

RESEARCH ARTICLE

Conformational diversity of the Goodpasture antigen, the noncollagenous-1 domain of the $\alpha 3$ chain of collagen IV

Juan J. Calvete¹, Fernando Revert², Mario Blanco², Javier Cervera², Céline Tárrega², Libia Sanz¹, Francisco Revert-Ros², Froilán Granero^{2, 3}, Enrique Pérez-Payá², Billy G. Hudson³ and Juan Saus²

¹ Consejo Superior de Investigaciones Científicas, Instituto de Biomedicina de Valencia, Valencia, Spain

² Centro de Investigación Príncipe Felipe, Valencia, Spain

³ Department of Medicine, Vanderbilt University, Nashville, TN, USA

The noncollagenous-1 domain of the $\alpha 3$ chain of collagen IV networks of basement membranes is the target of an antibody-mediated inflammatory response in Goodpasture autoimmune disease. This domain when excised from basement membranes by bacterial collagenase digestion exists in two molecular forms, M_H and M_L , that differ in cleavage site and mobility in SDS-PAGE. In the present study, M_H and M_L were shown to also differ with respect to epitope exposure, susceptibility to endoprotease digestion, and redox states of specific cysteine residues, as determined by MS. Moreover, M_H and M_L assemble to form different quaternary structures, critically influencing pathogenic epitope(s) exposure and autoantibody binding. Collectively, our findings reveal that M_H and M_L are conformational isomers stabilized by a distinct disulfide bond connectivity, and coexist in basement membranes. The hitherto unrecognized conformational diversification of the Goodpasture autoantigen may be of relevance in pathogenesis.

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1 Introduction

Goodpasture (GP) disease is mediated by autoantibodies (GP antibodies) that target the noncollagenous-1 (NC1) domain of the $\alpha 3$ chain of collagen IV networks [$\alpha 3$ NC1 or $\alpha 3$ (IV)NC1], a major component of basement membranes. GP antibodies typically deposit in a linear manner along the alveolar and glomerular basement membranes, causing a rapidly progressive glomerulonephritis and often lung hemorrhage (see [1] for a review).

Among the six α chains ($\alpha 1$ – $\alpha 6$) existing in collagen IV, the $\alpha 3$ chain interacts with an $\alpha 4$ and an $\alpha 5$ chain to yield a triple-helical protomer, which self-assembles through end-to-end connections to form a supramolecular network that scaffolds specific basement membranes. At the C terminus, two protomers are linked head-to-head by interactions of their trimeric NC1 domains, forming a stable NC1 hexamer complex, the native form of the autoantigen targeted by the GP antibodies. The NC1 hexamer complex can be excised from the insoluble collagen IV network with bacterial collagenase for *in vitro* studies.

Under *in vitro* conditions, the hexamer complex dissociates into monomer and dimer subunits upon treatment with protein denaturants, such as acid and SDS. The chain identities and the quaternary structure of these subunits are known. The epitopes for GP antibodies are located within the $\alpha 3$ NC1 monomer and dimer subunits and are structurally sequestered within the hexamer complex, unavailable for binding of antibodies. The *in vivo* unmasking of the cryptic

Correspondence: Dr. Juan Saus, Centro de Investigación Príncipe Felipe, Avd. del Saler, 16 Camino de las Moreras, 46013 Valencia, Spain

E-mail: jsaus@cipf.es

Fax: +34-96-3289701

Abbreviations: GP, Goodpasture; NC1, noncollagenous-1; TBM, testis basement membrane

epitopes by putative pathogenic factors such as oxidative stress is thought to be critical in the mechanism underlying the etiology and pathogenesis of the disease.

Two forms of the $\alpha 3$ NC1 have been extracted from bovine basement membranes both of which bind GP antibodies. They differ in mobility in SDS-PAGE gels and in the site of cleavage by bacterial collagenase, indicating a structural distinction in the autoantigen that may be of importance in pathogenesis [2]. Here, the nature of the structural difference was further characterized by differential antibody binding and MS. The two forms, designated M_H and M_L , were shown to differ in epitope exposure, susceptibility to proteases, and redox state of specific cysteine residues. The findings are consistent with M_H and M_L as being conformational isomers, stabilized by distinct disulfide bonds. This structural diversification may be of relevance in pathogenesis as it critically impacts pathogenic epitope(s) exposure in the quaternary structure.

2 Materials and methods

2.1 Bovine testis $\alpha 3$ (IV)NC1 preparation

Bovine $\alpha 3$ (IV)NC1 was prepared from testis basement membrane (TBM) as described [3]. The $\alpha 3$ (IV)NC1 (7–10 μ g) was subjected to Western blotting under nonreducing conditions, and stained with Ponceau. The two major polypeptides were either excised and the N-terminal sequence determined using an Applied Biosystem Procise 494 Sequencer or they were individually extracted as described [4] and used for Western blot studies.

2.2 In-gel enzymatic digestion and PMF

Polypeptides separated by SDS-PAGE procedures were subjected to automated digestion with sequencing grade enzymes, including a mixture of bovine pancreas trypsin (Roche) and α -chymotrypsin (Sigma) at a final concentration of 20 ng/ μ L in 50 mM ammonium bicarbonate, pH 8.3, using a ProGest digester (Genomic Solutions) following manufacturer's instructions. Digestions were done with or without prior reduction with DTT (10 mM for 15 min at 65°C) and carbamidomethylation with iodoacetamide (50 mM for 60 min at room temperature). The tryptic peptide mixtures were dried in a SpeedVac and the samples were dissolved in 5 μ L 50% ACN/0.1% TFA, and subjected to PMF. To further confirm disulfide bond assignments, digestion mixtures of nonreduced polypeptides were dissolved in 5 μ L 10 mM HEPES pH 9.0, reduced by addition of DTT (100 mM) and incubation at 65°C for 15 min. The reduced digestion mixtures were then freed from reagents using a C18 Zip-Tip pipette tip (Millipore) activated with 70% ACN and equilibrated in 0.1% TFA. Following protein adsorption and washing with 0.1% TFA, the proteins were eluted with 3 μ L 70% ACN/0.1% TFA and used for PMF.

For PMF analysis, 0.85 μ L of the digests were spotted onto a MALDI-TOF sample holder, mixed with an equal volume of a saturated solution of CHCA (Sigma) in 50% ACN containing 0.1% TFA, dried, and analyzed with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer, operated in delayed extraction and reflector modes. The peptide mass fingerprint obtained from each electrophoretic band was compared with the expected proteolytic digest of the bovine $\alpha 3$ (IV)NC1 domain (Swiss-Prot accession code Q28084 amino acids 227–471) using the program PAWS (Proteometrics, available at <http://prowl.rockefeller.edu/>). All searches were constrained to a mass tolerance of 100 ppm. A tryptic peptide mixture of *Cratylia floribunda* seed lectin (Swiss-Prot accession code P81517) prepared and previously characterized in our laboratory was used as mass calibration standard (mass range, 450–3300 Da).

2.3 Chemical modification of cysteine residues

For carbamidomethylation of sulfhydryl groups, the protein bands of interest were excised from a CBB-stained SDS-PAGE gel, dehydrated by incubation in ACN for 30 min, and rehydrated overnight at room temperature in 300 mM HEPES, pH 9, followed by incubation for 1 h at room temperature in the same buffer containing 50 mM iodoacetamide. For labeling cysteine residues engaged in the formation of disulfide bonds, the carbamidomethylated proteins were reduced at 65°C for 15 min in the presence of 50 mM DTT in 300 mM HEPES, pH 9, containing 5 M guanidinium hydrochloride, and the newly exposed sulfhydryl groups were pyridylethylated by the addition of 100 mM 4-vinylpyridine and incubation for 1 h at room temperature.

2.4 CID by MS/MS

For peptide sequencing, the protein digest mixture was subjected to ESI-MS/MS analysis using a QTrap mass spectrometer (Applied Biosystems) [5] equipped with a nanospray source (Protana, Denmark). Doubly charged ions selected after enhanced resolution MS analysis were fragmented using the Enhanced Product Ion with Q_0 trapping option at 250 amu/s across the entire mass range. For MS/MS experiments, Q_1 was operated at unit resolution, the Q_1 – Q_2 collision energy was set to 35 eV, the Q_3 entry barrier was 8 V, the linear IT Q_3 fill time was 250 ms, and the scan rate in Q_3 was 1000 amu/s. CID spectra were interpreted manually or using the online form of the MASCOT program at <http://www.matrixscience.com>.

2.5 Antibodies

The production of antibodies against the N terminus of bovine $\alpha 3$ (IV)NC1 domain (aSP) or towards the native and assembled form of bovine $\alpha 3$ (IV)NC1 (Mab3 previously Mab17) has been previously reported [2, 6]. The $\alpha 3$ (IV)NC1-specific mAbs Mab30C, Mab175 and Mab189 were raised

against bacterial randomly folded human recombinant $\alpha 3(\text{IV})\text{NC1}$, whose production was previously reported [7]. For these purposes, the recombinant material analyzed by SDS-PAGE under reducing conditions was excised from the gel and used for mice immunization. All the mAbs were monospecific in Western blot studies using recombinant proteins representing each of the six human $\alpha(\text{IV})\text{NC1}$ domains.

2.6 Immunoprecipitation procedures

TBM hexamer was precipitated essentially as described [8, 9] using affinity-purified GP antibodies (GP_a) or human control IgG (IgG) and the corresponding immunoprecipitates analyzed by Western blot. Supernatants from control IgG studies were further precipitated with Mab3 and similarly analyzed.

3 Results and discussion

3.1 Chemical and immunochemical properties of $\alpha 3(\text{IV})\text{NC1}$ isoforms

Collagenase treatment of bovine TBM yielded the two $\alpha 3\text{NC1}$ polypeptides, designated M_H for monomer of higher M_r , which contains four Gly-X-Y triplets at the N terminus, and M_L for monomer of lower M_r , which contains one Gly-X-Y triplet (Fig. 1A), both of which corresponded to the different digestion products previously characterized [2]. Consistently, M_H but not M_L reacted with aSP, a rabbit polyclonal antibody raised against the residues 4–17 of M_H [2], whereas the Mab175 mAb recognizing an epitope comprising residues 103–117 of human $\alpha 3\text{NC1}$ domain [8], and which is also present in bovine homologous domain, reacted with the two polypeptides (Fig. 1A).

The structural relationship of M_H and M_L was explored by SDS-PAGE analysis under nonreducing or reducing conditions (Fig. 1B). Interestingly, when the polypeptides were analyzed under nonreducing conditions, the difference in molecular mass was ~ 2.1 kDa, a value significantly higher than the ~ 0.9 kDa expected for the longer collagenous extension on M_L (Fig. 1A), and which was observed when M_H and M_L were reduced (Fig. 1B). These data suggest that the apparent M_r differences between nonreduced M_H and M_L species depend on two different structural features: one relying on primary structure and other on tertiary structure. Possible differences in tertiary structure were further investigated using Mab189 and Mab30C (Fig. 1C). Their relative binding towards nonreduced M_H and M_L was significantly different, a similar binding of Mab189 and Mab30C to M_H contrasted with high binding of Mab189 and virtually no binding of Mab30C to M_L . Interestingly, these differences in reactivity were abolished when M_H and M_L were reduced. These results suggest that M_H and M_L have distinct tertiary structures that are stabilized by disulfide bonds.

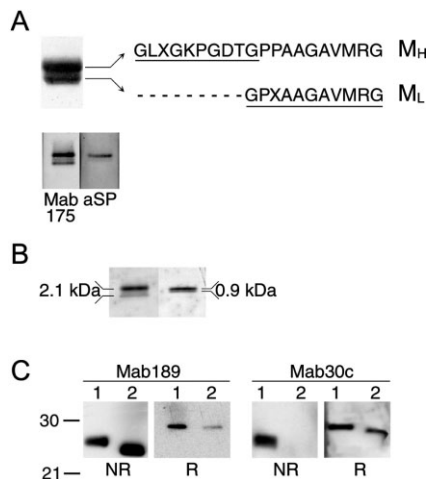


Figure 1. Evidence for conformational diversification in isolated bovine $\alpha 3(\text{IV})\text{NC1}$ domain. (A) Bovine $\alpha 3(\text{IV})\text{NC1}$ monomers were analyzed by Western blot under nonreducing conditions, Ponceau S stained (top) and either the N-terminal sequence of membrane-bound M_H and M_L determined (sequence underlined) or probed with the indicated antibodies (bottom). (B) The same material was analyzed by SDS-PAGE under nonreducing (NR) or reducing (R) conditions and silver stained. Indicated are M_r differences between the two polypeptides. (C) M_H or M_L were subjected to Western blot analysis carried out under the indicated redox conditions and $\alpha 3(\text{IV})\text{NC1}$ -specific antibodies.

3.2 MS analysis of M_H and M_L isomers

The tertiary structural differences between M_H and M_L could be associated to differences in the pairing of cysteine residues forming disulfide bonds or in the redox states of specific cysteine residues. This was initially investigated by MALDI-TOF PMF of in-gel digestions of the reduced and carbamidomethylated M_H and M_L polypeptides. The fingerprints were almost identical (Fig. 2), except for the distinct presence of ions at m/z 1827.6, 3931.1, and 3971.8 in M_H , and 1442.8 and 3521.3 in M_L . Due to their large mass, the ions at m/z 3931.1 and 3971.8 (M_H) and 3521.3 (M_L) could not be characterized by MS/MS analysis. Furthermore, these ions could not be assigned solely on their molecular masses, suggesting that the corresponding peptides may carry structural modifications. All other ions were unambiguously assigned to $\alpha 3(\text{IV})\text{NC1}$ domain using the PAWS program and MS/MS analysis (Table 1).

The ion of 1827.6 Da was identified by MS/MS as the N-terminal polypeptide 1–19 of M_H containing three hydroxyproline (p) residues ($^1\text{GLKGGKpGDTGppAAGAVMR}^{19}$) (Table 1). The peptide of 1442.8 Da from M_L was also sequenced by MS/MS and corresponded to AHGQDLGTSCSLQR present in bovine $\alpha 1(\text{IV})\text{NC1}$ domain. This finding along with the fact that another minor peptides at m/z 1924.9 and 1924.6 present in the proteolytic maps of M_L and M_H corresponded to $\alpha 1(\text{IV})\text{NC1}$ [ILYHGYSL-

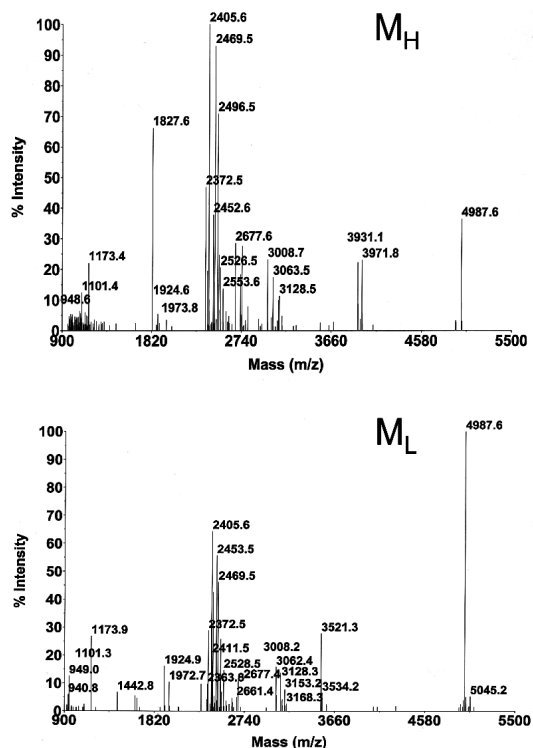


Figure 2. Mass fingerprints of reduced and carboxyamidomethylated M_H and M_L polypeptides. Shown are mass fingerprints of trypsin and chymotrypsin digests of reduced and carboxyamidomethylated M_H or M_L . Amino acid sequence assignments done by combining MALDI-TOF MS and CID-MS/MS analysis are shown in Table 1.

Table 1. Assignment of MALDI-TOF ions generated by degradation of the reduced and carboxyamidomethylated higher (M_H) and lower (M_L) electrophoretic bands of monomeric bovine $\alpha 3(\text{IV})\text{NC1}$ with a mixture of trypsin and chymotrypsin. Fragment limits refer to the amino acid sequence numbering in M_H . CM-C, carbamidomethylated cysteine; M-ox, methionine sulfoxide; OH-P, hydroxyproline

$M+H^+$	M_H	M_L	M_H and M_L	Remarks
948.5			115–122	
1101.5			227–236	
1173.4			185–194	CM-C ¹⁹¹
1827.6	1–19			OH-P ⁶ , OH-P ¹¹ , OH-P ¹²
2372.5			195–214	CM-C ¹⁹⁷
2405.6			113–134	CM-C ¹²³ , CM-C ¹²⁶
2407.1			174–194	CM-C ¹⁷⁹ , CM-C ¹⁹¹
2453.5			72–91	CM-C ⁸⁰ , CM-C ⁸⁶
2469.5			72–91	CM-C ⁸⁰ , CM-C ⁸⁶ , M ⁷⁵ -ox
2496.5			200–219	
2526.5			58–79	CM-C ⁶⁸
2677.6			154–180	CM-C ¹⁷⁹
3128.3			155–184	CM-C ¹⁷⁹ , M ¹⁶⁰ -ox
4987.6			26–71	CM-C ³⁵ , CM-C ⁶⁸

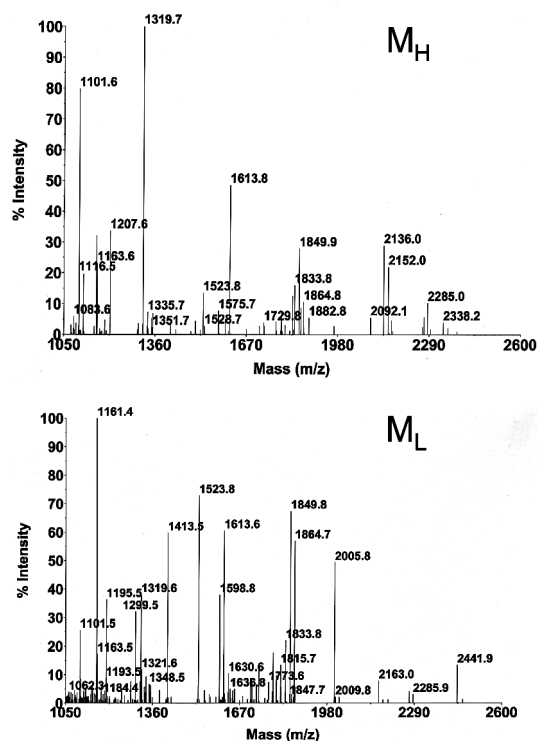


Figure 3. Mass fingerprints of nonreduced M_H and M_L polypeptides. The study was similar to that described in Fig. 2, but performed on nonreduced M_H and M_L . The corresponding peptide assignments are shown in Table 2.

LYVQGNR], and still another peptide at m/z 1207.6 present in the proteolytic map of non-reduced M_H (Fig. 3 below) corresponded to $\alpha 4(\text{IV})\text{NC1}$ [SLLYLEGQER], revealed minor presence in our samples of NC1 material from these other collagen IV chains which was not detectable by N-terminal sequencing.

In contrast, MALDI-TOF analysis of nonreduced M_H and M_L yielded different spectra (Fig. 3). The quasimolecular $M+H^+$ ions were matched as above within the amino acid sequence of the bovine $\alpha 3$ chain of collagen IV, and the assignment is displayed in Table 2. Although M_H and M_L shared a number of peptides, many other ions were distinctly present in the respective spectra (Table 2), suggesting that M_H and M_L were cleaved at different peptide bonds because they possess different conformations.

The NC1 domain is composed of two in-tandem homologous subdomains, each of which contains six cysteine residues engaged in the formation of three disulfide bonds (pattern I-VI, II-V, and III-IV) in the crystal structures of $\alpha 1/\alpha 2$ hexamer from human placenta or bovine lens capsule [10, 11], and which correspond to Cys³⁵-Cys¹²⁶, Cys⁶⁸-Cys¹²³, Cys⁸⁰-Cys⁸⁶ (N-terminal subdomain), and Cys¹⁴⁵-Cys²⁴⁰, Cys¹⁷⁹-Cys²³⁷, and Cys¹⁹¹-Cys¹⁹⁷ (C-terminal subdomain) in bovine M_H . Noteworthy, CID-MS/MS analysis of the fragments generated by degradation of nonreduced M_L and M_H

Table 2. Assignment of MALDI-TOF ions generated by degradation of the nonreduced higher (M_H) and lower (M_L) electrophoretic bands of monomeric bovine $\alpha 3(IV)NC1$ with a mixture of trypsin and chymotrypsin. SH, sulfhydryl group; M-ox, methionine sulfoxide; OH-P, hydroxyproline

$M+H^+$	M_H	M_L	M_H and M_L	Remarks
402.1	243–245			
725.8	20–25			
948.5			115–122	
1101.5			227–236	
1116.5	185–194			C^{191} -SH
1161.4		10–21		OH-P ¹¹ , OH-P ¹²
1163.5			205–214	
1195.5		174–184		C^{179} -SH
1299.5		78–88		C^{80} - C^{86}
1319.6			205–215	
1321.6		157–169		M^{160} -ox
1348.5		209–219		
1351.7	20–31			
1413.5		189–200		C^{191} - C^{197}
1523.8			155–169	M^{160} -ox
1575.7	157–172			M^{160} -ox
1598.8		174–188		C^{179} -SH
1613.8			78–91	C^{80} - C^{86}
1630.6		89–102		
1801.8		98–114		
1817.8			98–114	1M-ox
1833.8			98–114	2M-ox
1849.8			98–114	3M-ox
1864.8			189–204	C^{191} - C^{197}
2005.8		78–94		C^{80} - C^{86}
2136.0	150–169			
2152.0	150–169			M^{160} -ox
2285.9			189–207	C^{191} - C^{197}
2338.2	72–91			C^{80} - C^{86}
2441.9		78–97		C^{80} - C^{86}

showed that ions at m/z 1195.5 (in M_L) and 1116.5 (in M_H) contained reduced Cys^{179} and Cys^{191} , respectively (Fig. 4). However, whereas Cys^{179} was consistently found only in reduced state in replicated digests of M_L , Cys^{191} in M_H was found in both reduced and forming a disulfide bond with Cys^{197} (Table 2).

To check the redox state of all 12 cysteine residues, reactive sulfhydryl groups in M_H and M_L were labeled by carbamidomethylation and subsequently cystine reduction was followed by pyridylethylation of the new exposed sulfhydryls prior to proteolytic degradation and PMF analysis. Using this approach, we found that Cys^{179} and its partner Cys^{237} are the only cysteine residues containing free sulfhydryl groups in M_L , and no evidence was found that these exist as a disulfide bond. In contrast, Cys^{191} and its partner Cys^{197} exist in both reduced and disulfide bond forms in M_H (Table 3). Finally, the evidence also suggests that the later cysteine residues exist exclusively as disulfide bond in M_L . With the exception

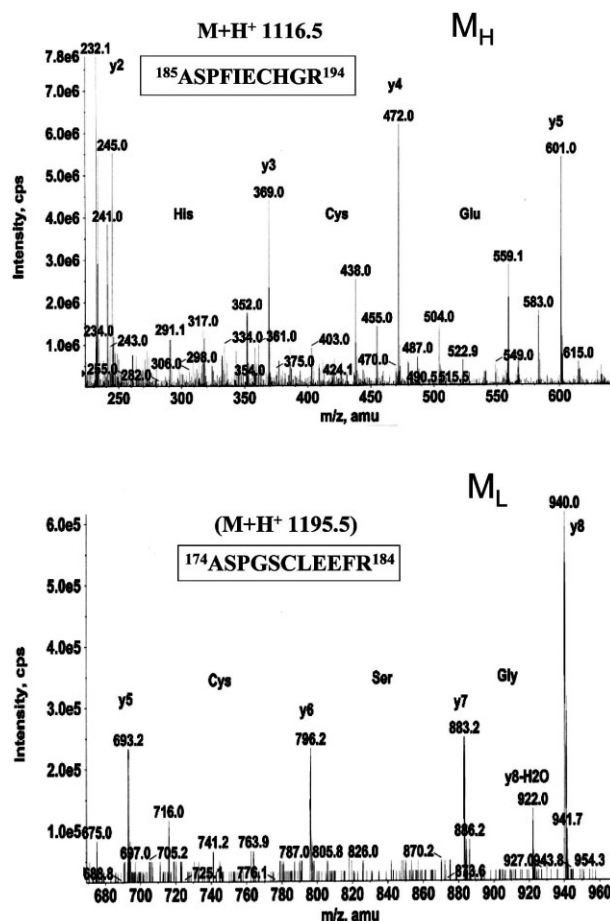


Figure 4. Cys^{191} in M_H and Cys^{179} in M_L exist in a reduced form. Detail of the MS/MS spectra of the doubly charged ions at m/z 559.3 and 598.7, which correspond to the MALDI-TOF ions at m/z 1116.5 and 1195.5 in Table 2, showing that the daughter sequence-specific y -ions confirm to the redox state of Cys^{191} in M_H and Cys^{179} in M_L , respectively.

of Cys^{237} and Cys^{240} in M_H , for which we could not find direct evidence on their redox state, the rest of cysteine residues in both M_H and M_L exist as disulfide bonds. Nevertheless, we found that Cys^{179} and Cys^{145} (the partners of Cys^{237} and Cys^{240}) are engaged in disulfide bond formation in M_H , which, in turn, supports that Cys^{145} - Cys^{240} and Cys^{179} - Cys^{237} also exist in M_H . Collectively, these results indicate that the redox state of certain cysteine residues in the C-terminal subdomains differ between M_H and M_L , but that all the cysteine residues in the N-terminal subdomain of M_H and M_L exist as disulfide bonds, as summarized in Fig. 5.

The opening of the Cys^{191} - Cys^{197} and Cys^{179} - Cys^{237} disulfide bonds are expected to have different consequences on the tertiary structure of the $\alpha 3NC1$ domain as deduced from the crystal structure of homologous domains (Fig. 6). The reduction of the Cys^{191} - Cys^{197} linkage is not expected to significantly affect the conformation of the domain as it is located in a peripheral loop, whereas reduction of the Cys^{179} - Cys^{237}

Table 3. Assignment of MALDI-TOF ions containing Cys residues which were generated by degradation of the double-labeled (carboxyamidomethylation of sulfhydryl groups in the native polypeptides followed by reduction and pyridylethylation) higher (M_H) and lower (M_L) electrophoretic bands of monomeric bovine $\alpha 3(IV)NC1$ with a mixture of trypsin and chymotrypsin. CM-C: carbamidomethylated cysteine; PE-C: pyridylethylated cysteine

$M+H^+$	M_H	M_L	Remarks
1116.6		235–242	CM-C ²³⁷ , PE-C ²⁴⁰
1175.6	185–194		CM-C ¹⁹¹
1221.6	185–194	185–194	PE-C ¹⁹¹
1228.8	195–204		CM-C ¹⁹⁷
1251.6		174–184	CM-C ¹⁷⁹
1300.7	174–184		PE-C ¹⁷⁹
1491.7		66–77	PE-C ⁶⁸
1511.8	195–206	195–206	PE-C ¹⁹⁷
1565.8		80–91	PE-C ⁸⁰ , PE-C ⁸⁶
1826.1	78–91	78–91	PE-C ⁸⁰ , PE-C ⁸⁶
2327.8	26–46	26–46	PE-C ³⁵
2330.3	162–184		PE-C ¹⁷⁹
2463.6	51–72	51–72	PE-C ⁶⁸
2776.7	47–71	47–71	PE-C ⁶⁸
3051.8	123–149		PE-C ¹²³ , PE-C ¹²⁶ , PE-C ¹⁴⁵
3214.4		44–72	PE-C ⁶⁸
3494.1	20–50		PE-C ⁶⁸
3623.6		185–214	PE-C ¹⁹¹ , PE-C ¹⁹⁷
3779.8		185–215	PE-C ¹⁹¹ , PE-C ¹⁹⁷
4608.2		115–154	PE-C ¹²³ , PE-C ¹²⁶ , PE-C ¹⁴⁵

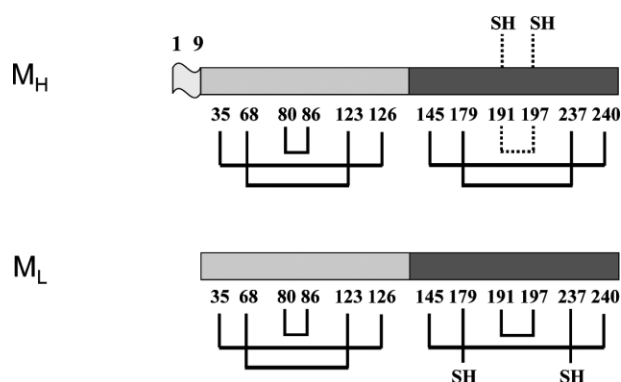


Figure 5. Schematic representation of bovine M_H and M_L $\alpha 3(IV)NC1$ polypeptides. Schematic drawing of primary structure features of the M_L and M_H polypeptides of the $\alpha 3(IV)NC1$ domain isolated from TBM, highlighting the presence of the N-terminal polypeptide extension in M_H , and the disulfide bonds and free cysteines within the N- and C-terminal subdomains of M_L and M_H . Solid lines denote cysteines only found in a reduced form in M_L , and dashed lines and brackets indicate cysteines found in both reduced and oxidized form in M_H . SH, sulfhydryl group. Cysteine residues are numbered according to their position in M_H .

bond may disrupt structural constraints linking two distant parts of the domain. This might explain at least in part the co-migration of the two redox isoforms of M_H and the abnormal migration of M_L in SDS-PAGE gels. It is also conceivable that such structural perturbation may have addi-

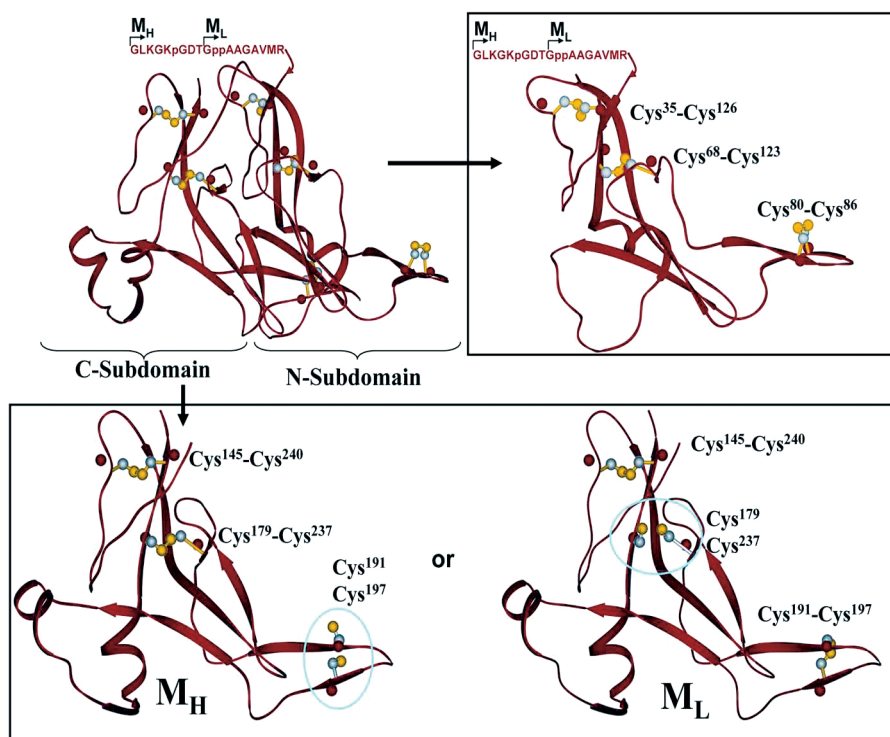


Figure 6. Structural representation of bovine M_H and M_L $\alpha 3(IV)NC1$ polypeptides. Structural model for M_H and M_L based on the crystal structures of homologous $\alpha 1$ and $\alpha 2$ NC1 domains (PDB accession codes 1M3D, 1T60, and 1T61), showing the distinct structural features. The pairs of cysteines whose redox state differentiate M_H from M_L are circled.

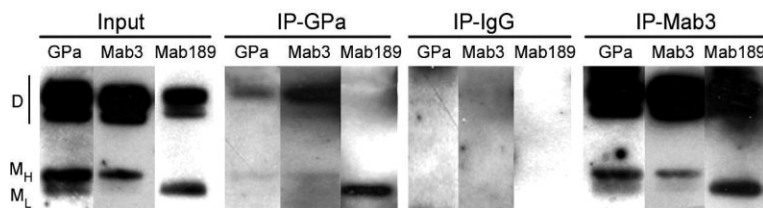


Figure 7. Differential exposure of pathogenic epitope(s) in M_H and M_L at the quaternary structure level. TBM hexamer (Input) or the indicated immunoprecipitates thereof (IP) were analyzed by Western blot using the indicated antibodies. Denoted are the position of dimers (D) and M_H and M_L .

tional consequences, *i.e.*, exposure of epitopes, as pointed out by the different antibody reactivity and also by the distinct susceptibility to endoprotease digestion displayed by M_H and M_L .

3.3 Evidence for conformational diversification of the $\alpha 3(IV)NC1$ domain in the quaternary structure

To assess the biological significance of our findings, the presence of $\alpha 3NC1$ conformers in the quaternary structure was investigated. NC1 hexamer purified under non-denaturant conditions from TBM was subjected to precipitation using GP antibodies (Fig. 7). Western blot analysis of monomer residing in the hexamer revealed that both GP and Mab3 antibodies reacted more with M_H , whereas Mab189 showed more reactivity with M_L (Input, Fig. 7) reflecting the relative higher abundance of M_H and the specificity of Mab189 antibodies for M_L (Fig. 1). In contrast, material in the dimer region (D) showed two major molecular species both of which reacted with all three antibodies. Analysis of immunoprecipitates revealed that the pathogenic antibodies pulled down preferentially minor M_L polypeptide, and were comparatively less efficient in precipitating the more abundant M_H and dimer components (IP-GPa). The specificity of GP antibodies precipitating M_L was further confirmed by demonstrating that, under similar experimental conditions, human IgG from control individuals could not precipitate NC1 material (IP-IgG), whereas Mab3 antibodies precipitated all molecular species (IP-Mab3). These findings reveal that M_H and M_L have a different quaternary structure, and this critically impacts on epitope(s) exposure mediating GP disease. It has been reported that *in vitro* the GP antibodies bind to the hexameric form of the autoantigen and extract its monomeric form [8]. More recent evidence indicates that the hexamer contains at least two subpopulations, one composed solely of monomers in which the pathogenic epitope(s) is accessible to the autoantibodies and other composed of monomer and dimers in which the epitope(s) is not available for autoantibody binding [9]. Collectively, all these findings suggest that M_H and M_L are assembled in different quaternary structures, one containing M_L , which can be breached by the autoantibody and undergo binding, and others containing dimers and M_H , which is resistant to disruption and autoantibody binding.

Our findings establish the existence of conformational diversity within the $\alpha 3(IV)NC1$ domain isolated from natural source. Moreover, the differential collagenase processing of

the $\alpha 3(IV)$ chain when assembled in the basement membranes, and the differential exposure of the GP epitope in the resulting $\alpha 3NC1$ polypeptides prior to quaternary structure dissociation, point to the existence of conformational diversity also in the natural source.

4 Concluding remarks

Our data indicate that the redox state of specific cysteines is one of the structural features associated to the susceptibility of the $\alpha 3(IV)NC1$ domain to acquire related but distinct conformations. Conceivably, triggering events, *i.e.*, oxidative stress, could have structural consequences within the $\alpha 3(IV)NC1$ domain, which activate the immune system and cause GP disease. In this hypothesis, the autoimmune response is envisioned as a legitimated reaction of the immune system against conformations of the autoantigen that differ from the normal. Such “aberrant” polypeptides can be retrotranslocated from the ER to the cytoplasm to undergo degradation by the ubiquitin-proteasome system [12], and potentially induce MHC class I immune responses. In line with an “aberrant conformation autoantigen” hypothesis for GP disease, GP patients have been shown to possess disease-associated CD8⁺ autoreactive T cells specifically recognizing residues 10–18 of the $\alpha 3(IV)NC1$ domain [13]. This non-conventional MHC class I immune response for an extracellular component might represent a collateral phenomenon resulting from retro-translocation of aberrant $\alpha 3(IV)NC1$ conformers most of which, however, reach the basement membrane and induce the production of pathogenic autoantibodies through an MHC class II immune response.

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