CMLS, Cell. mol. life sci. 53 (1997) 51–60
1420-682X/97/010051-10 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Quaternary structure-dependent idiotope and antigen binding of a monoclonal antibody specific for conformational epitope on type II collagen

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Received 29 July 1996; received after revision 13 September 1996; accepted 18 October 1996

Abstract. We previously generated a monoclonal antibody (mAb) against a putative pathogenic epitope on native type II collagen (CII) for the induction of collagen-induced arthritis in mice (mAb1), and an anti-idiotypic mAb which appears to possess the internal image of the CII epitope (mAb2). In the present study, the structural basis of the antigen/mAb1 and mAb1/mAb2 interactions was examined. When partially SH-reduced mAb1 was analysed on Western blots, only fragments containing both heavy (H) and light (L) chains were recognized by mAb2. When mAb2 was partially SH-reduced, only fragments containing both H and L chains were recognized by mAb1. H and L chains were separated from mAb1 in a reduced, denatured condition, and each chain and a mixture of the two were refolded. mAb2 reacted specifically to the renatured whole IgG molecule of mAb1, but not to the refolded L or to H chains. Recombinant single chain Fv (scFv) generated from mAb1 and mAb2 had properties of the original mAbs, whereas genetically constructed chimeric scFvs, consisting of V_H from mAb1 and an irrelevant V_L, or V_L of mAb1 and an irrelevant V_H, did not react either to CII or to mAb2. Thus, interactions among CII, mAb1 and mAb2 appear to depend on quaternary structures containing different protein subunits. These observations support the internal image property of the mAb2. In addition, this dependency on quaternary structure for recognition of proteins may also be relevant to other protein-protein interactions.
Key words. Protein-protein interaction; higher-order structure; anti-idiotypic antibody; type II collagen; autoimmune arthritis.

Abbreviations. Ab = antibody; CIA = collagen-induced arthritis; CII = type II collagen; Ab1 = Ab against external antigens; Ab2 = anti-idiotypic Ab; mAb = monoclonal Ab; scFv = single chain Fv; V_H = heavy chain variable region; V_L = light chain variable region; CDR = complementarity-determining region; 2ME = 2-mercaptoethanol; ABC = avidin/biotinylated alkaline phosphatase complex; CBB = Coomassie brilliant blue; H chain = heavy chain; L chain = light chain; HEL = hen egg-white lysozyme.

Autoantibodies have a critical role in the pathogenesis of type II collagen-induced arthritis (CIA) that is regarded as an animal model of rheumatoid arthritis [1–3]. The pathogenic antibodies (Abs) are reactive against native type II collagen (CII), but not against denatured collagen, and it is therefore likely that they recognize the triple helical structure of α1(II) chains [1, 3].

A series of monoclonal Abs (mAbs) specific for CII has been generated in our laboratory [4]. One of these mAbs, termed 1-5, is of particular interest because this particular mAb has the potential to induce polyarthritis in DBA/1J mice previously immunized with CII [5], and a rabbit anti-idiotypic Ab to this mAb, but not to the others, partially replaced the antigen CII for the induction of CIA [6]. A monoclonal anti-idiotypic Ab, termed 8-4-1, has also been established [7]. Although this mouse anti-idiotypic Ab, which is different from the xenogenic rabbit anti-idiotypic Ab, is incapable of inducing arthritis, immunization with mAb 8-4-1 coupled with a carrier protein elicited an Ab response against native CII, and the intravenous administration of the mAb 8-4-1 resulted in an idiotype suppression, and concomitantly decreased the susceptibility to CIA [7]. These results suggested that mAb 1-5 reacts with a pathogenic epitope on CII, and that mAb 8-4-1 may resemble the structure of the epitope. It is therefore important to delineate the interaction between the two mAbs, since useful information on the pathogenic conformational epitope of CII can probably be acquired.

The antigen binding site of Abs resides at the variable (V) domain, and consists of amino acids from complementarity-determining regions (CDR) of heavy (H) and light (L) chains. Anti-idiotypic Abs (Ab2) are specific for the V domains of other Abs (Ab1). Ab2 is classified into three categories as Ab2α, Ab2β and Ab2γ, according to location of the idiotope on Ab1 [8]. Although all Ab2s react to the V domain of Ab1, Ab2α is incapable of inhibiting the binding of Ab1 to the antigen. Both Ab2β and Ab2γ react to the antigen-binding site of Ab1 and inhibit the binding of Ab1. The mode of interaction between Ab2β and Ab1 is similar to that seen between Ab1 and antigen, while that between Ab2γ and Ab1 differs. Therefore, Ab2β possesses antigen-binding sites functionally and, possibly, conformationally similar to the antigenic epi-

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topes for Ab1 [8–10]. Thus this class of Ab2 is also referred to as the internal image of antigen [8].

We used various approaches to examine the requirement of H- and L-chain V domains (termed V_H and V_L, respectively) of the two mAbs with regard to their binding capacities. We found that mAb 1-5 required both V_H and V_L to react with a conformational epitope on triple helical CII and anti-idiotypic mAb 8-4-1, which also needed both V_H and V_L to interact with mAb 1-5. V-region genes of the two mAbs were cloned and sequenced, but the primary structures yielded no information indicating specific interactions. Thus, higher-order structures of the two mAbs and CII were absolutely required for their recognition. The mode of interaction between mAbs 1-5 and 8-4-1 was immunochemically indistinguishable from that between mAb 1-5 and CII, thereby supporting the proposal that this anti-idiotypic mAb functionally and, possibly, conformationally resembles the original epitope on native CII. The work we have described herein also provides a relatively simple methodology to distinguish between quaternary structure dependence and tertiary structure dependence of protein-protein interactions.

Materials and methods

Antibodies. Mouse mAbs 1-5 and 2-60 (both are IgG2a, k) were established from DBA/1J mice by immunization with native human CII [4]. Two anti-idiotypic mAbs were used; one is specific for mAb 1-5, termed 8-4-1 (IgG1, k) [7], and the other is specific for mAb 2-60, termed 2-3-1 (IgG1, k) [4]. The mAbs were purified from mouse ascites by ammonium sulfate precipitation followed by protein A affinity chromatography. The binding pair of mAbs 1-5 and 8-4-1 are designated mAb1 and mAb2, respectively, in this report. A biotinylated mAb specific for mouse kappa L chain (clone LO-MK-1), and a cocktail of mAbs (clones LO-MG-7, LO-MG1-2 and LO-MG3-7) specific for mouse gamma H chain of all IgG subclasses were purchased from Zymed (South San Francisco, CA). A purified mAb to a peptide tag (designated E-tag) located at the carboxyl terminus of recombinant scFv-Abs was purchased from Pharmacia Biotech (Uppsala, Sweden). The presence of E-tag (amino acid sequence in the one-letter code: GAPVPYPDPLEPR) allowed detection of scFv-Abs, using this specific mAb. mAb1, mAb2 and anti-E-tag mAb were biotinylated in our laboratory with sulfo-NHS-biotin (Pierce, Rockford, IL).

Collagen preparations. Human CII purified from costal cartilage, as described in [11], was stored in a lyophilized form at 4 °C. Type I collagen purified from human placenta was commercially obtained (Fuji Pharmaceuticals, Takaoka, Japan). The lyophilized compounds were dissolved in 0.1 M acetic acid at 4 °C at a concentration of 2 mg/ml. Denaturation of CII was carried out by incubating the solution at 56 °C for 30 min, and the solution was quickly chilled on ice. These collagen solutions were further diluted in appropriate buffers and used for assays described below.

Purification of H and L chains from mAb1 and *in vitro* renaturation of separated chains. Complete reduction of mAb1 (1 mg/ml) was achieved in a denatured condition by incubation in 8 M urea solution (0.584 M Tris-HCl, 8 M urea, 5.37 mM EDTA, pH 8.6) containing 0.3 M 2-mercaptoethanol (2ME) for 2 h at 40 °C, under a nitrogen atmosphere. The free sulfhydryl groups were reversibly protected by adding 1 g of cystamine to the mAb solution, followed by incubation for 4 h at 40 °C. The reduced mAb1 was applied to a column (15 mm diameter, 2 m length) of Sephacryl S200 (Pharmacia) equilibrated with 10% acetic acid containing 6 M guanidine-HCl. The elution was monitored by tryptophyl fluorescence intensity at 350 nm on excitation at 280 nm. Two peaks corresponding to H chain and L chain, respectively, were obtained. The two fractions were dialysed against 10% acetic acid to remove guanidine and then lyophilized to evaporate acetic acid. The purified H chain and L chain were dissolved in the 8 M urea solution.

Renaturation of the separated chains and a mixture of the two was performed as described elsewhere [12]. In brief, 2ME was added to the solutions to give a final concentration of 70 mM, and the mixture was incubated for 90 min at 40 °C to deprotect the sulfhydryl groups. Oxidized glutathione was weighed and added at 16.2 mg for 1 ml solution (13 mM) and incubated for a further 20 min. The samples were dialysed against the dialysing buffer (0.1 M Tris-HCl, 7 mM 2ME, 1.3 mM oxidized glutathione, 1 mM EDTA, pH 8.0) with 8 M urea. Renaturation was carried out by gentle removal of urea from the solution at 4 °C, by diluting the dialysing buffer containing 8 M urea to the dialysing buffer with no urea, as described for renaturation of hen egg-white lysozyme (HEL) [12]. The renatured materials were dialysed against PBS (10 mM PO_4, 150 mM NaCl, pH 7.4).

Recombinant scFv production. Recombinant scFv-Abs were generated from mAb1 and mAb2 using a Recombinant Phage Ab System (Pharmacia) according to the manufacturer's instruction. The principle of the system is described in detail elsewhere [13], and the structure of the gene is given in figure 1. Soluble scFv-Ab was extracted from the periplasm of the bacterium. scFv-Ab1 (scFv-Ab derived from mAb1) was confirmed by binding capacity to CII and mAb2, and scFv-Ab2 was confirmed by binding to mAb1 and by inhibition of the association between mAb1 and CII. Chimeric scFv-Abs were constructed from the scFv-Ab1 and another scFv (scFv103) specific for HEL, which was generated from an anti-HEL mAb, LKS103 (unpublished). At first we constructed genes encoding V_H alone or V_L alone of LKS103. The scFv103 gene was digested with *Eco*81I at 3′ end of the framework 4 region of V_H, and with *Not*I

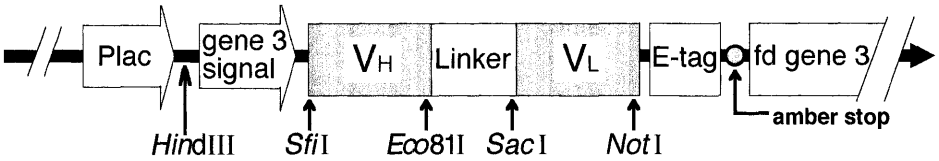

Figure 1. Construction of the scFv gene. Restriction enzyme sites used are denoted by arrows. The amber stop codon is not recognized in *E. coli* strain TG1 but is in strain HB2151, thus an scFv-Ab is produced, under control of the lac promoter (Plac) as a fusion protein with the filament fd in the former strain but as a soluble protein in the latter.

at 3′ end of the framework 4 of V_L, to remove the linker and V_L sequences (fig. 1), and was then ligated; the resultant gene product containing the entire sequence of V_H of mAb LKS103 was designated V_H103. V_L103 was obtained by digesting the scFv103 gene with *Sfi*I and *Sac*I to remove the V_H and linker sequences, followed by ligation. The scFv-Ab1 gene was digested with *Eco*81I and *Not*I, and the fragment containing the linker and V_L was introduced into the V_H103 expression vector. A fragment containing V_H of Ab1 and linker genes obtained by digestion with *Hin*dIII, and *Sac*I was also introduced into the V_L103 expression vector. The two chimeric scFv-Abs were designated scFv-L15/H103 and scFv-H15/L103, respectively.

DNA sequencing of V-region genes from scFv-Ab1 and Ab2. Each cloned scFv gene was again separated into nucleotide fragments containing V_H and V_L genes, respectively, by digestion with restriction enzymes; *Hin*dIII and *Sac*I were used to obtain the V_H gene, and *Sac*I and *Not*I were used in case of V_L gene (fig. 1). The V_H and V_L gene fragments were ligated into the multicloning site of an M13mp19 vector derivative containing a *Not*I site (unpublished), which was pretreated with either of the pairs of restriction enzymes, the same as for the V-region genes. DNA sequencing of each recombinant gene cloned in the M13 vector was carried out using Taq dye primer cycle sequencing kits (Perkin-Elmer, Applied Biosystems Division, Foster City, CA) with DNA sequencer model 373A (Applied Biosystems). Two clones were sequenced for each mAb. Figure 2 shows the deduced amino acid sequences of those V genes. The V segments, D and J segments were classified according to Kabat et al. [14].

Enzyme-linked immunosorbent assay (ELISA). Antigen specificity of mAb1 and mAb2 was analysed using ELISA. Microtitre ELISA plates (Nunc, Roskilede, Denmark) were coated with native CII (5 μg/ml) in 0.05 M sodium carbonate buffer, pH 9.6 or with mAb1 (3 μg/ml) overnight at 4 °C, followed by blocking with nonfat dry milk (2% in PBS).

Biotinylated mAb1 or mAb2 were incubated with different amounts of unconjugated mAbs or collagen preparations for 30 min in PBS containing 0.05% Tween 20 (PBST), then the mixtures were added to the plates. After 1 h of incubation, the plates were washed in PBST and incubated for another 1 h with a solution of avidin/biotinylated alkaline phosphatase complex (termed ABC reagent, Pierce) prepared in PBST 30 min prior to being added to the plates. The plates were washed in PBST, then incubated with the substrate of *p*-nitrophenyl phosphate (1 mg/ml in 0.05 M sodium carbonate buffer, pH 9.6, 2 mM $MgCl_2$). The absorbance at 405 nm of each well was recorded using an automated ELISA reader (Bio-Rad, Richmond, CA). To determine the antigen-binding capacities of recombinant scFv-Abs, plates were coated with either CII, mAb1 or mAb2. Serially diluted periplasmic extracts obtained from *Escherichia coli* cells producing scFv-Abs were preincubated with PBST containing 2% nonfat dry milk for 15 min to avoid nonspecific absorption, then added to the coated ELISA plates. After 1 h of incubation, biotinylated anti-E-tag (1 μg/ml) was added and further probed using the ABC reagent.

Western blot analysis. To achieve complete SH-reduction of mAbs, 10 μg of mAbs were incubated at 100 °C for 5 min in 40 μl of 0.05 M Tris-HCl buffer (pH 7.0) containing 2ME (0.14 M) and SDS (0.1%). For limited reduction, mAbs were incubated at 37 °C for various periods with varied concentrations of 2ME, with or without 0.1% SDS. After the reduction, 40 μl of *N*-ethylmaleimide (0.3 M, dissolved in the Tris buffer) was added to each reaction mixture to inactivate the reducing agents. Treated samples were subjected to duplicate slab gels of discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) (1 μg protein/lane). One of the gels was developed using Coomassie brilliant blue (CBB), while the other gel was overlaid with a sheet of Immobilon-P membrane (Millipore, Bedford, MA), and the resolved proteins were electrophoretically transferred to the membrane in the presence of 2 mA/cm^2 current for 1 h in a semidry transfer unit (Bio-Rad, Transblot SD). The membrane was saturated with a blocking buffer (5% nonfat dry milk in 0.01 M Tris-HCl, 0.15 M NaCl, 0.05% Tween, pH 7.4, termed TBST) overnight at 4 °C. The membrane was then cut into pieces and probed with biotinylated mAbs, followed by ABC reagent, as described for ELISA. After extensive washing in TBST, the sheets were incubated in the BCIP/NBT substrate [15].

Results

Antigen specificity of mAb1 and mAb2 in ELISA. To determine the antigen specificity of mAb1 and mAb2,

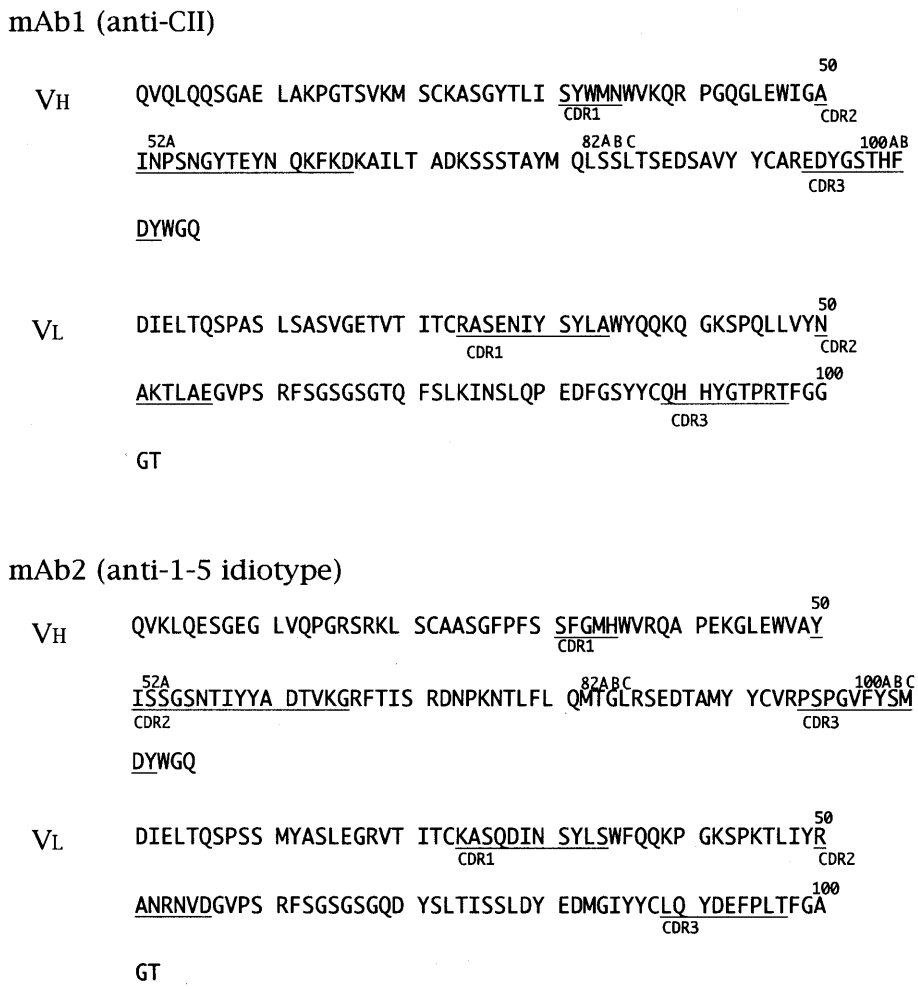

Figure 2. Translated amino acid sequences (one-letter code) for V_H and V_L regions of mAb1 (anti-CII, 1-5) and mAb2 (anti-1-5 idiotype, 8-4-1). The CDR regions are underlined. The V segments, D, and J segments were classified according to Kabat et al. [14]. The anti-CII mAb1 used a V_H segment belonging to the protein subgroup IIB, alternatively V_H1 (J558) of the V_H gene family [32], a D segment of DFL16 and J_H2 for VDJ recombination. The V_L segment belonged to the Vk protein group V (or Vk12 gene family [33]), and Jk1 was used for the VJ recombination. Several replacing mutations were observed, most frequently in the CDR2 of the two chains, and there were a few silent mutations. mAb1 probably used the same or a closely related germ line V_H segment previously reported for anti-CII mAbs [34], although V genes of those mAbs were unmutated, and their CDR3 structures have not been determined. The V_L genes of those mAbs (Vk21) were unrelated to that of mAb1. The most significant homology for the V_H was observed with an anti-DNA mAb [35]: 88% and 93% homology in the amino acid sequence, with and without CDR3, respectively. The V_L amino acid sequence including CDR3 showed 97% homology with an anti-human interleukin 2 receptor mAb [36], and 92% homology with another anti-DNA mAb reported in the V_H study mentioned above [35]. In spite of the striking homology in the primary structures, mAb1 showed no reactivity either to single- or double-stranded DNA (data not shown). The mAb2, 8-4-1, possessed a V_H segment belonging to the protein subgroup 'miscellaneous', or V_H5 (7183) of the V_H gene family [32], and used an undefined D segment and J_H4. The V_L segment belonged to the Vk protein group V (or Vk 9 gene family [33]), and Jk5 was used. The nucleotide sequence data reported in this paper will appear in the NCBI nucleotide sequence database with the accession numbers U69538, U69539, U69540 and U69541, respectively.

ELISA was performed. The reaction of biotinylated mAb1 to native CII immobilized on ELISA plates was inhibited by soluble native CII, but not by CII preincubated at 55 °C for 30 min (denatured CII) nor by native type I collagen (fig. 3A, closed symbols). Thus, mAb1 was specific for an epitope expressed on native CII. The anti-idiotypic mAb2, as well as mAb1 itself, inhibited the reaction, whereas another anti-CII mAb (2-60) of epitope specificity distinct from mAb1 and an anti-idiotypic mAb for 2-60 (2-1-3) were without effect (fig. 3A, open symbols). The reaction of biotinylated mAb2 to the plate-coated mAb1 was inhibited by free mAb1 and mAb2, but not by the other pair of anti-CII mAb and anti-idiotypic mAb (fig. 3B). This reaction was also blocked by native CII, but not by denatured CII. From these results, it was concluded that mAb1 was specific for native CII, mAb2 was the anti-idiotypic Ab specific for mAb1 and mAb2 reacted to the antigen-binding site of mAb1. Together with our previous finding that immunization of mice with the mAb2 elicits a response of

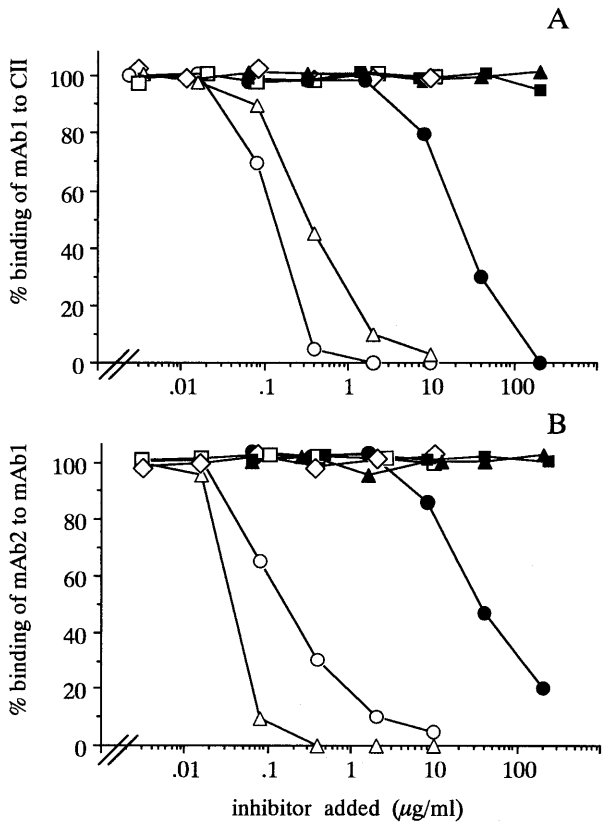

Figure 3. Antigen specificity of mAb1 and mAb2. (*A*) Reaction of biotinylated mAb1 to CII on the solid phase was inhibited by various mAbs (—○—, mAb2; —△—, mAb1 —□—, 2-1-3; —◇—, 2-60), or various collagen preparations (—●—, native CII; —■—, denatured CII; —▲—, native type I collagen). The mixture of biotinylated mAb1 (0.5 μg/ml) and various concentrations of the inhibitors was added to plates coated with CII. The reaction was followed by incubation with ABC reagent. (*B*) Reaction of biotinylated mAb2 to mAb1 on the solid phase was inhibited by the various reagents described above. The symbols and experimental conditions were the same as described above, except for the concentration of biotinylated mAb2 (0.1 μg/ml). The x-axes indicate the concentration of inhibitors in the mixtures, and the y-axes indicate percentage binding of biotinylated mAbs to the antigen-coated plates, which was calculated as A_{405} with inhibitor/average A_{405} of quadruplicate wells without inhibitor but with the biotin conjugates.

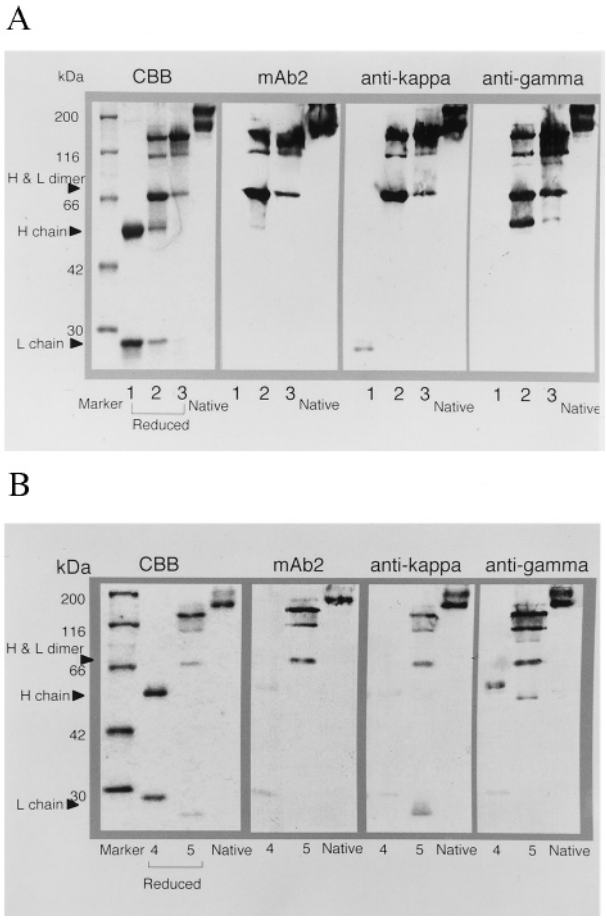

Figure 4. Reaction of mAb2 to partially reduced mAb1. (*A*) mAb1 was incubated in a buffer containing 2ME (0.14 M) and SDS (0.1%) at 100 °C for 5 min (lane 1), at 37 °C for 1 h (lane 2), or without SDS at 37 °C for 1 h (lane 3). The reducing reaction was terminated by adding *N*-ethylmaleimide (0.3 M). The reduced mAb1 and untreated native mAb1 were subjected to SDS-PAGE, followed by protein staining with CBB or by Western blots using biotinylated mAbs indicated at the top. (*B*) mAb1 was incubated in the buffer containing 2ME (0.14 M) and 0.1% SDS (lane 4), or without SDS (lane 5) at 37 °C for 15 h. Protein staining with CBB and Western blotting were carried out as described above. The numerals on the left are the molecular weights of marker proteins. The compositions of deducible bands are also indicated on the left.

anti-CII Ab with the mAb1 idiotype [7], it seemed plausible that the mAb2 was Ab2β that mimicked the epitope for mAb1, which was expressed on native CII.

Reaction of mAb2 to partially reduced mAb1 in Western blot analysis. mAb1 was reduced at 100 °C for 5 min in the presence of SDS, or was partially reduced at 37 °C for 1 h in the presence or absence of SDS, and analysed by SDS-PAGE followed by Western blots (fig. 4A). Temperature and the presence of SDS during the incubation greatly influenced the efficiency of reduction; incubation at 37 °C with SDS resulted in five major bands in protein staining with CBB (lane 2), but only three major bands without SDS (lane 3), whereas complete reduction was achieved by incubation at 100 °C for 5 min with SDS, shown by the two bands of H- and L-chain monomers (lane 1). The bands with an apparent molecular mass of 70 kDa in lanes 2 and 3 were considered to be heterodimers of H and L chains because of the molecular weight and the results of Western blots. Two bands at approximately 130 kDa and 100 kDa in these lanes were also composed of two chains, although the precise compositions were not deduced. mAb2 reacted to these bands composed of two immunoglobulin chains, but not to either of the monomers.

When we incubated mAb1 at 37 °C for 15 h, the mAb was completely separated into monomers of L and H chains in the presence of SDS (fig. 4B, lane 4), whereas in the absence of the detergent, multiple bands appeared

(lane 5). Lane 5 appeared to contain monomers of the H and L chains. On Western blots, one band at approximately 50 kDa was detected only by the H-chain-specific mAb, not by the L-chain-specific one, suggesting that this is the H-chain monomer. Another band smaller than 30 kDa, detected by the L-chain-specific mAb but not by the H-chain-specific one, was the L-chain monomer. The mAbs specific for H or L chains appeared to recognize reduction-sensitive, conformation-dependent epitopes residing in each chain, since they did not react to monomers obtained under complete reducing conditions (lanes 1 and 4). Furthermore, the monomers' bands in lane 5 migrated faster than corresponding monomers in lane 4, obtained in the presence of SDS. These results suggested that the H- and L-chain monomers in lane 5 retained native conformations. Nonetheless, the anti-idiotypic mAb2 did not react to either of the monomers with the conserved conformations but only to bands with molecular masses exceeding 70 kDa, which were simultaneously recognized by the two chain-specific mAbs.

Reaction of mAb1 to partially reduced mAb2. An experiment similar to that for figure 4, but in the reverse direction, was done to explore the structural requirement of mAb2 for interaction with mAb1. mAb2 was first reduced under the same conditions as for mAb1 in figure 4A (fig. 5A). It showed a markedly different sensitivity to reduction from that of mAb1; the L chains were quickly dissociated from the immunoglobulin molecule and separated into the L-chain monomer and the H-chain homodimer, even in the absence of SDS (lanes 3). No band corresponding to the heterodimer of H chain and L chain was obtained. These bands composed of L chains alone (monomer) and H chains alone (homodimer) obtained by the two mild reducing conditions were recognized by mAbs specific for each chain, but not by mAb1. Only a weak reaction of mAb1 was observed at a minor band of approximately *Mr* 130 K (lanes 3). This position was also weakly stained by both chain-specific mAbs. Completely reduced H and L chains (lanes 1) were again not recognized by either of the chain-specific mAbs.

Since only a limited amount of the L chain was associated with the H chain after reduction of mAb2 for 1 h even without SDS, we lowered the concentration of 2ME and shortened incubation period in the absence of detergents (fig. 5B, lanes 4 and 5). Here, a more intense reaction of mAb1 was obtained, although the overall results were similar to those for figure 5A; mAb1 only reacted to bands composed of both H and L chains of mAb2, not to the L-chain monomer or to the H-chain dimer, while these were all detectable with the two chain-specific mAbs.

It was thus suggested that both H and L chains of the mAb1 and mAb2 are required for mutual recognition. However, there remains the possibility that conformations of the idiotopes might reside in only one of the chains, and be more fragile than other conformational epitopes. To rule out the possibility, the structural requirement for the idiotope expression, which could be a concomitant of the antigen-binding capacity of Ab, was further investigated using different techniques.

Idiotope expression was recovered on mAb1 reconstructed from separated H and L chains, but not on either subunit. mAb1 was completely denatured and reduced, and its H and L chains were isolated by gel filtration chromatography under denaturing conditions (fig. 6A). Purification of the two subunits was confirmed by SDS-PAGE under reducing conditions followed by CBB staining (fig. 6B, lanes a and c), and by Western blotting with chain-specific mAbs following nonreducing SDS-PAGE (lanes h, j, k and m). When the mixture was renatured, the major band appeared at the same posi-

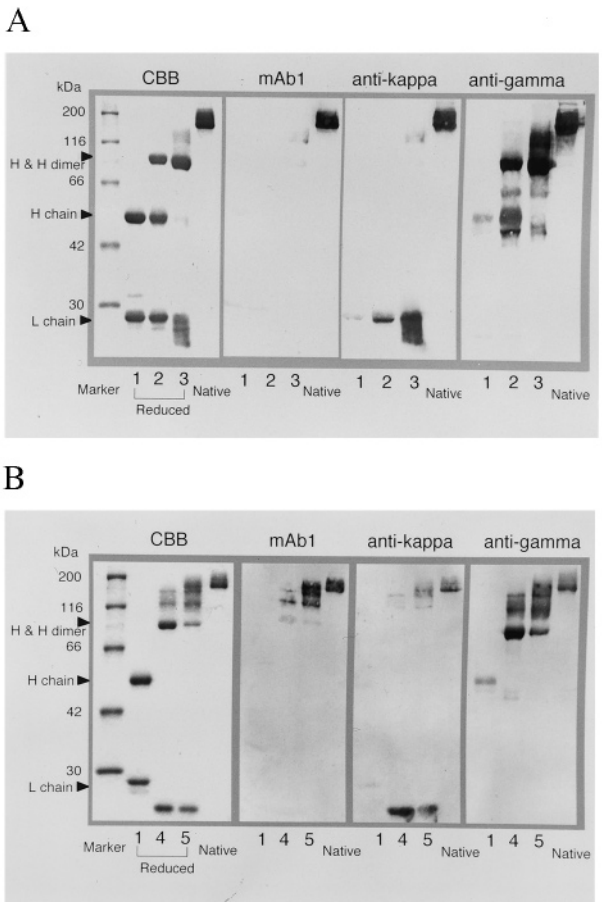

Figure 5. Reaction of mAb1 to partially reduced mAb2. (*A*) mAb2 was incubated in a buffer containing 2ME (0.14 M) and SDS (0.1%) at 100 °C for 5 min (lane 1), at 37 °C for 1 h (lane 2), or without SDS at 37 °C for 1 h (lane 3). The reduced mAb2 and untreated native mAb2 were subjected to SDS-PAGE, followed by protein staining with CBB or by Western blots using biotinylated mAbs indicated at the top, exactly as in figure 4A. (*B*) mAb2 was incubated in buffer containing 2ME (0.14 M) and 0.1% SDS at 100 °C for 5 min (1), or with no SDS and 0.05M 2ME at 37 °C for 30 min (lane 4) or 5 min (lane 5). Protein staining with CBB and Western blotting were carried out as described above.

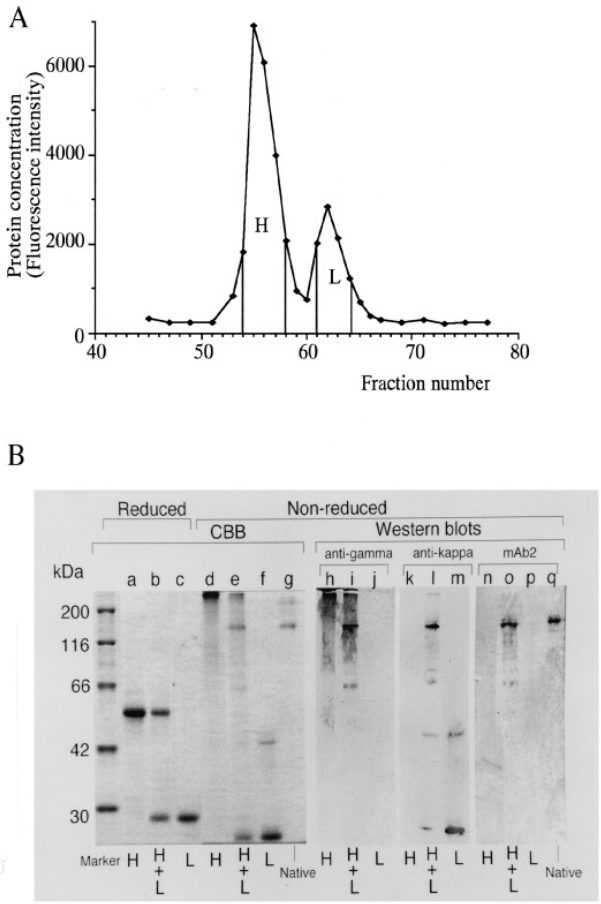

Figure 6. Purification of H and L chains from mAb1 and *in vitro* renaturation of the separated chains. (*A*) H chain and L chain of mAb1 were purified from the reduced mAb under complete denaturing conditions by gel filtration chromatography with Sephacryl S200 equilibrated with 10% acetic acid containing 6 M guanidine-HCl. The elution was monitored by fluorescence intensity at 350 nm on excitation at 280 nm. (*B*) Each of the chains (H, H chain alone; L, L chain alone) or the mixture of the two (H + L; ratio at 1 to 1 by volume of the column chromatography fractions) were renatured by gentle removal of urea from 8 M to 0 M in the presence of 2ME and oxidized glutathione. The samples were analysed by SDS-PAGE under reducing or nonreducing conditions and stained with CBB. The nonreducing SDS-PAGE was also followed by Western blot analysis and probing with the biotin-conjugated mAbs indicated at the top.

tion as native mAb1 (compare lanes e with g), thereby suggesting that renaturation had indeed occurred. The idiotope was simultaneously restored, as indicated by a positive reaction of mAb2 (lane o).

The separated L chain appeared to be well renatured in a monomeric form with some dimerization (lane f), and the renatured monomer migrated faster in the gel than did the denatured one (compare lane c with f). A conformational epitope for the anti-L chain mAb, which had been destroyed before renaturation (fig. 4), was also restored (lane m), whereas the renatured L chain was not recognized by mAb2 (lane p). The purified H chain did not become monomeric. Although it appeared as aggregates of a large size, as seen in lane d, a conformational determinant for the anti-H chain mAb was renatured on the aggregated H chain (lane h). Nonetheless, the mAb2 showed no reaction to the renatured H chain (lane n).

Reactivity of scFv-Ab and chimeric Abs. Recombinant scFv-Ab1 and scFv-Ab2 were produced by *E. coli* HB2151 cells, which were transformed with the cloned scFv genes, and the soluble scFv-Abs were extracted from the periplasmic component. The Ab activity in the periplasmic extracts was evaluated by indirect ELISA. As shown in figure 7, the scFv fragments possessed antigen specificities of their original mAbs. They were also capable of inhibiting interactions between CII and mAb1, and between mAb1 and mAb2 (data not shown). The idiotope expression on the scFv fragments was also confirmed on Western blots following nonreducing SDS-PAGE. scFv-Ab1 and scFv-Ab2 on the blots were specifically recognized by mAbs of the binding partners (fig. 7D).

Two chimeric scFv fragments constructed from scFv-Ab1 and scFv103, namely scFv-H15/L103 and scFv-L15/H103, were produced in the periplasmic compartments of transformed bacteria and were obtained in a soluble, monomeric form, as seen on Western blots with anti-E-tag mAb following SDS-PAGE under nonreducing conditions (fig. 8C). However, neither of the chimeric scFv reacted to CII and mAb2 in ELISA (fig. 8A, B) or in Western blots (fig. 8C). Thus, concomitant expression of the V_H and V_L domains was indispensable for the two correlated functional properties of Abs, i.e. antigen binding and idiotope expression.

Discussion

Idiotype expression that requires the contribution of both H- and L-chain V domains has been noted for other Ab systems using Western blot analysis [16–18]. However, the reduction-sensitive nature of idiotopes reported in these previous papers is not sufficient to conclude that these idiotopes require the association of both immunoglobulin chains, since many epitopes which reside in only one immunoglobulin chain can also be destroyed by reducing treatment. Indeed, such examples were seen in the present study on mAbs specific for respective chains (figs 4 and 5). These epitopes were expressed by tertiary structures of each immunoglobulin subunit, but not by quaternary structures. Therefore, to assess the structural requirement of our mAbs, we prepared limited reducing conditions so as not to affect the overall conformation of each chain of mAbs. Under the conditions used, where the two immunoglobulin chains were successfully separated yet with reactivity with the chain-specific mAb reagents fully retained, they showed no reactivity against the anti-idiotypic reagents.

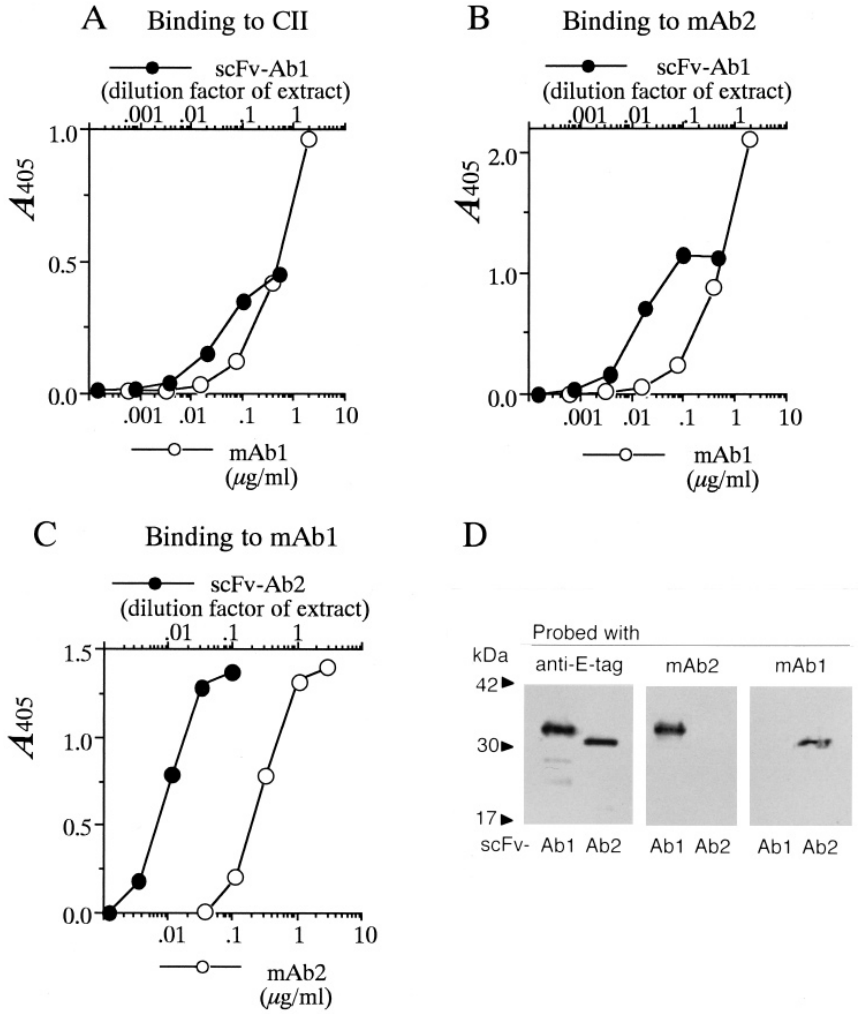

Figure 7. Antigen-binding capacities and idiotope expression of scFv fragments. Ab activity in the periplasmic extract of HB2151 *E. coli* cells producing scFv-Ab1 was evaluated in ELISA for the original antigen, native CII (*A*) or for anti-idiotypic mAb2 (*B*). Similarly, the periplasmic extract containing scFv-Ab2 was tested for binding capacity to mAb1 (*C*). Open circles show reaction of the biotinylated original mAbs and closed circles show reaction of the scFv fragments. scFv associated with plate-coated antigens were probed by biotinylated anti-E-tag (1 μg/ml), then followed by incubation with ABC reagent. Y-axes indicate A_{405}; x-axes indicate concentration of purified mAb (bottom) and dilution factor of the scFv containing periplasmic extracts (top). (*D*) Idiotope expression of the two scFv fragments was also analysed on Western blots. The two periplasmic extracts containing scFv fragments indicated at the bottom were resolved in SDS-PAGE under nonreducing conditions. The proteins were electrophoretically transferred to membrane sheets and probed with biotinylated mAbs indicated at the top, followed by incubation with an ABC reagent.

Hall et al. [19] investigated in depth the requirement of both immunoglobulin chains for an idiotope expression using a genetic technology, and concluded that both the H and L chains were required for idiotope expression. Doubly transfected myeloma cells with genes for L and H chains of the mAb, but not cells transfected with each gene, produced an idiotype-positive product. However, single gene products were not secreted but were located in the cytoplasm, and retention of the native tertiary structure was not confirmed. Furthermore, a chimeric Ab comprising the H chain of the mAb and the L chain of an irrelevant mAb weakly expressed the idiotope, whereas a single amino acid mutation at the L chain CDR3 abolished the idiotope expression. These results imply that the idiotope which was examined is primarily associated with the H chain, and that the determinant may be hindered or conformationally altered when paired with a particular L chain CDR3.

Thus, the structural requirement of idiotope expression should be assessed by different approaches. We incorporated a renaturation technique for denatured proteins in this study, since separation of the two immunoglobulin chains is achieved only under conditions of complete denaturation. When a mixture of the separated chains was refolded, the renatured IgG expressed the idiotope recognized by mAb2, whereas when each chain was refolded, neither was recognized by mAb2. Although the H chain tended to be aggregated when refolded in the absence of the L chain, reactivity with a mAb recognizing the tertiary structure of gamma H chains appeared to be fully recovered. This means that the native tertiary structure of the H chain was to some

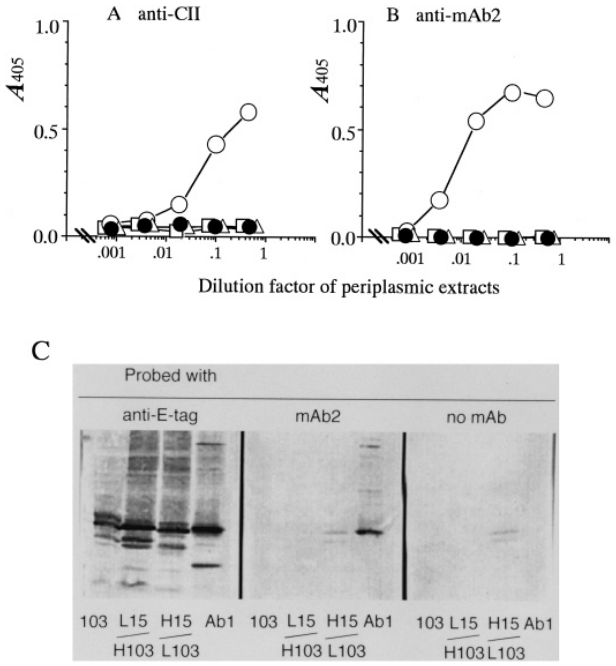

Figure 8. Antigen-binding capacities and idiotope expression of chimeric scFv fragments. Chimeric scFv-Abs consisted of mAb1–V_L and anti-HEL-V_H (L15/H103) and of mAb1-V_H and anti-HEL-V_L (H15/L103) were generated. Periplasmic extracts from the two chimeric scFv-Abs and from the two original scFv-Abs were tested in ELISA for anti-CII activity (*A*), and for anti-Ab2 activity (*B*). scFv associated with plate-coated antigens were probed by biotinylated anti-E-tag (1 μg/ml), followed by incubation with an ABC reagent. Open circles show the reaction of scFv-A1, closed circles are that of scFv103, open triangles are scFv-L15/H103, and open squares are scFv-H15/L103. (*C*) Idiotope expression and E-tag expression of the four scFv-Abs were also analysed on Western blots. The periplasmic extracts indicated at the bottom were resolved in nonreducing SDS-PAGE, transferred to membrane sheets, and incubated with biotinylated anti-E-tag (left), mAb2 (centre), or without Abs (right), followed by the incubation with an ABC reagent. Only the wild-type scFv-Ab1 was detected by mAb2. The faint reaction between scFv-H15/L103 and mAb2 was probably due to a nonspecific interaction between the scFv and the ABC reagent, since the same extent of reaction was also recorded in the absence of Abs. This chimeric scFv might have increased nonspecific binding characteristics.

extent restored in the isolated H chain after renaturation, but the idiotope was not expressed on the renatured H chain. Comparisons of Ab properties between wild type and chimeric recombinant scFv-fragments also supports the notion that the idiotope on mAb1 requires the contribution of both V_H and V_L domains. There remains the possibility that one of the two chains of mAb1 could weakly react to mAb2 and to CII. Nonetheless, our results clearly indicate the requirement of both chains of mAb1 for proper recognition of the CII epitope and the mAb2 idiotope.

Although there are strong arguments regarding the concept of idiotype mimicry [20, 21], it has been experimentally demonstrated that some of anti-idiotypic Abs structurally and functionally resemble external antigens (reviewed in refs 9 and 10). The most well documented example is the mAb 87.92.6 raised against an idiotope of a mAb toward reovirus type 3 hemagglutinin that interacts with the β-adrenergic receptor [22]. This Ab2 contains a sequence homologous to a portion of the viral antigen in its L chain CDR2, and synthetic peptides corresponding to the portion bind to both the β-adrenergic receptor and the antivirus Ab1 [23, 24]. A similar mode of antigen mimicry by anti-idiotypic Abs, i.e. a portion of Ab V region resembling an epitope of an external antigen, has been also noted for different antigen/Ab systems [25–27]. Differing from the earlier observations, we found no homology between mAb2 and CII at the primary amino acid sequence level (fig. 2). This was, however, not surprising: the characteristic amino acid sequence of collagens is the repeating (Gly-X-Y), but since glycine residues are not located on the surface of the native trimer molecule, they are unlikely to interact with Abs. A stereoscopic analysis rather than a linear sequential analysis needs to be done to examine the possible structural homology between mAb2 and CII, or the structural complementarity between mAb1 and mAb2, and between mAb1 and CII. Higher-order structural analyses, such as computer homology modelling, nuclear magnetic resonance and X-ray crystallographic studies are in progress in our laboratory using the pair of recombinant scFv-Abs. Preliminary results of the homology modelling study suggest that wide areas of the two mAbs derived from V_H and V_L are involved in their interaction (unpublished), similar to the behaviour of other anti-protein Abs revealed by X-ray crystallography [28–31]. Since all the properties of our mAb2 met the criteria of Ab2β, an internal image Ab, to elucidate further the mode of interaction between the two mAbs would provide important information on the pathogenic conformational epitope of CII, whose chemical property has not yet been fully characterized.

One major objective in current biochemistry is to reveal higher-order structures that determine interactions of proteins with their specific ligands. At present, great efforts are put to studying primary structures. This also holds true for research into antigen-Ab interactions. However, clinically important Abs and antigens which are potentially involved in the pathogenesis of or protection from diseases may not be characterizable by primary structural analyses. Higher-order structural analyses will be needed to understand fully the properties of epitopes. Our experience in the present work provides useful information on an immunochemical methodology to distinguish between quaternary structure dependence and tertiary structure dependence of protein-protein interactions.

Acknowledgements. We thank H. Ohtsuka and T. So for excellent technical assistance, and M. Ohara and M. Hirata for comments on the manuscript. This work was supported in part by a

Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (No. 07857135, No. 08877282).

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