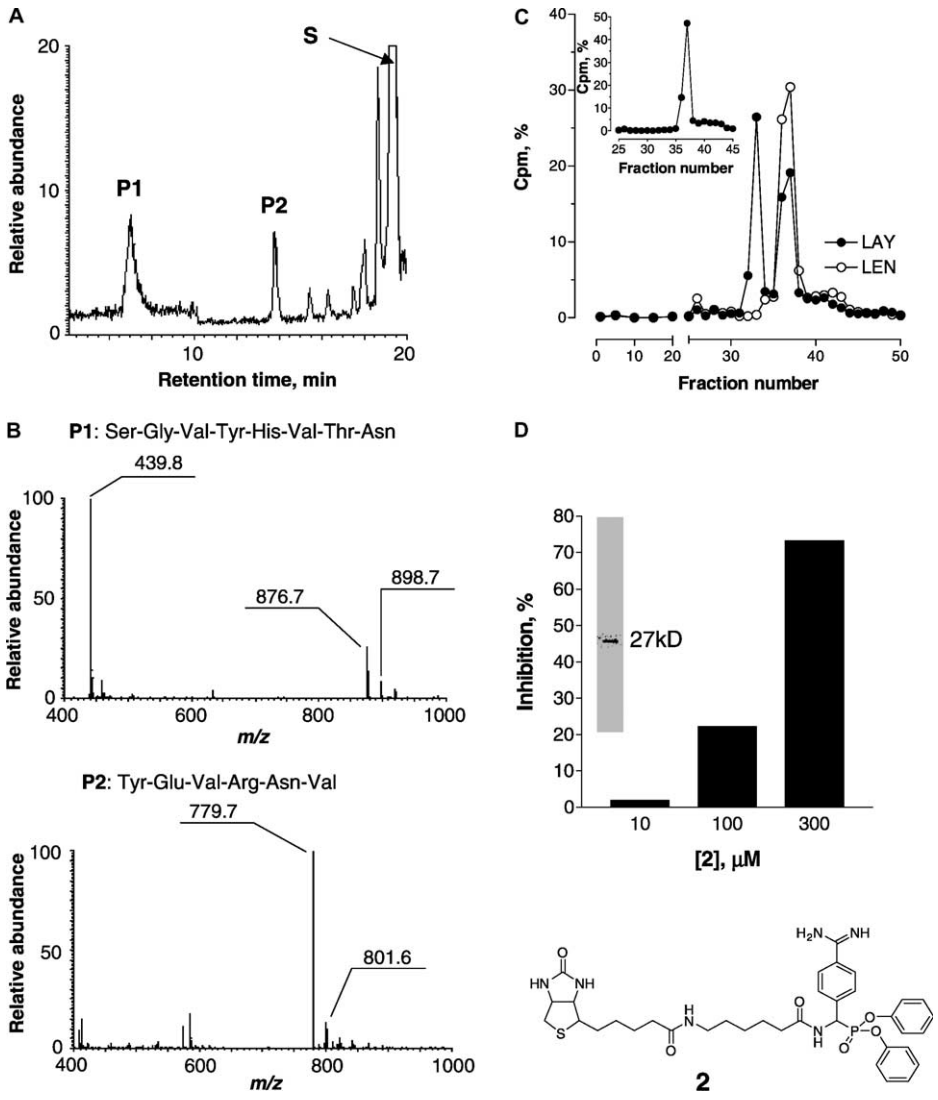

Figure 1. Hydrolysis of peptide **1** by LAY light chain and inhibition of the light chain activity by **2**. (A) Total ion current chromatogram of **1** incubated with light chain LAY. Two product peaks were detected at 7.0 min (P1) and 13.8 min (P2). S (19.3 min) denotes the substrate peptide **1**. All peaks other than P1 and P2 were also observed in control runs (**1** incubated without light chain and with control light chain LEN). Compound **1** (100 μM) was incubated with LAY light chain (0.5 μM) at 37 °C for 48 h. HPLC conditions: column, 238MS5215 C18 2.1×150 mm (Grace Vydac; Hesperia, CA); solvents, 0.2% formic acid in water: 0.2% formic acid in CH_3CN 95:5 for 5 min, 95:5 to 75:25 in 25 min; flow rate, 0.2 mL/min; detector, LCQ DECA XP plus mass detector (Thermo Electron; San Jose, CA). (B) Electro-spray ionization mass spectra of P1 and P2. P1: molecular mass determined from observed m/z values for quasi-molecular ions was 875.7, in good agreement with the calculated value for E1 peptide 198–205 ($C_{38}H_{57}N_{11}O_{13}$, 875.4; calcd m/z for $(M+2H)^{2+}$ 438.7, $(M+H)^+$ 876.4, $(M+Na)^+$ 898.4). P2: molecular mass observed was 778.7, in good agreement with the calculated value for E1 peptide 192–197 ($C_{34}H_{54}N_{10}O_{11}$, 778.4; calcd m/z for $(M+H)^+$ 779.4, $(M+Na)^+$ 801.4). (C) HPLC profiles of ^{125}**I-1** incubated with light chain LAY (•), LEN (○) or without light chain (inset). Incubation was at 37 °C for 48 h. HPLC conditions: column, Nova-Pak C18 3.9×150 mm (Waters; Milford, MA); solvent, 0.1% TFA in water: 0.1% TFA in 80% CH_3CN–water 95:5 for 10 min, 95:5 to 50:50 in 45 min, 50:50 to 0:100 in 5 min, 0:100 for 5 min; flow rate, 0.5 mL/min. Radioactivity of each fraction was determined with a Packard Cobra II Auto-Gamma counter (Downers; Grove, IL) and expressed as percentage of the total radioactivity recovered in factions 1–75. For clarity, data from every five fractions are shown for the first 25 fractions. % CPM values in the product fractions (#32–34) were 35% (LAY), 3% (LEN) and 1% (without light chain). Reduction of radioactivity in the substrate fractions (#36–38) after incubation with LAY light chain was 31%. (D) Inhibition of light chain-catalyzed cleavage of ^{125}**I-1** by **2**. ^{125}**I-1** incubated with LAY light chain (0.2 μM) at 37 °C for 45 h in the presence or absence of **2** (10, 100, 300 μM; structure, lower panel) was analyzed by HPLC and % inhibition was computed from the decrease of radioactivity in the product fractions (#32–34). Percent cleavage in the absence of **2** was 63%. Inset: A streptavidin-peroxidase stained blot of an SDS-electrophoresis gel showing the light chain—**2** adduct. The light chain LAY (0.2 μM) was incubated with **2** (200 μM) at room temperature for 4 h.

desirable. Improvement of k_{cat} may potentially be obtained by mutagenesis followed by selection for increased nucleophilic reactivity, as described recently using phage-displayed light chain libraries.[5d] Other groups have reported that the heavy chain of Abs provides major contributions in antigen binding.[23] A suitable method for deriving Abs with enhanced E1 specificity is the pairing of the light chain with the heavy chain of HCV neutralizing Abs specific for E1 residues 192–205. We have previously reported that pairing of a

catalytic light chain with its noncatalytic heavy chain partner directed to the neuropeptide VIP results in reduced K_m for VIP.[8]

Acknowledgements

We thank Robert Dannenbring for expert technical assistance, and Dr. A. Solomon (University of Tennessee, Knoxville, TN) for generously providing the light chain preparations from multiple myeloma patients. This work was supported by United States Public Health Service Grants AI058865 and AI46029. S.K.H.F. was supported in part by PHS grants HL079381 and AI47355.

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12. Bence Jones proteins with following IDs were used in this study: B6, BOR, BOS, Cagle, COL, EVE, FRE, HAS, HOU, JOH, LAY, LEN, LOC, MCP, Patterson, Rhea, RHY, ROB, TEW, UND, WAT, WIT, XOC, Bostic, rCDR-1, rLEN, rREI. Proteins with IDs starting with 'r' are recombinant VL domains. Amidase activity of these proteins except Bostic, Cagle, Patterson, rCDR-1, Rhea, and TEW is reported in Ref. 4b.
13. Ten light chains are from a phage-displayed lupus light chain library [GG-63 (Genbank accession#, AF352557), GG-71, SK-18 (AF329459), SK-38, SK-45, SK-135, SK-147, SK-159, SK-161, SK-L6] and one from a phage-displayed asthma light chain library [hk-14 (L43499)]. The catalytic activity of GG-63 and hk-14 is reported in Refs. 5d and 15, respectively.
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16. Observed m/z (electro-spray ionization): 546.6 $(M+3H)^{3+}$, 819.1 $(M+2H)^{2+}$; calcd for $C_{72}H_{109}N_{21}O_{23}$ 546.3 $(M+3H)^{3+}$, 818.9 $(M+2H)^{2+}$.
17. ^{125}I-**1** was prepared in the usual manner by chloramines-T oxidation (Ref. 4a) and purified by HPLC. HPLC analysis of the reaction revealed two radioactive peptides (t_R 35.5 and 36.5 min; column, Waters Nova-Pak C18 3.9×150 mm; solvent, 0.1% TFA in water: 0.1% TFA in 80% CH_3CN–water 95:5 for 10 min, 95:5 to 50:50 in 45 min, 50:50 to 0:100 in 5 min, 0:100 for 5 min; flow rate, 0.5 mL/min). The peptide contains two Tyr residues at which labeling can occur, Tyr192 and Tyr197. Multiply iodinated peptides display prolonged retention times (Mody, R.; Tramontano, A.; Paul, S. *Int. J. Pept. Protein Res.* **1994**, *44*, 441). The earlier eluting species with t_R 35.5 min was used in this study.
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