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Goodpasture antigen: Expression of the full-length α3(IV) chain of collagen IV and localization of epitopes exclusively to the noncollagenous domain

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Goodpasture antigen: Expression of the full-length α3(IV) chain of collagen IV and localization of epitopes exclusively to the noncollagenous domain.

Background. Tissue injury in Goodpasture (GP) syndrome (rapidly progressive glomerular nephritis and pulmonary hemorrhage) is mediated by antibasement membrane antibodies that are targeted to the α3(IV) chain of type IV collagen, one of five α(IV) chains that occur in the glomerular basement membrane. GP antibodies are known to bind epitopes within the carboxyl terminal noncollagenous domain (NC1) of the α3(IV) chain, termed the GP autoantigen. Whether epitopes also exist in the 1400-residue collagenous domain is unknown because studies to date have focused solely on the NC1 domain. A knowledge of GP epitopes is important for the understanding of the etiology and pathogenesis of the disease and for the development of therapeutic strategies.

Methods. A cDNA construct was prepared for the full-length human α3(IV) chain. The construct was stably transfected into human embryonic kidney 293 cells. The purified full-length r-α3(IV) chain was characterized by electrophoresis and electron microscopy. The capacity of this chain for binding of GP antibodies from five patients was compared with that of the human r-α3(IV)NC1 domain by competitive enzyme-linked immunosorbent assay.

Results. The r-α3(IV) chain was secreted from 293 cells as a single polypeptide chain that did not spontaneously undergo assembly into a triple-helical molecule. An analysis of GP-antibody binding to the full-length r-α3(IV) chain showed binding exclusively to the globular NC1 domain.

Conclusion. The full-length human α3(IV) chain possesses the capacity to bind GP autoantibodies. The epitope(s) is found exclusively on the nontriple-helical NC1 domain of the α3(IV) chain, indicating the presence of specific immunogenic properties. The α3(IV) chain alone does not spontaneously undergo assembly into a triple-helical homotrimeric molecule, suggesting that coassembly with either the α4(IV) and/or the α5(IV) chain may be required for triple-helix formation.

Key words: Goodpasture syndrome, recombinant protein, type IV collagen, autoimmune disease, NC1 domain.

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Goodpasture (GP) syndrome is an autoimmune disease characterized by rapidly progressive glomerular nephritis and pulmonary hemorrhage [1]. Tissue injury is thought to be mediated by antibasement membrane antibodies reacting with the glomerular basement membrane and the alveolar basement membrane. The target autoantigen in the basement membrane is known to be the α3(IV) chain of type IV collagen, commonly referred to as the "GP autoantigen" [2–4]. A knowledge of GP epitopes is important for the understanding of the etiology and pathogenesis of the disease and for the development of therapeutic strategies.

The complete primary structure of the human α3(IV) chain is known [5]. The chain is characterized by a long collagenous domain of 1410 amino acid residues that is interrupted by 23 short noncollagenous sequences and by a noncollagenous (NC1) domain of 232 residues at the carboxyl terminus. In the glomerular basement membrane, the α3(IV) chain exists in a supramolecular network along with the α4(IV) and α5(IV) chains of type IV collagen [6]. The network is characterized by loops and supercoiled triple-helical molecules that are stabilized by disulfide cross-links.

The study of GP-autoantibody binding has been focused on epitopes in the NC1 domain of the α3(IV) chain [2–4]. Whether epitopes also exist within the 1400-residue collagenous domain is unknown, as the α3(IV) chain is not amenable to isolation and purification from tissue as the NC1 domain. The α3(IV)NC1 domain was initially isolated as a 28 kDa protein from basement membranes by collagenase digestion. Its novel identity was established based on molecular properties and its capability to bind GP autoantibodies [2–4, 7, 8]. Subsequently, it was confirmed by molecular cloning [9–11]. The discovery of homologous NC1 domains that correspond to the α4(IV), α5(IV), and α6(IV) chain [12–14] raised the question of whether epitopes also exist within the NC1 domains of other members of the type IV family of six α(IV) chains. In a recent study of 58 GP patient

Table 1. Primers used in PCR amplification reactions

Product	Primer sequences
1st PCR of cDNA	A1 5′-GGCTCTGAGCTCTCTCCCACCATG-3′
	B1 5′-ACAATCACCGTATGAGCAGTGCC-3′
2nd PCR of cDNA	A2 5′-CGCGCTAGCCAAGGGTTGTGTCTG TAAAGAC-3′
	B2 5′-TCTATCGATGCTTCAGTGTCTTTT CTTCATG-3′
Correction fragment	A3 5′-CCCTGGCACACTTAAGATTAT CTCC-3′
	B3 5′-TCTATCGATGCTTCAGTGTCTTTT CTTCATG-3′
1st PCR of vector	A4 5′-CAAGCTAGCGGCCGCTCGAGAT GCATCTAGAGGGCCC-3′
	B4 5′-GCCGCTAGCTTGTCATCGTCGTC CTTGTAGTCGGCTAGTGGGGCTGC CAGAGCCCT-3′
2nd PCR of vector	A5 5′-AGCTCTAGAGTCATCGATGTTA ACCGCGGGCCCTATTCTATAGTGTC-3′
	B5 5′-CCCTCTAGATGCATCTCGAGC GGC-3′

sera, the α3(IV)NC1 domain was found to be the common autoantigen [15]. The antibodies of all patients reacted with the α3(IV)NC1 domain (85% exclusively). Additional limited reactivity with the α1(IV)NC1 and α4(IV)NC1 was found in 15 and 3% of the patients, respectively. In a study of 10 patients, using *E. coli* fusion proteins of the six α(IV)NC1 domains, all showed reactivity to the α3(IV)NC1 domain, and several sera showed additional reactivity to α2(IV), α4(IV), or α6(IV)NC1 domains [16]. Overall, the results *in vitro* establish that among the six α(IV)NC1 domains, the α3(IV)NC1 domain is the primary target for GP antibodies.

In this study, a cDNA construct encoding the full-length α3(IV) chain was successfully made, characterized, and expressed in human embryonic kidney 293 cells. The expressed chain allows for the determination of binding of GP autoantibodies and localization of epitopes. The results show that binding of GP autoantibodies is exclusively localized to the NC1 domain of the nontriple-helical, full-length α3(IV) chain, indicating the presence of specific immunogenic properties in this region. The results also suggest that the triple-helical assembly of the α3(IV) chain may require coassembly with either the α4(IV) chain, the α5(IV) chain, or both.

METHODS

DNA construction

An approximately 5.0 kb full-length α3(IV) cDNA (nucleotides 246 to 5174 according to the published sequence, GenBank X80031) was generated by nested polymerase chain reaction (PCR) amplification from human Marathon kidney cDNA library (Clontech, Palo Alto, CA, USA) by using Klentaq polymerase (Clontech) and primers (Table 1) designed according to the published sequence [5]. *Nhe* I and *Cla* I restriction enzyme sites were introduced at the 5′ and 3′ ends, respectively. The cDNA was ligated into a modified pRC-X expression vector into *Nhe* I and *Cla* I sites. The construction of this vector was based on the pRC/AC7 vector (kindly provided by Dr. Ernst Pöschl, Erlangen, Germany), containing the signal peptide of BM40 downstream of the cytomegalovirus (CMV) promoter [17]. The DNA sequence encoding the FLAG™ peptide (DYKDDDDK) and additional restriction enzyme sites (Fig. 1) for subcloning were introduced by primers (Table 1). The resulting pRC-X/α3(IV) construct was checked by restriction enzyme analysis and DNA sequencing. One PCR-related single-base mutation (T4968→C) was corrected by cutting the construct with *Afl* I and *Cla* I restriction enzymes and replacing the fragment with a new fragment generated by PCR amplification (Table 1).

Cell cultures and transfections

Human embryonic kidney 293 cells were grown in Dulbecco's modified Eagle's medium/F12 medium (Sigma Chemicals, St. Louis, MO, USA) medium containing 5% fetal bovine serum and 50 μg/ml ascorbic acid phosphate (Wako, Richmond, VA, USA). Transfections were done by using SuperFect reagent (Qiagen, Chatsworth, CA, USA) and 5 μg of pRC-X/α3(IV) construct. Selection of transfected cells was started with 250 μg/ml G418 (GIBCO, Grand Island, NY, USA) two days after transfection. Resistant clones were isolated and expanded. Stably transfected clones were screened for protein expression from serum-free medium by slot blotting and/or Western blotting using anti-FLAG antibody (Kodak, Rochester, NY, USA).

Protein purification

Medium from stably transfected 293 cell cultures was collected after 48 hours. Protease inhibitors ethylenediaminetetraacetic acid (EDTA, 1 mM) and phenylmethylsulfonyl fluoride (PMSF, 2 mM) were then added, and the medium was stored at −20°C. The medium was made 0.2 M with NaCl before passing through an anti-FLAG affinity chromatography column (Kodak). The bound FLAG-tagged recombinant α3(IV) chain was eluted from the column by competing FLAG-peptide (Kodak) at a concentration of 100 μg/ml in Tris-buffered saline. Protein samples were concentrated by ultrafiltration (Amicon, Beverly, MA, USA) and were stored at −70°C.

SDS-PAGE and Western blots

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 4 to 15% linear gradient gels [18] and was stained with Coomassie Brilliant Blue R250 or transferred onto nitrocellulose membranes. Anti-FLAG M2 (Kodak) and monoclonal anti-α3(IV)NC1, Mab3 (Wieslab Ab, Sweden) antibodies

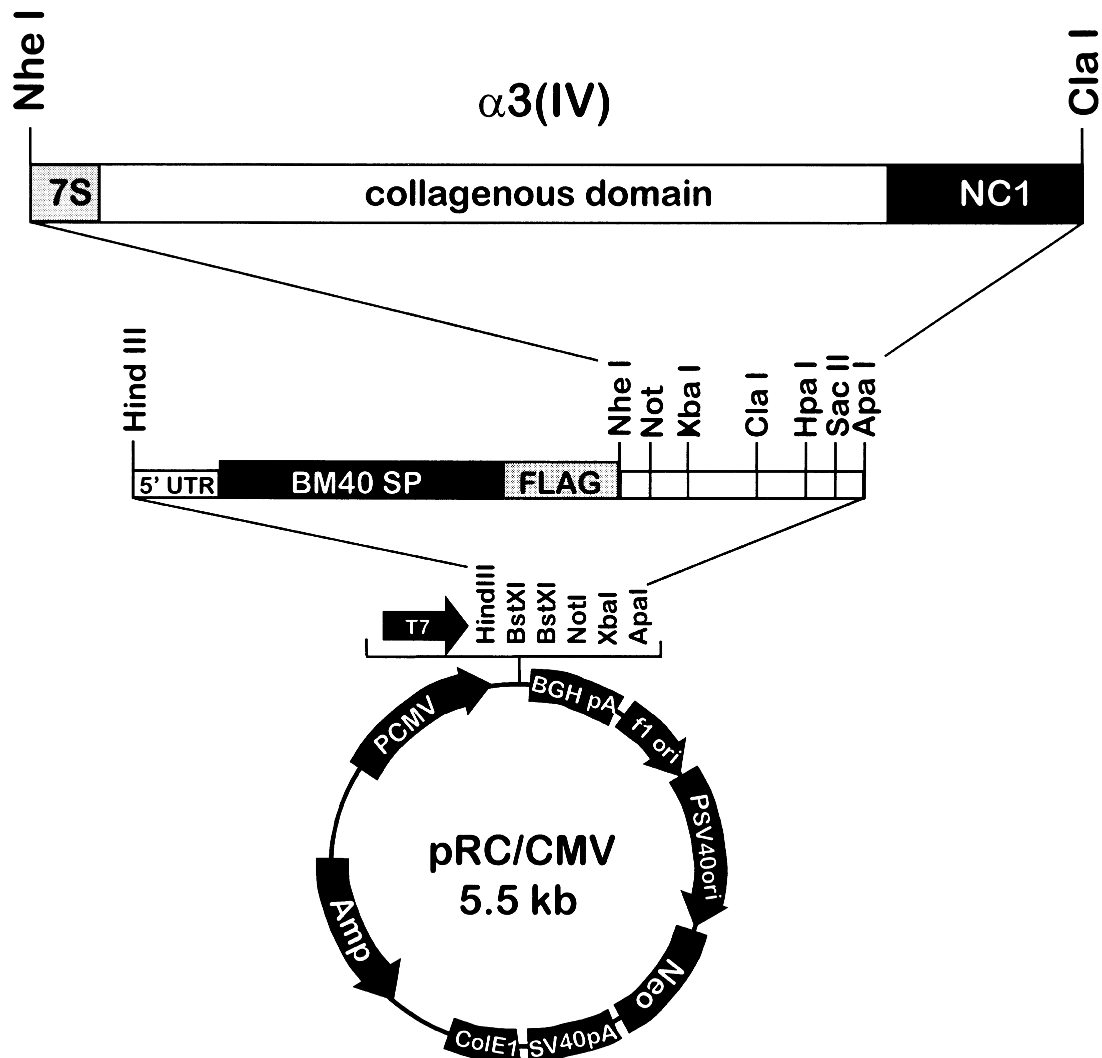

Fig. 1. Schematic showing the pRC/α3(IV) construct. The full-length α3(IV) cDNA was generated by polymerase chain reaction (PCR) and subcloned into *Nhe* I and *Cla* I restriction sites in the pRC-X expression vector, which was modified by adding BM40 signal peptide, FLAG peptide, and additional restriction sites.

were used in Western blots under conditions described previously [8].

Enzyme digestions

For collagenase digestion, the purified r-α3(IV) chain was ethanol precipitated and suspended into 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, containing 5 mM $CaCl_2$, 1 mM N-ethylmaleimide, and 2 mM phenylmethylsulfonyl fluoride. Bacterial collagenase was added at a 1:10 enzyme/protein ratio, and digestion was performed at +37°C for 18 hours. For pepsin digestion, recombinant protein was suspended in 0.5 M acetic acid and was incubated with pepsin at 1:10 enzyme/protein ratio for 18 hours at +4°C. After enzyme digestion, proteins were ethanol precipitated and analyzed by SDS-PAGE.

Expression of recombinant human α2(IV)NC1 and α3(IV)NC1 domains

Human r-α2(IV)NC1 and r-α3(IV)NC1 proteins were expressed in human embryonic kidney 293 cells and purified as described earlier [19].

Patient sera

Aliquots of plasmapheresis fluid of five GP patients were used in enzyme-linked immunosorbent assays. GP-IgG antibody (GP-1) sera was described earlier [8].

Affinity chromatography of Goodpasture autoantibodies

Recombinant α3(IV)NC1 domain at a concentration of 0.5 mg/ml was coupled to Affigel-10 matrix (Bio-Rad,

Table 2. Amino acid changes compared to the published primary structure of α3(IV) chain [5]

Base pair[a]	Amino acid
A 375 → C	Thr-72 → Pro
T 583 → C	Leu-141 → Pro
A 646 → G	Glu-162 → Gly
T 1360 → A	Ile-400 → Lys
G 1486 → T	Gly-442 → Val
C 1882 → T	Pro-574 → Leu
C 2903 → G	His-914 → Gln
T 3193 → G	Leu-1011 → Arg

[a] Numbering is based on the sequence in GenBank, X80031

Richmond, CA, USA) according to the manufacturer's protocol. Two milligrams of GP-1 was applied to the column. Unbound material was collected, and the bound material was eluted with 3 M potassium thiocyanate (KSCN). Both fractions were concentrated by Centricon-10 spin concentrators and were used in competitive ELISA assays.

Competitive enzyme-linked immunosorbent assay

Fifty nanograms of the r-α3(IV)NC1 or 200 ng of the r-α3(IV) chain were coated on 96-well plates (Nunc, Naperville, IL, USA) in 200 μl of 50 mM sodium carbonate, pH 9.5, coating buffer. Plates were washed with 150 mM NaCl and 0.05% Tween 20 buffer (washing buffer) and were blocked with 1% casein in 50 mM Tris-HCl, pH 7.4. Different concentrations of the r-α3(IV) chain, the r-α3(IV)NC1, the r-α2(IV)NC1, and albumin were preincubated with 1:100 dilution of five different GP sera, 1:25 dilution of unbound GP-IgG, or 1:50 dilution of bound GP-IgG in 50 mM Tris-HCl, pH 7.4, buffer before adding to the coated wells. Alkaline phosphatase-conjugated anti-human IgG at a dilution of 1:3000 was used as a secondary antibody, with disodium p-nitrophenyl phosphate as a substrate. Plates were read at 410 nm in a Dynatech MR 400 plate reader.

Electron microscopy

The recombinant α3(IV) chain was diluted with 0.15 M ammonium bicarbonate buffer to a concentration of 50 μg/ml, and an equal volume of glycerol was added. The sample was then sprayed onto freshly cleaved mica sheets. Rotating samples were shadowed at a 5 to 6 degree angle with 0.9 nm platinum and were then coated with carbon deposited at a 90° angle. The replicas were viewed with a JEOL JEM 100CX II electron microscope (JEOL, Tokyo, Japan), photographed at a magnification of ×19,000, and the prints of the negatives were enlarged fivefold.

Amino acid analysis

Amino acid analysis of the r-α3(IV) chain and mouse type IV collagen (Becton Dickinson, Mountain View, CA, USA) after hydrolysis was performed by the BioCore Facility at University of Kansas Medical Center.

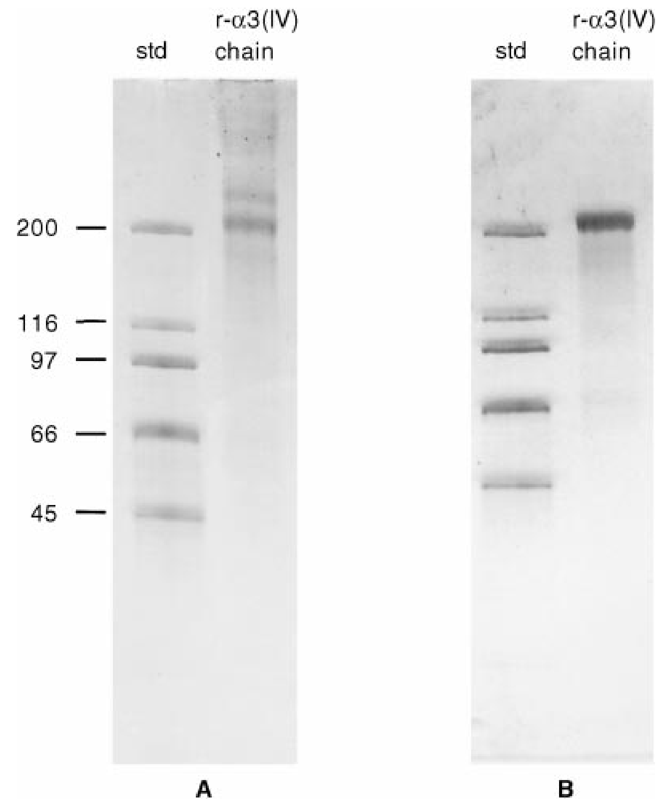

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of purified r-α3(IV) chain. The r-α3(IV) chain was purified from the culture medium by using FLAG-affinity chromatography. Purified samples were run on 4 to 15% gradient gels under nonreducing (*A*) and reducing (*B*) conditions and were stained with Coomassie Blue. Without reduction, the r-α3(IV) chain migrated as a double band with $M_r > 200,000$. After reduction with 20 mM dithiothreitol, the chain appeared as a single band, M_r approximately 250,000.

RESULTS

Preparation of a full-length construct of the human α3(IV) chain

The cDNA encoding a full-length human α3(IV) chain was generated from a kidney cDNA library by a nested PCR amplification strategy. This strategy was selected to avoid several modifications steps involved in generating full-length cDNA from the original partial cDNA clones [5], which lacked suitable restriction enzyme sites for subcloning. The full-length α3(IV) cDNA was then ligated into a pRC-X expression vector containing a FLAG sequence for affinity purification (Fig. 1) and sequenced. Nine sequence variations were found in the full-length α3(IV) cDNA that differed from the published sequence [5] and would encode variations in the amino acid sequence. The base pair changes were confirmed by repeating cDNA amplification from the same

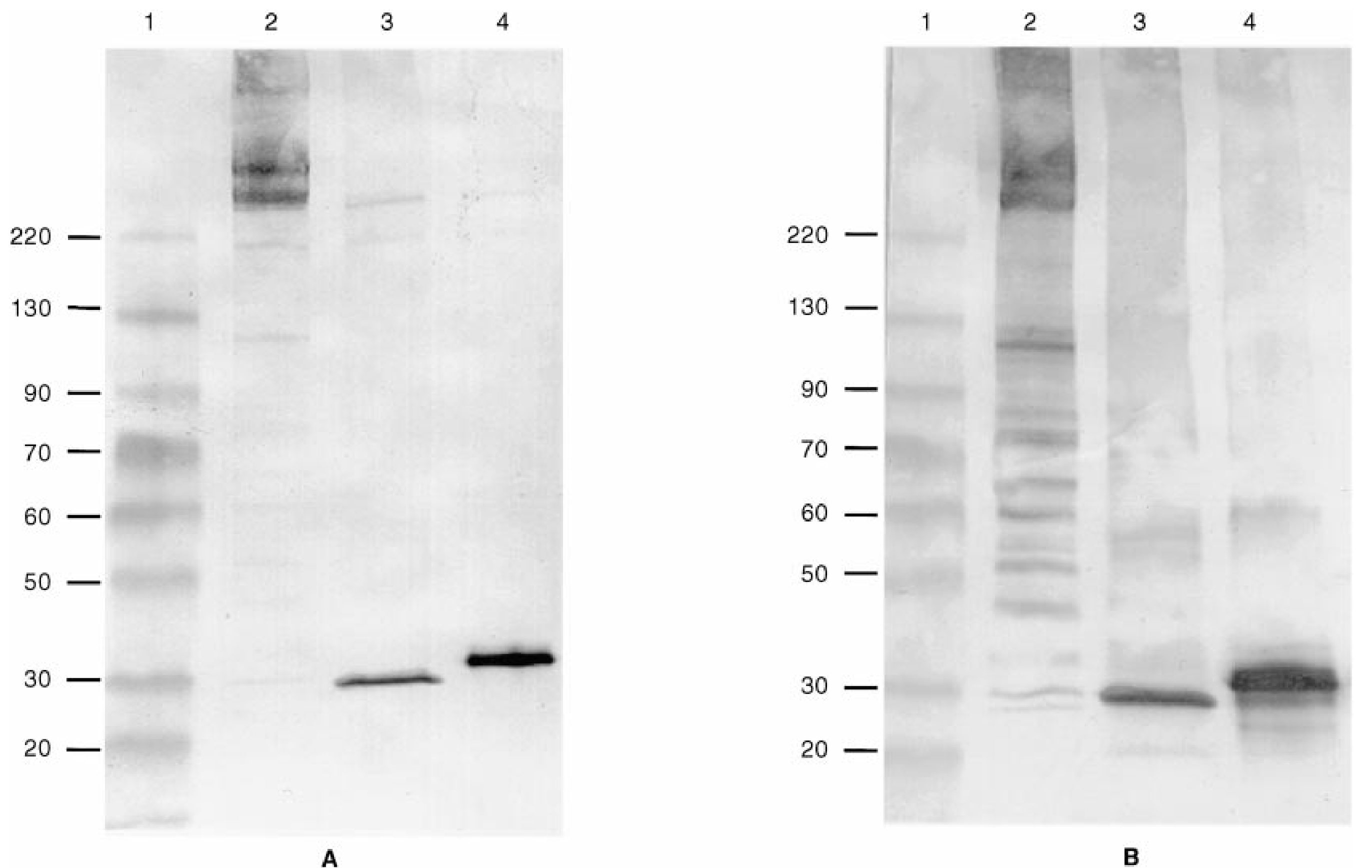

Fig. 3. Western blot analysis of the full-length and collagenase-digested r-α3(IV) chain. The r-α3(IV) chain and its collagenase digestion products were run on 4 to 15% gradient gels and were blotted with monoclonal antibody against α3(IV)NC1 (*A*) and Goodpasture antibodies (GP-1; *B*). Lane 1, molecular weight standard (Gibco); lane 2, r-α3(IV) chain; lane 3, collagenase-digested r-α3(IV) chain; and lane 4, r-α3(IV)NC1.

Table 3. Amino acid analysis of r-α3(IV) chain

Amino acid	% in α3(IV)	Hydroxylated %
HyL	2.62	28
Lys	6.70	
HyP	10.28	48
Pro	11.13	

library and by subsequent sequencing. The original cDNA clones (PCR1, AK20, AK12, and AK1) on which the published α3(IV) sequence is based were then resequenced. Eight of nine variations in questions were found to be present in original clones. Hence, the published α3(IV) sequence contains eight incorrect base pairs that resulted in incorrect amino acid residues (Table 2). Five of the sequencing errors were in the collagenous domain in Y positions, one in an X position of the Gly X-Y collagenous repeat sequence, and two were in interruptions of the collagenous sequence. The latter two were a substitution of glycine for glutamic acid 646 in interruption I, and a substitution of valine for glycine 1486 that increased the length of interruption VIII from one to four residues. The remaining sequence change (proline for serine 1603) most likely reflected a PCR-related mutation that occurred at a highly conserved amino acid position of the NC1 domain. This mutation was corrected by inserting a PCR-generated fragment with the correct sequence.

Expression and characterization of the r-α3(IV) chain

The full-length α3(IV) construct was stably transfected into human embryonic kidney 293 cells. Cell clones were tested for the r-α3(IV) chain expression by slot and Western blotting using anti-FLAG antibody. The r-α3(IV) chain was expressed as a fusion protein containing a short FLAG peptide (DYKDDDDK) at the amino terminus that allowed purification of recombinant protein by single-step FLAG-affinity chromatography (Kodak).

The r-α3(IV) chain appeared as a double band, with a $M_r > 200{,}000$, as observed by SDS-PAGE under nonreducing conditions, and after reduction of the disulfide bonds as a single band with M_r = approximately 250,000 (Fig. 2). The higher band seen on the gel under nonreducing conditions probably represents disulfide-linked di-

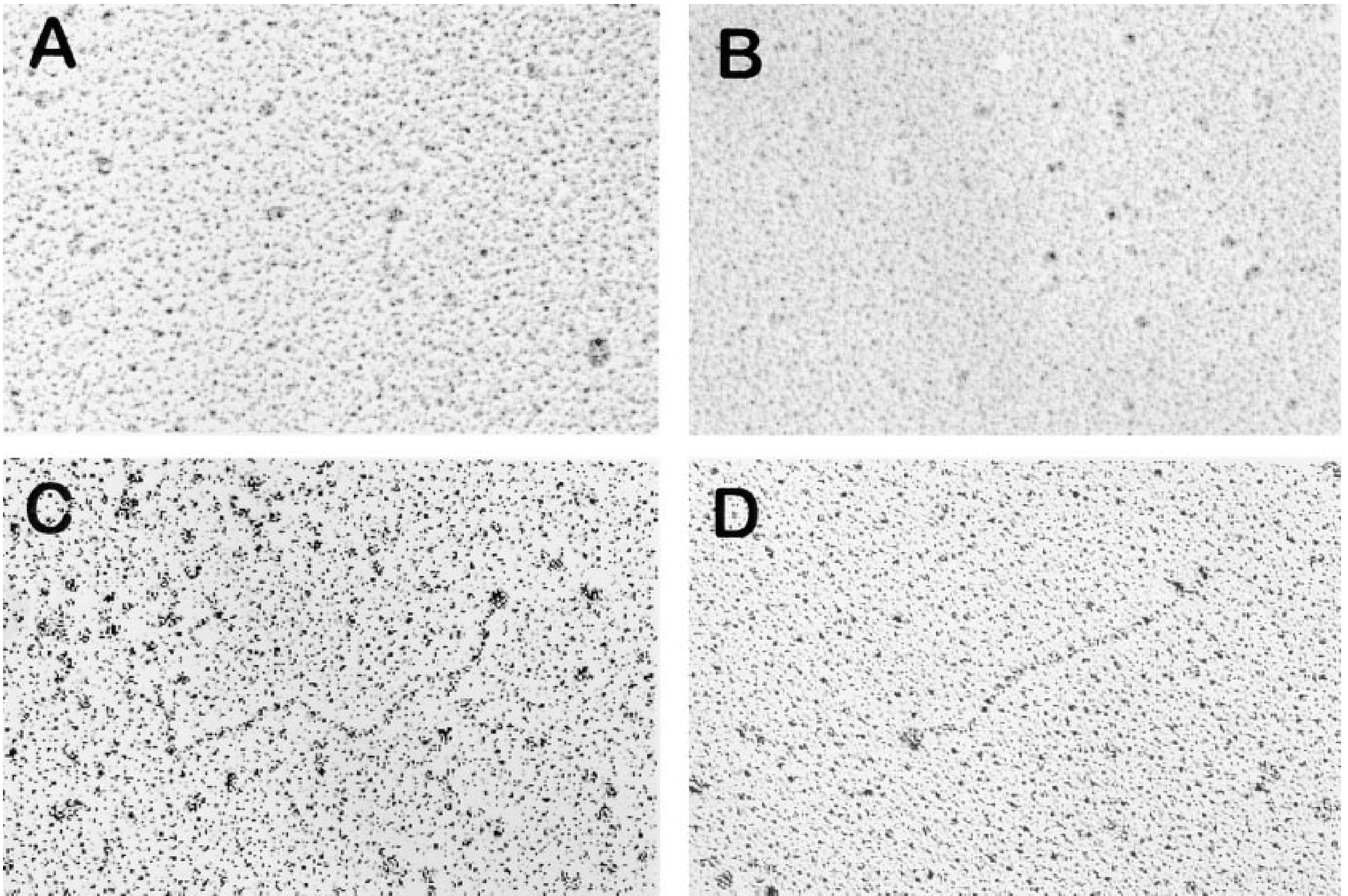

Fig. 4. Rotary shadowing electron microscopy of the r-α3(IV) chain. The r-α3(IV) chain exists mainly in nontriple-helical form, and only the globular NC1 domains were visible by electron microscopy (*A and B*). In a few cases, rod-like extensions from the globular domains, representing triple-helical domains, were observed (*C and D*).

mers. The r-α3(IV) chain is slightly larger in size than the recombinant mouse α1(IV) and α2(IV) chains expressed in Chinese hamster ovary (CHO) cells [20], which had M_r = 185,000 and 170,000, respectively. The size of the r-α3(IV) chain was estimated to be 1767 residues based on the electrophoretic mobility of standard proteins containing a known number of residues [21]. After collagenase digestion, the r-α3(IV) chain yielded the α3(IV)NC1 domain with M_r = 28,000. The size of this collagenase digestion product is slightly smaller than that of the recombinant α3(IV)NC1 on SDS-PAGE (Fig. 3) because the latter contains a FLAG peptide at the amino terminus. The full-length α3(IV) chain and its NC1 domain were both recognized by monoclonal antibody against α3(IV)NC1, as well as by the affinity-purified GP autoantibody (GP-1; Fig. 3 A, B, respectively), establishing the identity and purity of the expressed full-length r-α3(IV) chain. Amino acid analysis of the recombinant α3(IV) chain showed that approximately 48% of the proline residues and 28% of the lysine residues were hydroxylated (Table 3). Mouse type IV collagen was used as a control in the analysis, and it revealed 65% and 52% hydroxylation of the proline and lysine residues, respectively. The underhydroxylation of the recombinant protein might be a result of overexpression of the recombinant protein due to the strong cytomegalovirus promoter.

Rotary shadowing electron microscopy of the r-α3-(IV) chain revealed a high number of globular domains (NC1), which appeared in different sizes (Fig. 4). This indicates that the NC1 domain forms some aggregates such as dimers. Rod-like extensions to globular domains, representing triple-helical domains, were observed in only a few cases. The length of these triple helices was determined by the NIH Image program, and it varied between 340 and 380 nm, coming close to the lengths of type IV collagen molecules in native basement membranes. Pepsin digestion of expressed recombinant protein did not reveal any pepsin-resistant material (data not shown), indicating that the amount of triple-helical molecules, as observed by rotary shadowing electron microscopy, is very low. Presumably, these triple-helical

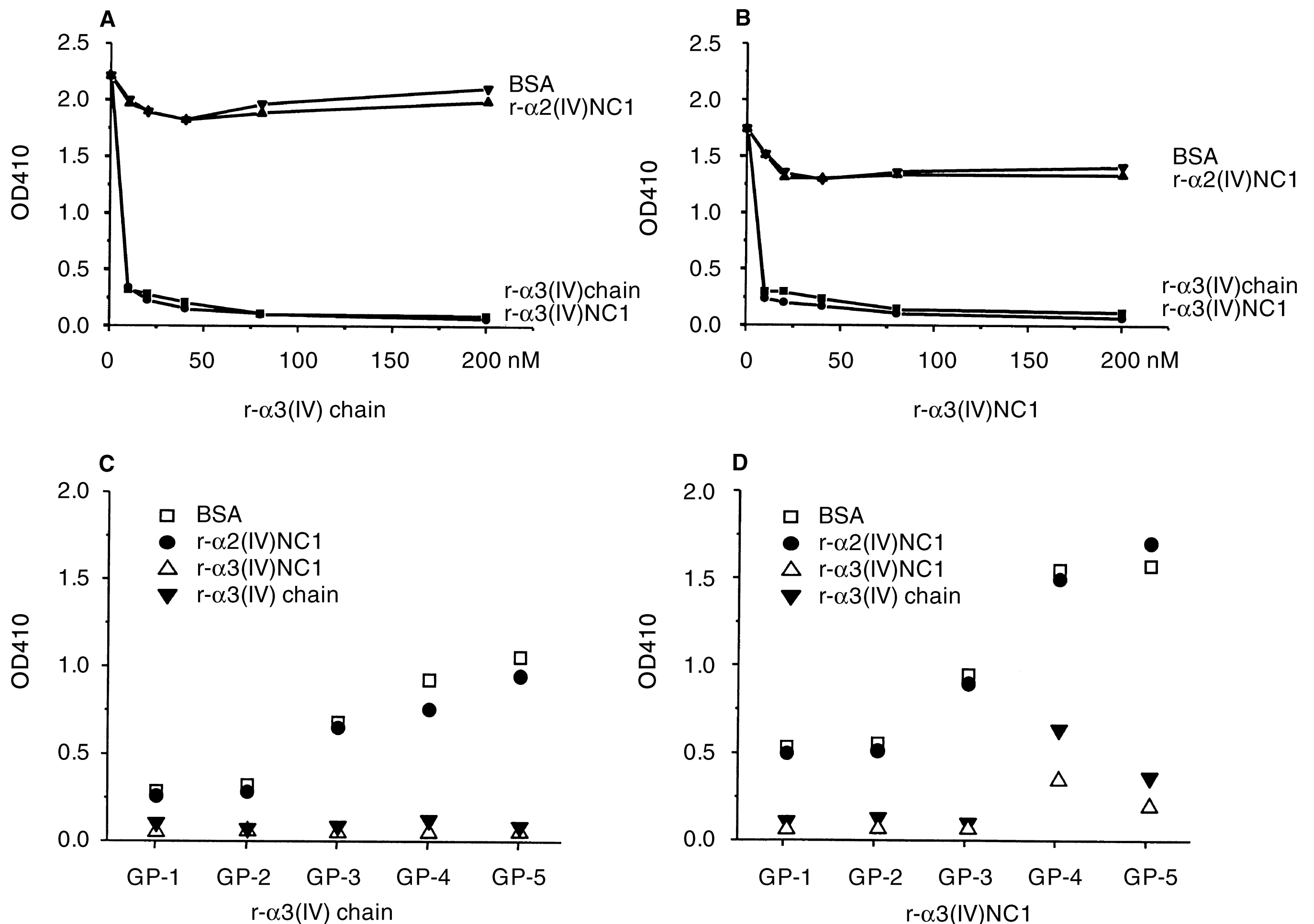

Fig. 5. Assessment of the binding capacity of Goodpasture (GP) antibodies to r-α3(IV) chain relative to r-α3(IV)NC1 by competitive enzyme-linked immunosorbent assay. The r-α3(IV) chain (*A and C*) and the r-α3(IV)NC1 (*B and D*) were coated on the plates. GP antibodies from one patient (GP-1) were preincubated with various concentrations of the r-α3(IV) chain and the r-α3(IV)NC1 before adding them to the coated wells (A and B), and five GP sera were preincubated with the r-α3(IV) chain and r-α3(IV)NC1 at a concentration of 80 nM before adding them to the coated wells (C and D). r-α2(IV)NC1 and albumin were used as controls. Color development was measured at 410 nM.

molecules represent α3(IV) homotrimers because no endogenous expression of α4(IV) or α5(IV) chains was detected by Western blot analysis (data not shown).

Goodpasture autoantibody binding to the r-α3(IV) chain

The capacity of the human full-length r-α3(IV) chain to bind GP autoantibodies was tested by competitive ELISA and compared with that of the r-α3(IV)NC1 domain. The r-α3(IV)NC1 domain has a similar binding capacity of GP autoantibodies as the native α3(IV)NC1 monomers isolated from tissues (Boutaud A, Gunwar S, Singh N, Netzer K-O, Sado Y, Ninomiya Y, Noelken ME, Hudson BG, manuscript in preparation). The r-α3(IV)NC1 completely inhibited binding of GP autoantibodies to the full-length chain over a wide range of concentrations (Fig. 5A). Likewise, the full-length chain completely inhibited binding to r-α3(IV)NC1 (Fig. 5B). The amount of inhibition was also determined for several GP sera (Fig. 5 C–D), and the results showed virtually complete inhibition when the full-length chain was compared with the r-α3(IV)NC1 domain and vice versa. These results demonstrate that the epitopes for GP autoantibodies are exclusively localized on the NC1 domain of the nontriple-helical α3(IV) chain.

To verify this conclusion further, antibodies from one serum were absorbed onto an affinity column composed of r-α3(IV)NC1. The bound and unbound fractions from this column were analyzed for their capacity to bind the full-length chain (Fig. 6 A–D). The bound antibodies, directed against the α3(IV)NC1 domain, showed identical inhibition against the full-length r-α3(IV) chain or the r-α3(IV)NC1 domain, whereas the unbound fraction contained no antibody reactivity against either the full-length chain or the NC1 domain. These results further

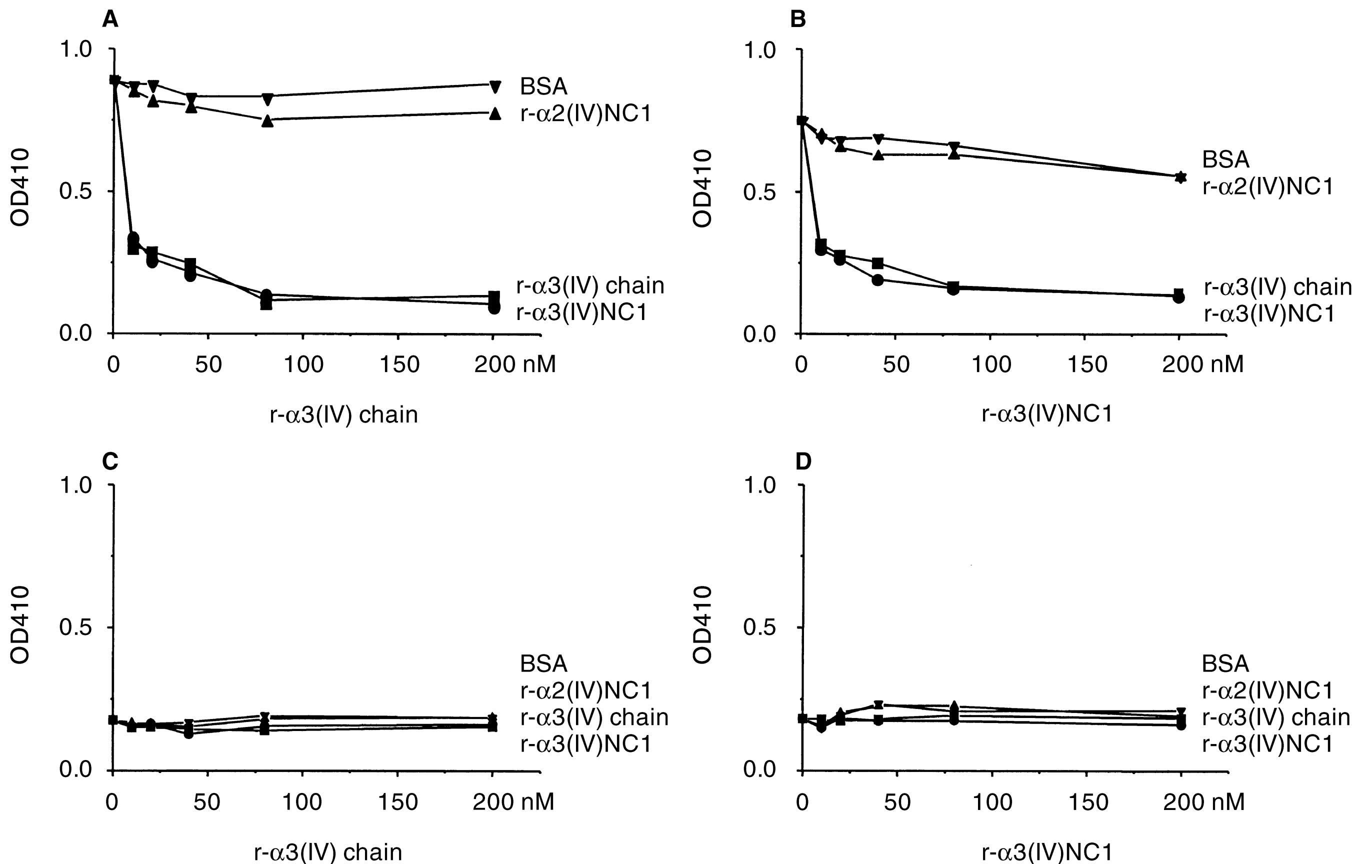

Fig. 6. The assessment of binding capacity of Goodpasture (GP) antibodies to the r-α3(IV) chain and the r-α3(IV)NC1 domain by binding of GP antibodies by r-α3(IV)NC1 affinity column. The r-α3(IV) chain (*A and C*) and r-α3(IV)NC1 (*B and D*) were coated on the plate. GP antibodies (GP-1), which bound to the r-α3(IV)NC1 affinity column (A and B) and unbound antibodies (*C and D*) were preincubated with various concentrations of r-α3(IV) chain, r-α3(IV)NC1, r-α2(IV)NC1, and albumin before adding them to the coated wells. Color development was measured at 410 nm.

demonstrate that the epitope for GP autoantibodies are exclusively directed to the α3(IV)NC1 domain.

DISCUSSION

In this study, the full-length human r-α3(IV) chain was expressed in 293 cells for the first time. As a collateral result of the assembly and sequencing of full-length α3(IV) cDNA, it was found that the previously published sequence [5] was incorrect in eight positions. The errors illustrate the difficulty of working with long DNA sequences of low complexity and point to cautious interpretation of sequence data.

Protein expression of the α3(IV) chain in eukaryotic 293 cells was feasible. Other examples for the successful expression of recombinant collagen molecules in 293 cells include type V and X collagen chains and their assembly into triple-helical molecules [22, 23]. However, in the case of the r-α3(IV) chain, only a very small fraction of chains formed triple-helical molecules, as observed by rotary shadowing electron microscopy. Accordingly, no pepsin resistance was found, indicating that the expressed chains exist mainly in nontriple-helical form. The triple-helix formation is believed to be driven by an interaction near the C-terminus; therefore, it is unlikely that the expression of the FLAG peptide at the N-terminus was interfering the trimer formation. The slight underhydroxylation of the recombinant protein in 293 cells, likely a result of the overexpression of the protein due to the strong CMV promoter, could be a reason for the absence of trimeric molecules. On the other hand, the report of expression of collagen X expressed in the same cell line showed a formation of triple-helical molecules and aggregates ranging from dimers to multimers despite the underhydroxylation [23].

In a study with recombinant expression of mouse α1(IV) and α2(IV) chains in CHO cells, it was noted that α1(IV) chains alone also were not able to form triple-helical molecules, but required coexpression of the α2(IV) chain [20]. In the case of the α3(IV) chain, it may be speculated that coexpression of the α4(IV) and/or the α5(IV) chain is likely required for triple-helix formation. This speculation is supported by the observations that (*a*) the α3(IV), α4(IV), and α5(IV)

chains *in vivo* coexist in a supramolecular network in basement membranes [6] and (*b*) mutations in the α5(IV) chain in patients with Alport syndrome cause disassembly of the α3(IV), α4(IV), and α5(IV) network [24–28]. Ultimately, the assembly of cDNA constructs of full-length α4(IV) and α5(IV) chains and their coexpression with other chains will be required to answer the ambitious question of the code for chain selection and association.

The expression of the full-length α3(IV) chain provided a novel strategy to determine whether epitopes for GP autoantibodies are localized to the long collagenous domain in addition to the NC1 domain. Our results with autoantibodies from five GP patients clearly establish that the epitopes are exclusively localized to the NC1 domain of the nontriple-helical form of the α3(IV) chain. Whether the collagenous domain or the NC1 domain possess additional epitopes formed by the assembly of the α3(IV), α4(IV), and α5(IV) chains in a triple-helical network, involving complex interactions between chains, triple helices, and NC1 domains, remains unknown. Clearly, however, epitopes in the α3(IV)NC1 domain are sufficient to induce the formation of autoantibodies that mimic virtually all of the characteristics of the pathogenic human GP autoantibodies [19, 29, 30]. Hence, these findings suggest that a potential therapeutic strategy for the selective removal of human GP autoantibodies from plasma is the use of affinity chromatography using the human r-α3(IV)NC1 domain and not the entire α3(IV) chain. The feasibility of affinity chromatography using r-α3(IV)NC1 has been established for this purpose [31].

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