Systemic lupus erythematosus: molecular cloning and analysis of recombinant monoclonal kappa light chain NGTA2-Me-pro-ChTr possessing two different activities—trypsin-like and metalloprotease

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Abstract

Polyclonal antibodies hydrolyzing myelin basic protein (MBP) can play an important role in the pathogenesis of multiple sclerosis and systemic lupus erythematosus (SLE). An immunoglobulin light chain phagemid library derived from peripheral blood lymphocytes of patients with SLE was used. The small pools of phage particles displaying light chains with different affinity for MBP were isolated by affinity chromatography on MBP-Sepharose. The fraction eluted with 0.5 M NaCl was used for preparation of individual monoclonal light chains (MLChs, 26–27 kDa). The clones were expressed in Escherichia coli in a soluble form; MLChs were purified by metal-chelating chromatography followed by gel filtration. In mammals, there are serine proteases and metalloproteases. These and many other enzymes usually have only one active site and catalyze only one chemical reaction. In contrast to canonical proteases, one MLCh (NGTA2-Me-pro-ChTr) efficiently hydrolyzed MBP (but not other proteins) and four different oligopeptides corresponding to four immunodominant sequences containing cleavage sites of MBP. The proteolytic activity of MLCh was efficiently inhibited only by specific inhibitors of serine-like (phenylmethanesulfonylfluoride, PMSF) and metalloproteases (EDTA). It was shown that MLCh possess independent serine-like and metal-dependent activities. The principal existence of monoclonal antibodies with two different proteolytic activities is unexpected but very important for the further understanding of at present unknown biological functions of human antibodies.

Keywords: hydrolysis of myelin basic protein, recombinant monoclonal light chain, similar amino acids of active center with serine-like and metalloproteases, systemic lupus erythematosus

Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system. Its etiology remains unclear, and the most widely accepted theory of MS pathogenesis assigns the main role in the destruction of myelin to the inflammation related to autoimmune reactions (1). Systemic lupus erythematosus (SLE) is a systemic autoimmune polyetiologic diffuse disease characterized by disorganization of conjunctive tissues with the paramount damage to skin and visceral capillaries (2). SLE and MS demonstrate some similarity in the development of medical, biochemical and immunological indexes including specific plaques in the brain (3). Similarly to MS patients, neuropsychiatric involvement occurs in about 50% of SLE patients and carries a poor prognosis [reviewed in (2)].

A special feature of autoimmune diseases including SLE and MS is high concentrations of auto-antibodies (antibodies to many different endogenous antigens) (4–7). Interestingly, the titers of antibodies against myelin basic protein (MBP) in SLE patients are 4.2-fold higher than in healthy individuals and only 2.1-fold lower than in MS patients (7). During the past two decades it has become clear that auto-antibodies from the sera of patients with different autoimmune diseases (AIDs) can possess enzymatic activities and that their occurrence is a distinctive feature of AIDs [reviewed in (4–7)].
Abzymes against transition chemical states of different reactions were studied intensively [reviewed in (4, 8–10)]. Similarly to artificial abzymes against analogs of transition states of catalytic reactions (4, 8–10), naturally occurring abzymes may be antibodies raised directly against enzyme substrates acting as haptenes and mimicking transition states of catalytic reactions (4–7). On the other hand, antidiotypic antibodies can be induced by a primary antigen and may show some of its features including the catalytic activity (11, 12).

Polyclonal natural IgG and/or IgA and IgM abzymes hydrolyzing DNA, RNA, polysaccharides, nucleotides, oligopeptides (OPs) and proteins from the sera of patients with several autoimmune and viral diseases including SLE and MS were revealed [reviewed in (4–7)]. It was shown that MBP-hydrolyzing activity is indeed an intrinsic property of IgGs and/or IgMs and IgAs of MS (13–15) and SLE patients (16, 17). Thus, MS and SLE patients can generate polyclonal abzymes attacking MBP in the myelin-proteolipid sheath of axons and play an important role in pathogenesis (7, 13–17).

The interesting goal of catalytic-antibody research is not only to study such abzymes but also to develop new patient and animal therapies that use the advantages offered by human and mammalian monoclonal catalytic antibodies. There are examples of monoclonal mouse IgGs hydrolyzing DNA (18–20). Bence-Jones proteins from patients with multiple myeloma should be considered as the first found natural human monoclonal abzymes (21). The MLCh of the anti-vasoactive intestinal peptide abzyme was expressed in bacteria, purified and found to possess an intrinsic VIP-hydrolyzing activity (20). Two MLChs from patients with multiple myeloma with prothrombinase activity were identified (22, 23). One MLCh from multiple myeloma patients specifically hydrolyzed gp120 protein (24). Recently MLChs capable of degrading the active site of the urease of *Helicobacter pylori* and eradicate the bacterial infection in a mouse stomach were obtained (25). A catalytic MLCh was obtained by immunizing with a peptide possessing a part of a sequence of a chemokine receptor, CCR-5, which is present as a membrane protein on the macrophage surface (26). Thirty-three kappa MLChs efficiently hydrolyzing DNA and demonstrating various physicochemical and enzymatic properties were obtained using an immunoglobulin light chain phagemid library derived from peripheral blood lymphocytes of patients with SLE (27, 28).

MLChs with metal-dependent proteolytic activity are of special interest. It was shown that Gp41 peptide antigen of the HIV-1 envelope was enzymatically degraded by the antibody light chain 41S-2-L (29). When EDTA was added in the induction period, it inhibited the degradation of TP41-1, thus ceasing the catalytic activity of 41S-2-L. In contrast, when EDTA was added after the induction period, only a small reduction in the catalytic activity was observed. These observations suggest that metal ions are important in stimulating catalytic activity early in the reaction. A metal-dependent heterodimer of immunoglobulin light chain variable domains that specifically hydrolyzes amyloid beta peptides was obtained (30).

Some healthy patients demonstrated abzymes with low proteolytic and polysaccharide-hydrolyzing activities (4–7). Healthy humans and patients with many diseases with insignificant autoimmune reactions usually lack abzymes or develop antibodies with very low DNase and some other activities, often on a borderline of the sensitivity of detection methods (4–7). At the same time, germline antibodies from healthy humans can express high-level promiscuous, amyloid-directed, and superantigen-directed activities and/or autoantigen-directed and microbe-directed specificities (31, 32).

It is known that the classical enzymes usually catalyze only one chemical reaction. For example, DNases are not able to hydrolyze RNA and vice versa RNases are not able to hydrolyze DNA. However, first it was shown that the recombinant c23.5 MLCh hydrolyses VIP and later that it has R-secretase-like activity producing the 1–16 and 17–40 amino acid fragments of beta-amyloid (33). The second MLCh (hk14) hydrolyzing VIP demonstrated carboxypeptidase-like activity, cleaving sequentially from the carboxyl terminus of beta-amyloid (33).

Many canonical proteases and metalloproteases usually have only one active site and specifically catalyze only one chemical reaction. We report in this article the first example of recombinant MLChs cloned from lupus patients using a phage library, which binds and hydrolyzes MBP and OPs corresponding to its four specific sequences (immunodominant sequences containing cleavage sites) demonstrating two different alternative proteolytic activities: serine-like and metal-dependent enzymatic activities.

**Methods**

**Materials and chemicals**

Most chemicals, proteins and the Superdex 200 HR 10/30 column were from Sigma while chelating Sepharose was from GE Healthcare. Human MBP was from the Department of Biotechnology, Research Center of Molecular Diagnostics and Therapy (Moscow). MBP-Sepharose was obtained by immobilizing of MBP on BrCN-activated Sepharose according to the standard manufacturer’s protocol.

**Amplification of phage library**

We have used human lupus kappa light chains library (from three patients; 10⁵ variants of different light chains); cDNA was cloned into the phagemid pCANTABHis6 vector after a leader sequence of the phage-coat protein gene pll of a filamentous *Escherichia coli* bacteriophage M13 between *SfiI* and *NolI* restriction sites using standard methods (34–37). This library was a gift from S. Paul and S. Plaque (University of Texas Houston Medical School, USA); all details of this library preparation were described earlier (35).

Amplification of the VCSM13 helper phage and determination of its titer were carried out according to (30). The amplification of the phage library was carried out as in (35–37).

**Chromatography of phage particles on MBP-Sepharose**

Preparations prepared using *E. coli* TG1 were exhausted from the main part of phage particles having affinity to DNA by chromatography on DNA cellulose according to (27, 28). Then preparations of phage particles (4 ml containing 2.5 × 10¹² phage particles) prepared using *E. coli* TG1 were loaded onto a MBP-Sepharose column (3 ml).
equilibrated with 20 mM Tris-HCl (pH 7.5) and the column was washed with the same buffer to zero optical density (A280). For the control, a similar solution of phage particles corresponding to a pCANTAB plasmid containing no library of light chains was used. The phage particles were eluted with the same buffer containing different concentrations of NaCl (0.01–3 M), and then with 50 mM glycine-HCl (pH 2.6) similarly to the purification of polyclonal antibodies (16, 17). Phage particles were collected, concentrated and each fraction was precipitated using PEG/NaCl as in (34, 35). The titters of phage particles were determined (see below) and each fraction was assayed for MBP-hydrolyzing activity using intact MBP and four OPs corresponding to the protein-specific immunodominant sequences containing cleavage sites (see below).

Preparation of monovalent phage particles

For preparation of soluble MLChs, we have used E. coli HB2151. An overnight culture of E. coli HB2151 (200 μl) was placed in a flask containing 80 ml of 2YT medium and this mixture was incubated with shaking at 37°C to A600 = 1.0. The cells were centrifuged for 10 min (4000 rpm), and the pellet was re-suspended in 80 ml of 10 mM MgSO4 for receiving a solution containing cells in the initial concentration. The preparation of phage particles eluted from MBP-Sepharose with 0.5 M NaCl was diluted 100-fold in 2YT, and 10 μl of this solution was added to 90 μl of the E. coli cells. The mixture was incubated for 30 min at 37°C and uniformly distributed over a Petri dish with agarized 2YTL containing 40 μg ml⁻¹ ampicillin; the dish was incubated overnight at 37°C. For further analysis, 72 of 440 individual colonies from two dishes were randomly chosen.

To propagate individual colonies of phages, the material from each colony was grown overnight at 37°C in a Petri dish as described above. The cells were scraped to a vial containing 1.5 ml 2YT medium supplemented with ampicillin (50 μg ml⁻¹), and the mixture was shaken at 37°C to A600 = 0.6. Then 1.5 ml of 2YT and isopropyl β-D-1-thiogalactopyranoside to the final concentration of 2 mM were added, and the mixture was shaken at 37°C overnight. The suspension was centrifuged (1 min, 12,000 rpm); the supernatant containing phage particles was collected and used to obtain monovalent phages.

Purification of MLChs

Supernatant (50 ml) containing every MLCh was dialyzed twice for 3 h at 4°C against 1 l of H2O and then overnight against buffer A consisting of 50 mM Tris-HCl (pH 7.2), 0.5 M NaCl and 1 mM DTT. The solution obtained was first applied on a column with Sephadex G-75 for removal of different hydrophobic compounds and then on a HiTrap™ chelating Sepharose column (1 ml) charged with Ni²⁺ and equilibrated in buffer A for affinity chromatography. After loading MLChs, the column was washed with the same buffer to zero optical density of the eluate. The bound MLChs were eluted with a gradient of imidazole (0–1 M) in 50 mM Tris-HCl (pH 7.2). The optical density was measured in all fractions. The fractions containing MLChs were dialyzed against 20 mM Tris-HCl (pH 7.5) and then concentrated. According to SDS-polyacrylamide gel electrophoresis (PAGE) analysis, these preparations contain small admixtures of several proteins (probably from medium, E. coli cells or phage particles) interacting with HiTrap™ chelating Sepharose. For isolation of homogeneous preparations of MLCh, we used gel filtration. Fast protein liquid chromatography gel filtration of this preparation was performed on a Superdex 200 HR 10/30 column equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.3 M NaCl as was described in (16, 17). Before gel filtration, the MLCh samples were incubated in buffer A containing 2.0 M MgCl2 for 30 min at 20°C. The fractions containing 26–27 kDa MLCh were collected. About 0.3–0.8 mg of MLChs was obtained from one liter of the medium, depending on the preparation. Only fractions corresponding to the MLChs (containing no proteins admixtures) demonstrated MBP-hydrolyzing activity.

In order to protect the MLChs from bacterial contamination, they were sterilized by filtration through a Millex filter (pore size 0.2 μm) and then concentrated in sterile condition and used for analysis. Incubation of standard bacterial medium with MLChs preparations did not lead to a formation of colonies. In this article, we have analyzed only one MLCh (NGTA2-Me-pro-ChTr) demonstrating high MBP-hydrolyzing activity.

Western blotting and ELISA

The NGTA2-Me-pro-ChTr was analyzed by western blotting onto a nitrocellulose membrane using horseradish peroxidase conjugated with mouse antibodies against light chains of human antibodies as in (13, 14, 16, 17). The interaction of the MLChs with MBP was also analyzed using standard ELISA plates with immobilized MBP as described in (13, 14, 16, 17). After adsorption of the MLChs and a consecutive treatment of samples with horseradish peroxidase conjugated with mouse antibodies against light chains of human antibodies, the reaction mixtures were incubated with tetraethyl benzidine and H2O2. The reaction was stopped with H2SO4, and the optical density (A405) was determined. The relative content of anti-MBP MLChs in the samples was expressed as a difference in the relative absorption at 450 nm between experimental and control samples; controls with MBP but without MLChs were used.

Proteolytic activity assay

The reaction mixture (10–40 μl) for analysis of MBP- or OP-hydrolyzing activity of MLCh, containing 20 mM Tris-HCl (pH 7.5), 0.5–1.0 mg ml⁻¹ (28–56 μM) MBP or 0.33–1.0 mM of one of four different OPs, and 0.001–0.01 mg ml⁻¹ of MLCh, was incubated for 0.1–24 h at 30°C. OP17 (X-ENPVVHFFKNVTPTP), OP19 (X-LRSFWSGAEGQLPGFYG), OP21 (X-YLASAS TMDHARHGFLPRHRR) and OP25 (X-AQGTLISKIFKLGR DSRSGPMARR) OPs corresponding to four known IgG-dependent-specific cleavage sites of MBP (15–17) and containing the fluorescent residue 6-O-(carboxymethyl)fluorescein ethyl ester (R) on its N-terminus were used.

The MBP cleavage products were analyzed by SDS-PAGE in 12% or 4–15% gradient gels with Coomassie R250 staining. The gels were imaged by scanning and quantified using GelPro v3.1 software. Finally, the activities of the MLCh preparations were determined as a decrease in the percentage

Recombinant monoclonal chain with protease activity

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of MBP converted from the initial to hydrolyzed forms taking into account incubation of MBP in the absence of antibodies.

The cleavage products of different OPs were separated by thin-layer chromatography (TLC) on Kieselgel F60 plates using the acetic acid–n-butanol–H₂O (1:4:5) system. The plates were dried and photographed. To quantify the intensities of the fluorescent spots after TLC, OPs incubated without MLCh were used as controls. Photographs of the plates were imaged by scanning and quantified using GelPro v3.1 software. All quantitative measurements (initial rates) were taken under the conditions of the pseudo-first order of the reaction within the linear regions of the time courses (15–40% of MBP or hydrolysis of OPs) and dependence of the rate on MLCh concentration.

pH dependencies were analyzed using different buffers (50 mM): MES-NaOH (pH 5.4–6.6), Tris-HCl (pH 6.0–8.6) and glycine-NaOH (pH 9.0–10.0). In some cases, MgCl₂, MnCl₂, CuCl₂, CoCl₂, NiCl₂, ZnCl₂ or CaCl₂ at different concentrations were used. At analysis of a possible type of proteolytic activity of MLCh (0.3–0.5 mg ml⁻¹), it was pre-incubated for 30 min at 25°C with one of specific inhibitors of different proteases: iodoacetamide (10 mM), phenylmethylsulfonylfluoride (PMSF) (1 mM) or EDTA (10–100 mM), and aliquots of these mixtures were then added to the standard reaction mixture. Dependences of MLCh activity on the concentrations of PMSF and EDTA were also analyzed. The substrate specificity of NGTA1-Me-pro was analyzed using MBP, human serum albumin, human milk lactoferrin and four different OPs (see above).

In gel assay of protease activity
Analysis of MBP-hydrolyzing activity of NGTA2-Me-pro-ChTr after SDS-PAGE was performed similarly to (13, 14, 16, 17). MLCh (5–7 μg) was pre-incubated at 30°C for 30 min under non-reducing conditions (50 mM Tris-HCl, pH 7.5, 1% SDS and 10% glycerol). After standard SDS-PAGE electrophoresis of MLCh to restore its MBP-hydrolyzing activity, SDS was removed by incubation of the gel for 1 h at 30°C with 4 M urea and washed 10 times (7–10 min) with H₂O. Then 2- to 3-mm cross sections of longitudinal slices of the gel were cut up and incubated with 50 μl of 50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl for 4–6 days at 4°C to allow protein refolding and eluting from the gel. The solutions were removed from the gels by centrifugation and used for assay of MBP hydrolysis as described above. Parallel control longitudinal lanes were used for detecting the position of MLCh on the gel by Coomassie R250 staining.

Determination of kinetic parameters
The Kₘ and Vₘₐₓ (Kᵥₘₐₓ) values were calculated from the dependencies of V versus [MBP] or [OP] by least-squares non-linear fitting using Microlab Origin v5.0 software and presented as linear transformations using a Lineweaver–Burk plot (38). The concentration of MBP was varied in the 30–200 mM range; 10 mM MLCh was used. Errors in the values determination were within 10–15%. The results are reported as mean ± S.E. of at least 2–3 independent experiments.

cDNA sequence
Determination of nucleotide sequence corresponding to NGTA1-Me-pro was performed using polymerase chain reaction similarly to (35); M13 reverse (5′-GGAAACAG CTATGACCATG-3′) and FdSeq1 (5′-GAATTTTCTGTATAGG-3′) primers were used. Determination of nucleotide sequence VL-fragments of DNA was performed using the automatic sequenator CEO™ 2000XL DNA Analysis System (Beckman) and special kit “F” CEQ DTCs. The nucleotide sequence and corresponding amino acid sequence of MLCh were analyzed using base data IgBLAST and V-base. The nucleotide sequence of recombinant MLCh reported in this paper has been submitted to the GenBank (gb-admin@ncbi.nlm.nih.gov); Data Bank with accession number NGTA2_sgen nuc_1, KP342057 for NGTA2-Me-pro-ChTr.

The lack of appreciable homology of different OPs was verified using ClustalW2 (http://www.ebi.ac.uk/tools/msa/clustalw2/). The percent of sequence homology was calculated using lalign (http://www.ch.embnet.org/software/LALIGN_form.html). The analysis of MLCh gene type was performed using BLASTN 2.2.27+ server (http://www.ncbi.nlm.nih.gov/igblast/igblast.cgi). Comparison of the complementary DNA sequence of this clone indicated the closest germline VL gene IGKV1-5*01 (86.2% of identity), IGKV1-5*03 (87.9%), IGKJ4*02 (91.2%), IGKJ4*01 (95.7%) and IGKJ1*01 (100%).

The spatial structures of OPs with minimal energy were calculated using a web service PEP-FOLD (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/). The analysis of molecular dynamics of OP spatial structures was performed using the special program Rosetta (Rosetta@home; http://boinc.bakerlab.org/).

Results
Preparation and analysis of recombinant light chains
Polyclonal anti-MBP antibodies from the sera of SLE and MS patients are usually very heterogeneous in their affinity for MBP and can be separated into many subfractions by chromatography on MBP-Sepharose (13, 14, 16, 17). Therefore, we first separated phage particles containing no cDNA of light chains (E. coli TG1) containing a pool of various MLChs with different affinity for MBP by chromatography on MBP-Sepharose (Fig. 1). The complete pool of phage particles containing MLChs bound with MBP-Sepharose was distributed between 10 peaks eluted during chromatography (Fig. 1A) and all fractions, corresponding to new small pools obtained were active in the hydrolysis of MBP (Fig. 1B). At the same time, we have not observed any detectable protein peaks having remarkable affinity for MBP-Sepharose after similar affinity chromatography of phage particles corresponding to pCANTAB plasmid containing no cDNA of light chains (Fig. 1A). It means that the pools of MLChs of all 10 fractions of phage particles with different affinity to MBP contain not only inactive but also catalytically active light chains with MBP-hydrolyzing activity.

For preparation of individual colonies corresponding to recombinant MLChs, we have used E. coli HB2151 and phage particles eluted from MBP-Sepharose with 0.5 M NaCl; this fraction demonstrated relatively high titer and catalytic activity in hydrolysis of intact MBP (peak 7, Fig. 1). The phage particles of this fraction were growing on two Petri dishes with agar and separated colonies were used for preparation of individual MLChs.
At the end of recombinant MLChs, there is a sequence of six histidine residues; this hexapeptide interacts efficiently with Ni\textsuperscript{2+} ions (35). One recombinant MLCh (NGTA2-Me-pro-ChTr, below marked as MLCh) demonstrating relatively high MBP-hydrolyzing activity and five single colonies without activity were used for purification of MLChs by chromatography on HiTrap\textsuperscript{TM} chelating Sepharose charged with Ni\textsuperscript{2+} ions, followed by gel filtration.

The electrophoretical homogeneity of ~26- to 27-kDa NGTA1-Me-pro and a control preparation of an equimolar mixture of five MLChs without activity (inact-MLCh, below marked as MLCh) demonstrating relatively high MBP-hydrolyzing activity and five single colonies without activity were used for purification of MLChs by chromatography on HiTrap\textsuperscript{TM} chelating Sepharose charged with Ni\textsuperscript{2+} ions, followed by gel filtration.

The affinity chromatography of phage particle preparation on MBP-Sepharose: (→) and (→→), absorbance at 280 nm of the material corresponding to phage particles with and without kappa light chains cDNA, respectively (A). Concentrations of NaCl used and relative titers of phage particles corresponding to different peaks are shown. The bars (B) indicate the relative activity of 10 small pools of phage particles corresponding to peaks 1–10 eluted from the sorbent with different concentrations of NaCl and an acidic buffer (pH 2.6) (A) in the hydrolysis of MBP: the reaction mixtures containing MBP (0.7 mg ml\textsuperscript{−1}) and 5 × 10\textsuperscript{7} plaque-forming units were incubated for 6 h at 37°C. For details, see Methods.

Fig. 1. Affinity chromatography of phage particle preparation on MBP-Sepharose: (→) and (→→), absorbance at 280nm of the material corresponding to phage particles with and without kappa light chains cDNA, respectively (A). Concentrations of NaCl used and relative titers of phage particles corresponding to different peaks are shown. The bars (B) indicate the relative activity of 10 small pools of phage particles corresponding to peaks 1–10 eluted from the sorbent with different concentrations of NaCl and an acidic buffer (pH 2.6) (A) in the hydrolysis of MBP: the reaction mixtures containing MBP (0.7 mg ml\textsuperscript{−1}) and 5 × 10\textsuperscript{7} plaque-forming units were incubated for 6 h at 37°C. For details, see Methods.

Fig. 2A. MBP-hydrolyzing and five single colonies without activity were used for purification of MLChs by chromatography on HiTrap\textsuperscript{TM} chelating Sepharose charged with Ni\textsuperscript{2+} ions, followed by gel filtration. The electrophoretical homogeneity of ~26- to 27-kDa NGTA1-Me-pro and a control preparation of an equimolar mixture of five MLChs without activity (inact-MLCh, below marked as MLCh) demonstrating relatively high MBP-hydrolyzing activity and five single colonies without activity were used for purification of MLChs by chromatography on HiTrap\textsuperscript{TM} chelating Sepharose charged with Ni\textsuperscript{2+} ions, followed by gel filtration.

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Fig. 2D. lane 3). There was no observed detectable hydrolysis of control nonspecific proteins: human serum albumin and human lactoferrin (Fig. 2D, lanes 5 and 7). Thus, in contrast to human trypsin, chymotrypsin and metalloproteases, MLCh is very specific toward a globular protein substrate. It means that MLCh preparations do not contain admixtures of any canonical proteases.

It was previously shown that in contrast to polyclonal MS IgGs, MBP-hydrolyzing abzymes from SLE patients are more sensitive to EDTA and less sensitive to PMSF, which is a specific inhibitor of serine-like proteases (13, 14, 16, 17). Figure 3A demonstrated that NGTA2-Me-pro-ChTr is not sensitive to specific inhibitors of thiol-like (iodoacetamide) and acidic-like (pepstatin) proteases. At the same time, pre-incubation of MLCh with specific inhibitor of serine-like proteases leads to decrease in its activity for 42 ± 4%. Human and mammalian intact polyclonal antibodies are known to interact with different metal ions and they do not completely lose intrinsically bound metal ions during the standard procedure of their purification (45). The addition of EDTA to MLCh containing only intrinsically bound Me\textsuperscript{2+}-ions led to a decrease in its activity for 58±5% (Fig. 3A). And average Me\textsuperscript{2+}-dependent proteolytic activity of MLCh containing only intrinsically bound Me\textsuperscript{2+}-ions was approximately 1.4-fold higher than its serine-like activity.

We have compared the effect of different metal ions on the proteolytic activity of MLCh in the hydrolysis of MBP (Fig. 3B). It was shown that in contrast to canonical metal-independent serine-, thiol- and acidic-like proteases, seven different external metal ions activate NGTA2-Me-pro-ChTr in the following order: Ca\textsuperscript{2+} ≥ Mn\textsuperscript{2+} ≥ Mg\textsuperscript{2+} = Co\textsuperscript{2+} = Ni\textsuperscript{2+} ≥ Cu\textsuperscript{2+} ≥ Zn\textsuperscript{2+} (Fig. 3B). Many known canonical human metalloproteases are calcium-dependent cysteine proteases (46, 47) or Zn\textsuperscript{2+}-dependent enzymes (48), while their activity in the presence of other metal ions is absent or significantly lower. MLCh is not a cysteine protease demonstrating maximal activity in the presence of Ca\textsuperscript{2+} and Mn\textsuperscript{2+}, but the effects of five other metal ions on its activity are to some extent comparable (Fig. 3B). In addition, serine-like activity of MLCh is approximately 2.2- to 3.4-fold lower than its Me-dependent activity in the presence of different external metal ions.
In contrast to all human canonical proteases having one pronounced pH optimum, polyclonal catalytic IgGs from the sera of individual MS and SLE patients demonstrate in the MBP hydrolysis quite distinct pH dependencies demonstrating from one to four-five pH optima within a wide range of pH values (5–10, 13, 14, 16, 17). Taking into account inhibition of MLCh by PMSF and EDTA, it was reasonable to expect a possibility of the existence of two optimal pH values. After treatment of MLCh with PMSF, its metalloprotease activity was maximal at pH 6.5–6.6 (Fig. 3C). In the presence of EDTA (or after dialysis against EDTA), serine-like protease activity demonstrated pH optimum at 7.4–7.5. The existence of two optimal pHs might be a consequence of accidental agglomeration of two different clones on Petri dishes. To obtain repeatedly chosen single colonies of phages, the phage material corresponding to this clone was re-grown in Petri dishes and five new single clones were randomly chosen. Purified MLCh preparations corresponding to five new single mono-colonies demonstrated the same two values of pH optima. It means that NGTA2-Me-pro-ChTr was single after the first step of its selection and that the recombinant MLCh is characterized by two different pH optima in the hydrolysis of MBP.

We cannot exclude that there may be only a single catalytic site, but the binding of metal ions at noncatalytic sites can change the MLCh conformation and as a consequence activate the serine protease-like catalytic site. Therefore, we have analyzed the inhibition of serine-like and metal-dependent activities of a nondialyzed preparation of MLCh containing intrinsic metal ions by PMSF at different concentrations. Figure 3D shows that the increase in PMSF concentration leads to complete suppression of MLCh activity in the conditions corresponding to serine-like activity (pH 7.5 in the presence of 50 mM EDTA). At the same time, in the absence of external metal ions at pH 7.5, the conditions corresponding to serine-like activity and metal-dependent activity (provided by intrinsically bound metal ions, Fig. 3C) the activity decreases only by approximately 50% (Fig. 3D). Addition of Ca²⁺ ions results in the increase of total MLCh activity and the decrease of the relative level of serine-like in comparison with that for metal-dependent activity (Fig. 3B). The total activity of MLCh in the presence of Ca²⁺ ions at pH 6.6 (optimal conditions for Me²⁺-dependent activity) was decreased only by approximately 30% (Fig. 3B). This supports the idea that serine-like and metal-dependent activities are substantially independent.

Fig. 2. SDS-PAGE analysis of MBP- and BSA-hydrolyzing activity in the presence of EDTA (A) and in the presence of CaCl₂ (B), as well as the homogeneity of NGTA2-Me-pro-ChTr (7 μg) using a reducing 5–16% gradient gel followed by silver staining (C, lane 1); the arrows (C, lane 2) indicate the positions of molecular mass markers. After electrophoresis, the gel was incubated under special conditions for renaturation of MLCh. The relative MBP- and BSA-hydrolyzing activities (%) were revealed using the extracts of many fragments (2- to 3-mm in length) of one longitudinal slice of the gel; reaction mixtures either contain 0 (A) or 2 (B) mM CaCl₂. The activity of MLCh corresponding to a complete hydrolysis of 0.5 mg ml⁻¹ MBP after 24 h of incubation of 25 μl reaction mixture containing 15 μl of the gel extracts was taken for 100%. The average error in the initial rate determination did not exceed 7–10%. SDS-PAGE analysis of hydrolysis of MBP (0.5 mg ml⁻¹) incubated for 6 h alone (lane C), with 0.1 mg ml⁻¹ inact-MLChmix (lane 1) or with 0.01 mg ml⁻¹ NGTA2-Me-pro-ChTr in the absence of external metal ions (lane 2) and in the presence of 2 mM CaCl₂ (lane 3). Hydrolysis of control proteins (0.5 mg ml⁻¹) by different MLCh (0.05 mg ml⁻¹) for 6 h: human serum albumin (HSA) and lactoferrin by inact-MLChmix (lanes 4 and 6, respectively) and NGTA2-Me-pro-ChTr (lanes 5 and 7, respectively) was analyzed. HSA and lactoferrin were incubated alone (lanes C). Lane C1 corresponds to proteins with known molecular masses.
It is obvious that NGTA2-Me-pro-ChTr corresponds to antibody against only one of four known immunodominant regions containing cleavage sites of MBP and therefore it is reasonable to suggest that it will bind and hydrolyze only one of four OPs. However, an unexpected result was obtained. MLCh was to some extent unspecific and efficiently hydrolyzed three of four OPs (Fig. 3E). The relative MLCh activity decreased in the following order: OP21 > OP19 > OP25 >> OP17; there was no effective hydrolysis of OP17 (Fig. 3F).

Anti-MBP abzymes from the sera of MS (15) and SLE (16, 17) patients hydrolyze MBP at several clustered sites localized within four known immunodominant regions of human MBP. In addition, polyclonal anti-MBP IgGs from the sera of MS and SLE patients cannot hydrolyze short peptide Pro-Phe-Arg-MCA, and they hydrolyze very slowly (only after 3–5 days of the incubation) Boc-Val-Leu-Lys-MCA and Boc-Ile-Glu-Gly-Arg-MCA OPs as well as longer nonspecific 20-mer OPs corresponding to immunodominant regions of HIV-1 integrase and reverse transcriptase (49). It means that anti-MBP IgGs exhibit specificity not only at the level of globular MBP but also at the OPs corresponding to its immunodominant regions.

Taking into account the absence of an exceptional specificity of MLCh in the hydrolysis of specific OPs, it was interesting to analyze a possible homology of four OPs corresponding to four different cleavage sites disposed in different parts of MBP (Fig. 4A). Figure 4B demonstrates the sequence alignment of the four OPs. The similarity between the four sequences is not absolute and only some positions fully coincide (marked with an asterisk), while several positions show good (marked with a colon) or moderate (marked with a dot) conservation.

**Fig. 3.** The relative MBP-hydrolyzing activity of NGTA2-Me-pro-ChTr after its pre-incubation with specific inhibitors of proteases of different types (A). MLCh (0.3 mg ml⁻¹) was pre-incubated in the absence of other components (control), in the presence of PMSF, iodoacetamide, pepstatin or EDTA, and then 1.0 μl added to 29 μl of standard reaction mixture containing MBP (A). The relative activity of MLCh before its pre-incubation with different inhibitors (control) was taken for 100%. Effect of EDTA (10 mM) and different metal ions (2 mM) on the relative activity of MLCh is shown (B). Dependences of the relative MBP-hydrolyzing activity of MLCh before its treatment, after pre-incubation with PMSF and EDTA on pH of reaction mixture are given (C). Dependences of the MBP-hydrolyzing activity of nondialyzed MLCh after its pre-incubation with PMSF in the conditions corresponding to serine-like activity (pH 7.5, 0.1 M EDTA), as well as serine-like + metal-dependent activity in the absence and in the presence of 2 mM CaCl₂, at pH 7.5 and 6.6 (D). All relative activities of MLCh before its pre-incubation were taken for 100%. The relative activity of MLCh in the hydrolysis of four different 1 mM OPs (E). For details, see Methods.
of physicochemical and structural properties. Thus, only partial homology between OPs was revealed. At the same time, in this case such a level of homology and the presence or absence of identical or at least similar cleavage sites in these OPs may be important for their effective hydrolysis by antibodies. Recently, all major and minor sites of SLE IgG-mediated proteolysis of four OPs corresponding to four MBP antigenic determinants were revealed (Fig. 4C) (49, 50). Interestingly, one cluster of the major sites of OP21 cleavage, which is the best substrate, contains two Ser residues and polyclonal IgGs hydrolyze this OP before and after serine residues (Fig. 4C).

The clusters of the major sites of relatively good substrates OP19 and OP25 are also contain Ser residues. However, in the protein sequence of the worst substrate OP17, there are no Ser residues and major sites of its cleavage are not similar to those for OP21, OP19 and OP25 (Fig. 4C). This may be one of the possible reasons why OP17 is the worst substrate.

It should be mentioned that the spatial structure of a substrate and its possibility for an adaptation to the optimal conformation after complexation with enzyme can play an important role in the catalysis (38). Therefore, we have analyzed possible spatial structures of the four OPs using de novo structure prediction of peptides with the lowest energy of physicochemical and structural properties.

**Fig. 4.** Position of four different sequences corresponding to four OPs in a complete protein sequence of MBP (A). Data of homology analysis between the sequences of OP17, OP19, OP21 and OP25 are shown (B). The amino acids identical between two pairs of OPs are marked with an asterisk (*), while nonidentical amino acids with highly conserved physicochemical properties are marked with a colon (:) and those with moderately conserved properties with a dot (•). All major and minor sites of proteolysis of four OPs corresponding to four MBP antigenic determinants in the case of polyclonal anti-MBP IgGs from the sera of SLE patients determined earlier (49, 50) (C). All sites corresponding to major products of the cleavage are shown by long arrows, whereas the minor ones are shown by diamonds.

**Fig. 5.** Spatial structures of OP17, OP19, OP21 and OP25, which are characterized by minimal energy. These structures were calculated using their de novo prediction using a web service PEP-FOLD. Only one structure with the lowest energy was found for OP17, OP19 and OP25, while OP21 is characterized by three alternative structures.
Three structures of OP21 with minimal energy are characterized by helix-loop or loop-helix-loop elements. The worst OP17 and relatively good OP25 substrates similarly to OP21 have elements of alpha-helix-loop structures. However, a good substrate, OP19, fits into a beta-sheet, and its structure is absolutely different in comparison with that for the best OP21 substrate (Fig. 5). The molecular dynamics of spatial structures of OPs was analyzed using the special program Rosetta. From the trajectory in the structure changes, it follows that OP21, OP19 and OP25 are highly dynamic structures, amino acid residues of which most of the time correspond to secondary structure and turns, or they are in the fixed state without secondary structure. During analysis of molecular dynamics, OP17 takes the majority of the time for secondary alpha-helix structure. Therefore, we can assume that the interaction of OP17 with the antibody may require unwinding of the alpha-helix, which should lead to a fine of a free energy during the formation of complexes. From the alpha-helix amphiphilic structure of OP17, it can also be assumed that OP17 can form multimeric forms that may also be possible reason for its lower activity. In general, fluctuations in the structure of the OP17 are much lower than for the three other OPs. Therefore, at first glance, the spatial structure of four OPs with minimal energy may not be the most important factor in the catalysis of their hydrolysis by MLCh. However, since the peptide structures are dynamic and can be changed to other structures with higher energy after the complexation with enzymes, it is possible that the tertiary structure of the OPs is still important for their antibody-dependent cleavage. Overall, it is reasonable to assume that all three factors including the partial homology between OPs, the presence or absence of similar cleavage sites, and the possibility of adaption of the spatial structure of OPs to optimize the catalysis can determine the efficiency of their hydrolysis by antibodies. Nevertheless, the presence of similar cleavage sites in three OPs may probably be the most important factor in the effectiveness of their hydrolysis.

The treated MLCh containing no intrinsic metal ions demonstrated two apparent $K_m$ and $k_{cat}$ values in the hydrolysis of MBP: at pH 6.5 in the absence of metal ions ($9.0 \pm 1.0 \mu M$, $8.6 \pm 0.6 \text{min}^{-1}$) and at pH 7.5 in the presence of CaCl$_2$ ($24.0 \pm 2.0 \mu M, 15.2 \pm 1.1 \text{min}^{-1}$) (Fig. 6A). The $K_m$ and $k_{cat}$ values for three best OP substrates were also determined: OP19 ($0.7 \pm 0.01 \mu M, 800 \pm 60 \text{min}^{-1}$), OP21 ($1.7 \pm 0.1 \mu M, 730 \pm 6.2 \text{min}^{-1}$) and OP25 ($1.8 \pm 0.1 \mu M, 340 \pm 3.0 \text{min}^{-1}$) (Fig. 6B).

**Nucleotide and amino acid sequences of MLCh**

We have determined the nucleotide sequence (Fig. 7A) and the corresponding amino acid sequence of NGTA2-Me-pro-ChTr (Fig. 7B). Comparison of the complementary DNA sequence of this MLCh clone indicated the closest germline VL genes (see above). Thus, NGTA2-Me-pro-ChTr is a typical light chain of antibodies.

Interestingly, it is only the same proteins (and enzymes) from different mammals that demonstrate a high level of homology (50–95%). The homology between different proteins with the same enzymatic functions is significantly lower. For example, we have estimated that the homology between four serine-like proteases (trypsin, chymotrypsin and elastase) varied from 31.4 to 34.5% (average value 33.5 ± 1.4) of identity. The protein sequences of 13 canonical human metalloproteases are known. The homology of MMP1 with other 13 MMPs (MMP2, MMP3, MMP7-MMP12, MMP14, MMP24, MMP25) varied in the range 33.4–61.5% (average value 43.6 ± 16.7). In addition, the relative level of homology between several pairs of MMPs is lower than 32% of identity: MMP2-MMP25 (31.3%), MMP9-MMP14 (31.4%), MMP9-MMP25 (30.6%) and MMP9-MMP24 (30.3%).

Since the protein sequence of the MLCh possessing MBP-hydrolyzing activity is a typical light chain, as well as trypsin-like and metal-dependent proteases, it should combine the elements of protein sequences specific for a V light chain and known proteases of the two types. Taking into account a low level of homology between different enzymes with the same enzymatic function, it was difficult to expect a high level of homology between NGTA2-Me-pro-ChTr and canonical trypsin-like or metalloproteases. The homology between the MLCh and trypsin (23.3%) or chymotrypsin (26.7%), as well as elastase (31.4%), was slightly lower than that between trypsin-like proteases (34.5–34.9% of identity). The homology between the MLCh and 13 classic human metalloproteases varied from 22.9 (MMP1) to 31.7% (MMP24) of identity,
Discussion

An extreme diversity of polyclonal IgG, IgA and IgM abzymes in their affinity for MBP was shown previously using different methods (4–7, 13, 14, 16, 17). Interestingly, when polyclonal IgGs were eluted from MBP-Sepharose by an NaCl gradient (0–3M), the antibody optical density and proteolytic activity were distributed all over the chromatography profiles (13, 14, 16, 17). Several fractions were eluted only with 2–3M MgCl2 or with acidic buffer (pH 2.6) in the conditions destroying strong immunocomplexes. We have expected a similar situation using chromatography on MBP-Sepharose in the case of separation of phage particles containing kappa light chains on their surfaces. Figure 1A shows the distribution of the phage particles (and their MBP-hydrolyzing activity) all over the profile of the chromatography on MBP-Sepharose. The data are indicative of the extreme diversity of SLE anti-MBP recombinant kappa light chains in their affinity for MBP. In this article for a preparation of individual recombinant MLChs, we have used phage particles eluted from MBP-Sepharose with 0.5M NaCl (Fig. 1A).

Comparison of the complementary DNA sequence of the clone indicated the NGTA2-Me-pro-ChTr closest to germline VL gene. As it was shown above, MLCh possesses not only serine-like but also metalloprotease activity. In principle, metalloproteases from different sources can be activated by different metal ions. For example, Ca2+ (51–53), Mg2+ (53), Zn2+ (54), Mn2+ (54), Cu2+ (55), Co2+ (54) have been reported as ions enhancing the activity of several bacterial alkaline proteases. In addition, some of bacterial alkaline proteases may be activated not only by one but also by several different metal ions (51–54). The calcium-dependent proteolytic system of mammals is composed of cysteine proteases named calpains (46, 47). In addition, mammals are characterized by many of Zn2+-dependent proteases (48, 56, 57).

MLCh is not a cysteine protease, since its activity does not depend on iodoacetamide (Fig. 3A). After a standard procedure of IgG purification, they contain intrinsically bound metal ions (45). PMSF and EDTA significantly suppress MLCh proteolytic activity, while addition of several different external metal ions leads to an increase in the activity: Ca2+ and Mn2+ are the best activators of MLCh (Fig. 3B). Thus, MLCh is in a greater extent similar to bacterial proteases, which can be activated by several different metal ions.

All known canonical mammalian proteases have one pronounced pH optimum. It was shown that after the treatment of MLCh with PMSF, the pH optimum of metalloprotease activity corresponds to 6.5–6.6 (Fig. 3C). After suppression of Me2+-dependent activity by EDTA, the maximum of serine-like protease activity was observed at 7.4–7.5 (Fig. 3C) and both of them were lower than those for human trypsin and chymotrypsin (7.8–8.0). In addition, PMSF in the presence of EDTA completely suppresses serine-like activity of MLCh (Fig. 3D).

In the absence and in the presence of external metal ions (at pHs 7.5 and 6.6; without EDTA), total activity (serine-like + metalloprotease) decreases only by approximately 50% and 30%, respectively (Fig. 3D). This indicates, that MLCh possess two independent serine-like and metal-dependent activities.

It is known that canonical proteases efficiently hydrolyze all proteins. After the separation on MBP-Sepharose, polyclonal SLE and MS IgGs hydrolyze only MBP (13, 14, 16, 17). Control proteins human serum albumin and human lactoferrin were not hydrolyzed by MLCh (Fig. 2D). MLCh hydrolyzes efficiently only MBP (Fig. 2C) and three of four different OPs corresponding to four different sites of MBP corresponding to its immunodominant regions (Fig. 3E). Since specific inhibitors of different proteases except PMSF and EDTA did not decrease remarkably the MBP-hydrolyzing activity of MLCh (Fig. 3A), it may be considered an unusual protease specifically recognizing and hydrolyzing MBP due to two alternative active centers with serine-like and metalloprotease activities.

It was shown that the hydrolysis of nonspecific OPs corresponding to MBP by anti-integrase abzymes of HIV-infected patients is caused by a partial homology between sequences of OPs and several fragments of integrase (58). Therefore, it was interesting to analyze a possible homology between different OPs of MBP.
MLCh efficiently hydrolyzes only three of them (Fig. 3E). Thus, MLCh-dependent hydrolysis of free MBP OPs can to some extent be a consequence of their partial homology. The presence or absence of identical or similar cleavage sites in these four OPs may also be important for their effective hydrolysis. Figure 4C demonstrates that the clusters of the major cleavage sites of OP21, OP19 and OP25 containing Ser residues are to some extent similar, whereas the major sites of OP17 containing no Ser are very different. This may be an important reason why OP17 is the worst substrate.

OP21 is a very dynamic substrate, since it is characterized by three different spatial structures with minimal energy containing helix-loop or loop-helix-loop elements (Fig. 5). Only one spatial structure with minimal energy was found for the worst OP17 and for relatively good OP25 substrates; similarly to OP21, they have elements of alpha-helix-loop structures. However, only one structure with minimal energy of the good substrate, OP19, contains a beta-sheet, and it is absolutely different with that for OP21 (Fig. 5). However, the peptide structures can be changed to other ones having higher energy after the complexation with enzymes. Therefore, it is reasonable to suppose that the partial homology between OPs, the presence or absence of similar cleavage sites and the possibility of adaption of the spatial structure of OPs to be optimal for the catalysis can work together to determine the relative rate of their hydrolysis by MLCh. From our point of view, the presence of similar cleavage sites in OPs may probably be the most important factor in the effectiveness of their hydrolysis. As it was shown previously, polyclonal anti-MBP IgGs from the sera of MS and SLE patients hydrolyze MBP at a 3- to 50-fold lower rate than those for different OPs (17, 49, 50). Transition from intact integrase to its OPs in the case of polyclonal anti-integrase IgGs from the sera of HIV-infected patients led to an increase in the rate of hydrolysis approximately 8- to 100-fold (39, 58). It is known that catalytic centers of proteolytic abzymes are usually located on the light chain, whereas the heavy chain is more often responsible for specific antigen recognition and increased antigen affinity for antibodies (4–7). Intact proteins usually interact with both light and heavy chains of abzymes, thus ensuring the specificity of the target protein recognition and its cleavage. At the same time, short OPs may interact mostly with the light chain, which possesses 100- to 1000-fold lower affinity for substrates (4–7, 17, 39, 48–50, 58). The increase in $k_{cat}$ may be a consequence of a higher affinity of intact antibodies compared with separated light chains for different substrates because of the interaction of globular protein substrates with both light and heavy chains of abzymes. The separation of the light chains can lead to a decrease in the lifetime of the existence of the complex and, as a consequence, to an increase in the turnover number and $V_{max}$ ($k_{cat}$) of the reaction catalyzed by light chains (4–7).

The affinity of polyclonal SLE and MS IgGs to MBP varied from 0.1 to 1.0 μM (7), whereas the affinity of MLCh to MBP is remarkably lower (9–24 μM) (Fig. 6). In addition, the affinity of three different OPs (0.7–1.8 mM) to MLCh is about 30- to 180-fold lower than that to MBP (Fig. 6B).

The catalysis mediated by artificial abzymes is usually characterized by relatively lower reaction rates than for canonical enzymes (4–7). The known $k_{cat}$ values for natural abzymes from autoimmune patients catalyzing many different chemical reactions vary in the range of $1 \times 10^{-5}–40 \text{min}^{-1}$ ([4–7] and refs therein). NGTA2-Me-pro-ChTr demonstrated two relatively high $k_{cat}$ values ($8.0 \pm 0.6$ and $15.2 \pm 1.1 \text{min}^{-1}$) in the hydrolysis of MBP, while these values in the hydrolysis of OPs are relatively very high (340–800 min$^{-1}$) (Fig. 6). Overall, depending on the sequence, the hydrolysis of peptides may be less specific in comparison with intact MBP.

Our previous findings showed that polyclonal antibodies from the sera of patients with SLE and MS can contain many monoclonal anti-MBP abzymes with very different enzymatic properties (13, 14, 16, 17). Here, we have described the first example of a recombinant MLCh with two combined serine-like and metalloprotease activities.

It is known that the presence of anti-MBP antibodies and abzymes is the main important diagnostic index for MS (13–15), but the sera of SLE patients also contain these antibodies and abzymes (16, 17). The established MS drug Copaxone appears to be a specific inhibitor of MBP-hydrolyzing abzymes (13, 59), which may indicate the involvement of these abzymes in pathogenesis of MS. In addition, the sera of patients with MS and SLE patients contain free light chains (7). Taking these data in account, we propose that extremely diverse intact antibodies and their free light chains with MBP-hydrolyzing activity may promote important neuropathologic mechanisms in MS and SLE pathogenesis.

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**References**

DNase monoclonal kappa light chains with different catalytic properties.


