Clin Exp Immunol 1998; **113**:17–27

Recombinant α-chains of type IV collagen demonstrate that the amino terminal of the Goodpasture autoantigen is crucial for antibody recognition

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(Accepted for publication 17 March 1998)

SUMMARY

Goodpasture's disease, an autoimmune disorder causing severe glomerulonephritis and pulmonary haemorrhage, is characterized by antibodies to the glomerular basement membrane (GBM). The principal target antigen has been identified as the carboxyl terminal non-collagenous (NC1) domain of the α3-chain of type IV collagen. Anti-GBM antibodies appear to recognize one major epitope that is common to all patients, and is largely conformational. We have analysed antibody binding to recombinant α(IV)NC1 domains using a construct and expression system shown to produce correctly folded antigen that is strongly recognized by autoantibodies. In this system, as with the native antigen, α3(IV)NC1 was bound strongly by antibodies from all patients, whereas the closely related α1(IV) and α5(IV)NC1 domains, similarly expressed, showed no such binding. A series of chimeric NC1 domains, between human α3(IV) and α1(IV), and between human and rat α3(IV), were expressed as recombinant molecules, and were recognized by autoantibodies to varying degrees. Strong binding required the presence of human α3(IV) sequence in the amino terminal region of both sets of chimeric molecules. This work strongly suggests that the amino terminal of α3(IV)NC1 is critical for antibody recognition, whereas the carboxyl terminal end of α3(IV)NC1 has a less important role.

Keywords type IV collagen glomerular basement membrane Goodpasture antigen autoantibody binding B cell epitope

INTRODUCTION

Goodpasture's disease is a rare autoimmune disorder, characterized by autoantibodies to the glomerular basement membrane (GBM), and often causing rapidly progressive glomerulonephritis and lung haemorrhage [1–3]. The pathogenicity of the autoantibodies has been shown by transfer experiments in primates, and is supported by clinical observations [4]. The aetiology of Goodpasture's disease is understood better than most forms of nephritis, making it a particularly good model in which to study mechanisms of autoimmunity.

The specific target of the autoantibodies, the Goodpasture antigen, has been localized to the non-collagenous (NC1) domain of the α3-chain of type IV collagen chain [5–8]. This is one of six related α(IV) chains, the ubiquitous α1(IV) and α2(IV) chains and the tissue-specific α3(IV)-α6(IV) chains [9]. A typical type IV collagen chain is $\approx$ 1700 residues in length, with a relatively short 7S domain at the amino end, a long collagenous region characterized by Gly-Xaa-Yaa repeats with short non-collagenous interruptions, and a non-collagenous (NC1) domain of 230 residues at the carboxyl terminus. Type IV collagen NC1 domains contain a highly conserved pattern of 12 cysteine residues, which is common to all known type IV collagen chains. These are believed to form six intramolecular disulphide bonds essential to the overall tertiary structure of the molecule [10]. Binding of Goodpasture antibodies to the autoantigen is conformationally dependent, as reduction of the disulphide bridges and denaturation results in abrogation of antibody binding [11,12].

The six α(IV)NC1 domains can be divided into two subgroups; the 'α1-like' group in which α3(IV)NC1 and α5(IV)NC1 show a homology of 74% and 82%, respectively, with α1(IV)NC1; and the 'α2-like' group in which α4(IV)NC1 and α6(IV)NC1 show a homology of 73% and 79%, respectively, with α2(IV)NC1 [13–16]. There is considerably less homology between the two subgroups, of between 54% and 64%. The α1(IV) and α2(IV) chains are expressed ubiquitously in basement membranes and their genes are arranged head-to-head on chromosome 13 with a common promoter [17]. The α3(IV) and α4(IV) chain genes share a similar arrangement on chromosome 2 [18–20], but are expressed only in

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certain specialized basement membranes, including those of the glomerular and the alveolar basement membranes [21,22]. Despite the high degree of homology between the α(IV)NC1 domains, only α3(IV)NC1 binds Goodpasture autoantibodies consistently, although some sera appear to bind other α(IV)NC1 domains weakly [23,24].

In this study, we investigated the epitope specificity of Goodpasture autoantibodies in the autoimmune response. B cell-mediated antigen presentation could be an important mechanism for both initiating [25] and perpetuating autoimmunity. Antigen uptake mediated by the specific immunoglobulin receptors on the surface of B cells allows them to be two orders of magnitude more efficient at stimulating T cells than other antigen-presenting cells (APC) exposed to the same concentration of antigen internalized non-specifically [26]. Furthermore, the specificity of the autoantibody may favour the presentation of particular peptides by APC (possibly peptides that would never reach 'significant' levels in normal circumstances), presumably by altering the proteolytic fate of parts, or the whole molecule [27]. These mechanisms are therefore of great potential importance in autoimmune diseases that are associated with autoantibodies.

Previous studies have investigated the Goodpasture epitope by studying synthetic peptides based on short linear sequences within the protein. Peptide inhibition studies suggested that the Goodpasture epitope was localized in the carboxyl region of the α3(IV)NC1 domain [28]. However, other authors were unable to demonstrate antibody binding to peptides derived from this region [29], but found consistent binding to several other peptides throughout the molecule. Research involving the expression of splicing variants of α3(IV)NC1 showed that the carboxyl region was not essential for antibody binding [30]. Our own studies comparing the sequence of the antigen in different species have suggested that the amino region was important for antibody recognition [31]. This work allowed us to identify regions of potential importance and eliminate others, but at best can only be a broad guideline.

Conclusions about conformational epitopes that are based on studies with synthetic peptides and malfolded polypeptides must necessarily be tentative, therefore we sought an alternative approach. In this study we adapted a mammalian expression system to produce a recombinant mini-collagen chain form of human α3(IV)NC1, previously shown to be expressed in insect cells [32], which has strong reactivity with patients' sera. We also expressed the closely related human α1(IV)NC1, human α5(IV)NC1, and rat α3(IV)NC1. We went on to generate chimeric molecules, involving the substitution of corresponding segments between homologous NC1 domains. These molecules are likely to retain their three-dimensional structure because of high sequence homologies between the different α(IV)NC1 chains and a highly conserved pattern of 12 cysteine residues which form disulphide bonds crucial to the tertiary structure of all type IV collagen chains. That the molecules do fold correctly has been confirmed by the success of this study. Human α1(IV)NC1 and rat α3(IV)NC1 have 74% and 91% amino acid homology with human α3(IV)NC1, respectively, and are therefore ideal molecules for homologous substitution. In this study we have expressed six chimeras of recombinant human α3(IV)/α1(IV)NC1 and of human/rat α3(IV)NC1, and used them to map the binding of autoantibodies to the Goodpasture antigen. This work defines the B cell epitope(s) of the intact molecule more clearly.

PATIENTS AND METHODS

Patients

Sera were taken from six patients diagnosed as having Goodpasture's disease, with linear antibody deposition on direct immunofluorescence of renal biopsies. The patients were generally representative of the Hammersmith Hospital series; three had pulmonary haemorrhage and nephritis and three had isolated anti-GBM nephritis. Circulating anti-GBM antibodies, from each patient, were detected by ELISA [33] and Western blotting against collagenase-solubilized human GBM [34].

Antibodies

Sera from the Goodpasture patients, stored at $-20°C$, were diluted 1:30 to detect native and recombinant antigen on nitrocellulose blots. The murine MoAb P1 has been described previously [35]. The murine MoAb W17 was kindly supplied by Dr J. Wieslander [36] (University Hospital, Lund, Sweden).

Preparation of collagenase-solubilized GBM

Glomeruli were isolated from fresh human kidney cortex by differential sieving. GBM (the native antigen) was extracted by sonication and subjected to digestion with collagenase as previously described [19]. Rat GBM was prepared by similar methods as previously described [37].

Construction of type IV mini-collagen chain gene and cloning into mammalian expression vector

All DNA manipulations were carried out using standard procedures [38]. A cDNA of the leader peptide, NH_2 terminus and 7S domain of the human α1(IV) chain (a 530-bp fragment), was joined in-frame to the NC1 domain of the human α3(IV) chain, effectively creating a mini-collagen chain gene [32]. This composite cDNA contained a *Sal*1 site at the 5′ end, a *Bam*H1 site at the junction of α1(IV)/α3(IV), and *Xho*1, *Kpn*1 sites at the 3′ end. The *Sal*1-*Xho*1 fragment, encoding IL-2, of the plasmid pBC12/CMV/IL-2 [39] was removed by restriction enzyme digestion and gel purification. The *Sal*1-*Xho*1 fragment of p530.α3(IV)NC1, encoding the α1(IV)/α3(IV) hybrid mini-collagen chain, was gel-purified and cloned into the *Sal*1/*Xho*1-cut pBC12/CMV/IL-2. The resultant plasmid was designated as pCMV 530-α3(IV)NC1.

Cloning of human α1(IV)NC1, human α5(IV)NC1 and rat α3(IV)NC1 cDNA fragments as mini-collagen chain genes

The human α3(IV)NC1 domain of the mini-collagen chain gene was sequentially replaced by the NC1 domains of human α1(IV), human α5(IV) and rat α3(IV). The *Bam*H1/*Hind*III fragment of the plasmid pHT-21 [40], encoding the human α1(IV)NC1, was subcloned into pGEM, and amplified with the oligonucleotide primers 5′ TTGCCA*GGATCC*ATGGGGCCT 3′ and 5′ TTGGTAC*CTCGAG*GCTTCATTA 3′ to introduce *Xho*I and *Kpn*I sites in the 3′ non-coding region. The *Bam*HI/*Xho*I α1(IV)NC1 cDNA fragment was then used to replace the α3(IV)NC1 cDNA fragment in the expression vector described above. The α5(IV)NC1 cDNA was amplified from reverse-transcribed RNA from human renal cortex using the primers 5′ CCATGGATGGTCCCCCT 3′ and 5′ CTCGAGACACTG-CATCCTAG 3′, and cloned into pUC12 cut with *Sma*I. The *Nco*I/*Xho*I fragment was subcloned into the same sites of an α3(IV)NC1 cDNA into which *Bam*HI and *Nco*I sites had been introduced in the collagenous region, and *Xho*I and *Kpn*I sites into

the 3′ non-coding region [32]. The *Bam*H1/*Xho*I fragment of this plasmid was then used to replace the α3(IV)NC1 cDNA fragment in the expression vector described above. The complete rat α3(IV)NC1 sequence was obtained from a rat kidney cDNA library (Clontech Labs, Basingstoke, UK) and was used to replace the α3(IV)NC1 cDNA fragment in the expression vector described above [41]. Polymerase chain reaction (PCR) amplification was performed using Taq (Perkin Elmer, Branchburg, USA) or Vent (New England Biolabs, Hitchin, UK) polymerases and 30 cycles of the following pattern in a Biometra Trio-thermoblock: 94°C for 1 min, 55°C for 1 min, 72°C for 2 min; followed by a final 10-min step at 72°C.

Construction of human α3(IV)/α1(IV)NC1 chimeras

The α3(IV)NC1 cDNA was divided into three regions based on the restriction enzyme sites *Pvu*II and *Eco*R1. These regions were designated as amino, central and carboxyl. The human α1(IV)NC1 cDNA sequence has a *Pvu*II site at the position precisely corresponding to that of human α3(IV)NC1. An *Eco*R1 restriction site, corresponding to that in the α3(IV)NC1 cDNA, was introduced into the α1(IV)NC1 cDNA by site-directed mutagenesis (Clontech), using the primer 5′ TGCCTGGAG*GAATTC*AGAAGTGCG 3′.

A modified vector was constructed by removing the *Pvu*II fragment of pUC19 and dephosphorylating its blunt-ended arms. This process removed the polylinker from the pUC19 vector without affecting its ability to be used as a cloning vector. The *Bam*H1/*Kpn*1 fragments of α3(IV)NC1 and α1(IV)NC1 were gel-purified, made blunt-ended using T4 DNA polymerase and nucleotide tri-phosphates (NTP), phosphorylated and cloned into the specially modified pUC19 vector, recreating the *Bam*H1 site. The common regions of α3(IV)NC1 and α1(IV)NC1 were exchanged to create six chimeric molecules (Fig. 1). The chimaeric α3(IV)/α1(IV)NC1 *Bam*H1/*Xho*1 fragments were then subcloned in *Bam*H1/*Xho*1-cut pCMV 530-α3(IV)NC1 (pCMV 530), and expressed in COS-7 cells. All constructs were verified by unique restriction enzyme digestion

Construction of human/rat α3(IV)NC1 chimeras

An identical cloning strategy was adopted to construct chimeras of human α3(IV)NC1 and rat α3(IV)NC1 domains using their *Bam*H1/*Xho*1 fragments. The rat α3(IV)NC1 cDNA has *Pvu*II and *Eco*R1 sites at the corresponding positions to those of human α3(IV)NC1. The *Bam*H1/*Kpn*1 fragments of human and rat α3(IV)NC1 were gel-purified, made blunt-ended using T4 DNA polymerase and NTP, phosphorylated and cloned into the specially modified pUC19 vector, recreating the *Bam*H1 site. The common regions of human and rat α3(IV)NC1 were exchanged to create six chimeric molecules. The chimeric human/rat α3(IV)NC1 *Bam*H1/*Xho*1 fragments were then subcloned into pCMV-530 vector and expressed in COS-7 cells.

Expression of recombinant α(IV)NC1 domains

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Paisley, UK) supplemented with 5% fetal calf serum (FCS). Cells were seeded at a density of $2–2{\cdot}5\times10^6$/75-cm^2 flask overnight, after which a subconfluent layer of cells was transfected with 5 μg of DNA using a DEAE–Dextran-mediated procedure [42]. The plasmid DNA was mixed with 1·9 ml of PBS and 100 μl of DEAE–Dextran (10 mg/ml). COS cells were rinsed with warm PBS (calcium- and magnesium-free), aspirated and the 2 ml transfection cocktail added. Flasks were incubated at 37°C for 30 min, with occasional gentle shaking to prevent drying. Culture medium (8 ml), supplemented with 100 μM of chloroquine, was added to each flask and incubation continued for 2·5 h, after which supernatants were aspirated and cells were treated with 7·5 ml of culture media containing 10% DMSO for 2·5 min. This was replaced by DMEM plus 5% FCS. Transfected cells were incubated for 72 h and supernatants were collected and stored at −20°C. The human and rat α3(IV)NC1 mini-collagen chains, along with the related α1(IV)NC1 and α5(IV)NC1 chains, were expressed in the same manner.

SDS–PAGE and Western blotting

COS supernatants were analysed by electrophoresis on 10% or 12·5% polyacrylamide gels containing SDS [43]. Gels were electroblotted onto nitrocellulose and membranes blocked with PBS/0·5% Tween for 1–2 h. Recombinant protein was detected using a 1:30 dilution of patients' serum (primary antibody) or MoAb in conjunction with alkaline phosphatase-conjugated anti-human or anti-mouse IgG (Sigma-Aldrich, Poole, UK). Colour was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

Cation exchange purification of recombinant protein

Cation exchange purification of recombinant protein was achieved using a bifunctional cation exchange resin, Bakerbond

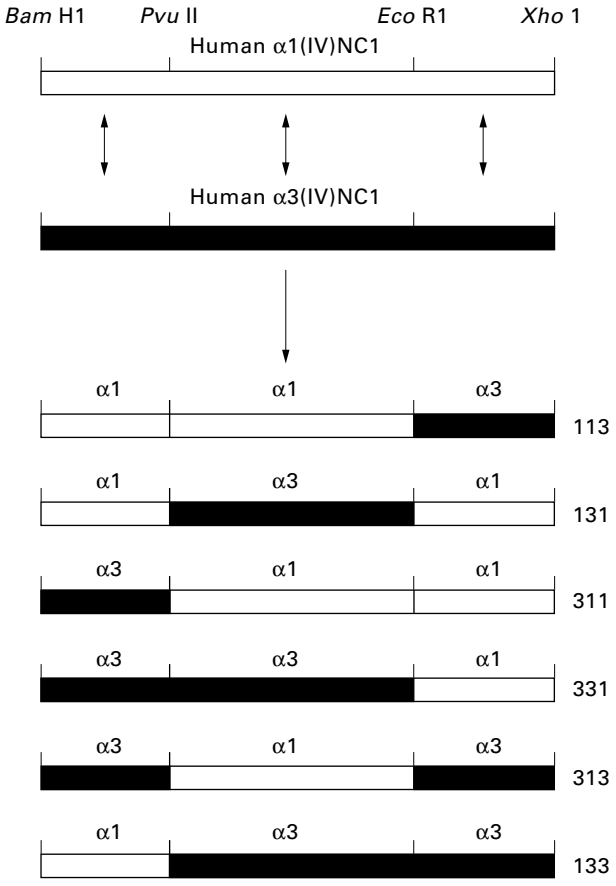


Fig. 1. Schematic diagram illustrating the derivation of chimeric human α3(IV)/α1(IV)NC1 domains. Exchange of corresponding regions of α3(IV)NC1 and α1(IV)NC1 domains to create six chimeric α3(IV)/α1(IV)NC1 domains. The constructs are described according to the origins of their amino, central and carboxyl regions, i.e. α331 was derived from the amino and central regions of α3(IV)NC1 and the carboxyl region of α1(IV)NC1.

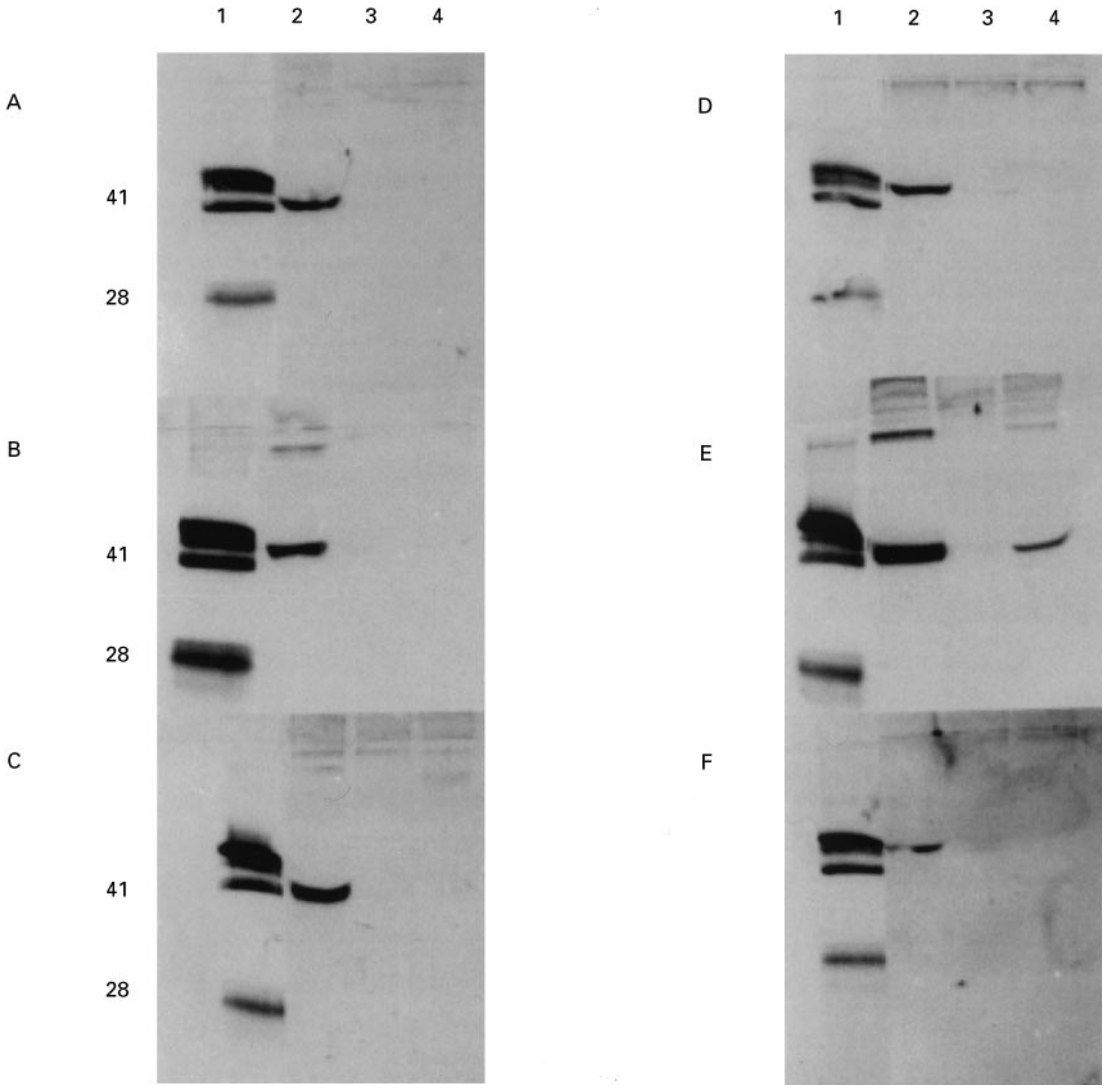


Fig. 2. Western blots showing binding of autoantibodies from six patients to recombinant α(IV)NC1 domains. COS supernatants from transfected cells were separated on 12·5% SDS–PAGE gels and electroblotted to nitrocellulose. Recombinant protein was detected with human autoantibodies from Goodpasture patients A–F. Lane 1 contained collagenase-solubilized (cs) human glomerular basement membrane (GBM) (20 μg) as a positive control. The recombinant protein in the other lanes was: lane 2, human α3(IV)NC1; lane 3, human α1(IV)NC1; lane 4, human α5(IV)NC1.

carboxysulphon. A 100×5 mm PEEK column was packed with carboxysulphon, and equilibrated in 0·05 M Tris–HCl buffer pH 7·3. Culture supernatant from transfected COS-7 cells was adjusted to pH 7·3 with 1 M acetic acid. Supernatant (20 ml) was then loaded onto the column at a rate of 1 ml/min and the column was washed for 20 min with Tris buffer. Cationic material bound to the column was eluted using a linear gradient of 0–100% of 0·5 M NaCl in 0·05 M Tris–HCl pH 7·3, over 30 min, and 1-ml fractions were collected. Loading of sample and eluting of fractions were carried out using a Waters 626 high pressure liquid chromatograph (HPLC) system. Fractions containing antigenic material were identified by SDS–PAGE and Western blot analysis. These were pooled, concentrated and desalinated on Centricon C30 (Amicon, Stonehouse, UK) columns and resuspended in 0·5 ml of TE buffer.

ELISA for detection of recombinant α3(IV)NC1

An ELISA for the detection of human or other mammalian GBM, and recombinant α3(IV)NC1, using anti-GBM antibodies, was developed for use in 96-well microtitre plates [44]. Collagenase-solubilized human GBM was coated at a concentration of 40 μg/ml in guanidine thiocynate as a positive control. Recombinant protein was diluted in 50 mM sodium carbonate buffer pH 9·8, and 100 μl were used to coat each well in duplicate. After overnight incubation at 4°C, the plate was washed three times in PBS–Tween (PBS/Tw) and then blocked for 1 h in 100 μl of blocking buffer containing PBS/Tw/1% bovine serum albumin (BSA). Primary antibody, in the form of sera from Goodpasture patients, was diluted in blocking buffer and 100 μl were incubated in each well at 37°C for 1 h. The plate was again washed three times, and 100 μl of 1:1000 dilution of secondary antibody, alkaline phosphatase-conjugated goat anti-IgG, in blocking buffer, were added to each well and incubated for 1 h at 37°C. The plate was washed three times, and developed with phosphatase substrate tablets; one tablet was dissolved in 5 ml carbonate buffer containing 5 mM $MgCl_2$ and

100 μl of this were added to each well. Colour was allowed to develop and the optical density (OD) was read at 405 nm using an Anthos htII ELISA plate reader.

^{35}S-radiolabelling of recombinant protein

Radiolabelling of recombinant protein was carried out in order to compare the protein expression levels of COS-7 cells that were transfected with different chimeric NC1 domain constructs. At 60–72 h after transfection, cell cultures were washed twice in cysteine- and methionine-free DMEM media (–FCS). Cells were then incubated in this medium for 20 min. This depleted the cells of their intracellular pools of sulphur-containing amino acids. The medium was removed by aspiration and 2 ml of fresh warm medium, containing 200 μCi of Promix (^{35}S-labelled methionine and cysteine; Amersham Life Science, Aylesbury, UK) were added to each 75-mm^2 flask. Cells were incubated at 37°C for 6 h, after which supernatants were collected and stored at −20°C. The supernatants were run on a 12·5% SDS–PAGE gel. The gels was then fixed in 10 volumes of glacial acetic acid:methanol:water in a ratio of 10:20:70 for 5 min. After a brief wash in distilled water, the gel was placed on saran wrap and a piece of 3MM blotting paper was placed over it and inverted. The gel was dried at 50°C for 90 min and then exposed to autoradiograph film for 48 h.

RESULTS

Characterization of recombinant human α3(IV)NC1 and related chains

COS-7 cells were transfected with 10 μg of plasmid DNA using standard DEAE–dextran techniques. Supernatants were collected after 72 h and examined by SDS–PAGE and Western blotting. Recombinant α3(IV)NC1 was detected in COS supernatants by patients' sera at the predicted molecular size of 41 kD. All six sera studied bound strongly to α3(IV)NC1. Recombinant α1(IV)NC1 was not recognized by patients' sera, while α5(IV)NC1 was weakly recognized by only one patient's serum (Fig. 2). Recombinant α3(IV)NC1 was also recognized by two MoAbs, P1 (data not shown) and W17 (Fig. 5). These two MoAbs have been raised to human and bovine GBM, respectively, and shown to bind to native human α3(IV)NC1 [21,35,36,45]. Recombinant α1(IV)NC1 and α5(IV)NC1 were not recognized by P1 or W17. Recombinant rat α3(IV)NC1 was detected, at the predicted molecular size of 42 kD, by human autoantibodies, but binding was considerably weaker than that to human α3(IV)NC1 (Fig. 3).

Characterization of chimeric human α3/α1(IV)NC1 domains

Chimeric human α3(IV)/α1(IV)NC1 domains were produced in order to locate the site of autoantibody binding within the α3(IV)NC1 domain. Substituting regions of human α1(IV)NC1 for corresponding regions of human α3(IV)NC1 provides a means of determining the critical sequences for antibody binding, since α1(IV)NC1 is not recognized by patients' sera. The NC1 domains of human α3(IV) and α1(IV) were divided into three regions, designated as amino, central and carboxyl regions, which were shuffled using the common restriction sites to manufacture six chimeric NC1 domains of α1(IV)/α3(IV). Each of these was subcloned into the expression vector pCMV 530 as described. The chimeras were defined according to the origins of their regions, e.g. α331 was derived from the amino and central regions of α3(IV)NC1 and the carboxyl region of α1(IV)NC1 (Fig. 1).

The binding of autoantibodies to the recombinant α3(IV)/α1(IV) chimeras was remarkably consistent (Fig. 4). All six sera showed the highest avidity for α331 and α333. Binding was not significant unless the chimera had the α3(IV) amino terminal region. The presence of an adjacent α3(IV)NC1 central region appeared to augment binding slightly. In contrast, the α3(IV) carboxyl region contributed very little to recognition; the reactivities of α333 and α331 were similar, whereas a striking contrast was observed in the reactivities of α333 and α133. A small degree of heterogeneity was observed in binding patterns between patients; two sera showed a moderate degree of reactivity for α113. Interestingly, the binding pattern observed with the MoAb P1 to the chimeric molecules was similar to that observed with patients' autoantibodies (not shown). Of the six chimeras, α331 was the only chimera bound by the MoAb W17 (Fig. 5). It was shown subsequently (see radiolabelling experiment) that constructs encoding α131 and α133 did not produce any detectable recombinant protein.

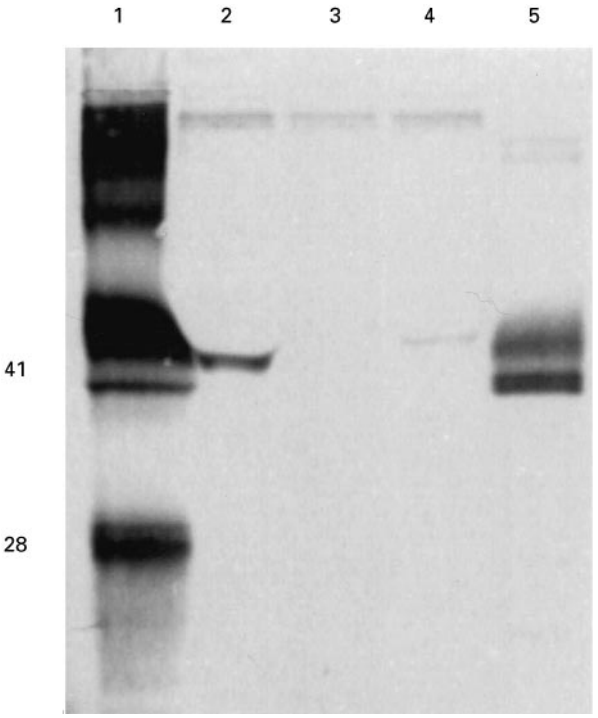


Fig. 3. Western blot showing binding of human autoantibodies to recombinant human and rat α(IV)NC1 domains. COS supernatants from transfected cells were separated on 12·5% SDS–PAGE gels and electroblotted to nitrocellulose. Recombinant protein was detected with human autoantibodies from Goodpasture patients. Lanes 1 and 5 contained collagenase-solubilized (cs) human and rat glomerular basement membrane (GBM), respectively, as positive controls. The recombinant protein in the other lanes was: lane 2, human α3(IV)NC1; lane 3, human α1(IV)NC1; and lane 4, rat α3(IV)NC1.

Characterization of recombinant α331 chimera

As the α331 chimera appeared to have similar autoantibody binding properties to the recombinant Goodpasture antigen, α3(IV)NC1, it was decided to investigate its binding characteristics in greater detail. To this end, an equivalent volume of COS-7 cell supernatant containing recombinant α331 or α3(IV)NC1 was purified by cation exchange chromatography and concentrated on

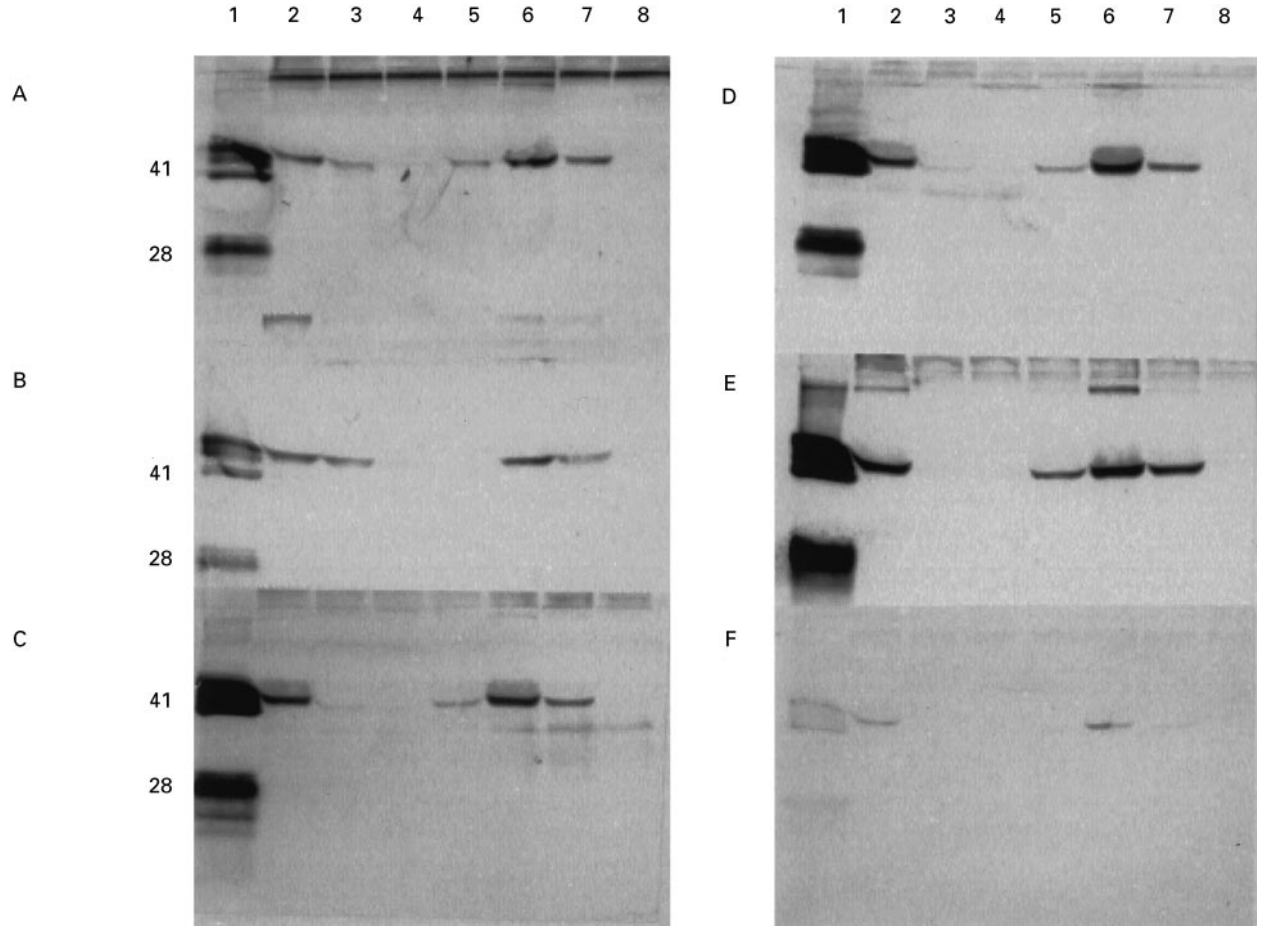
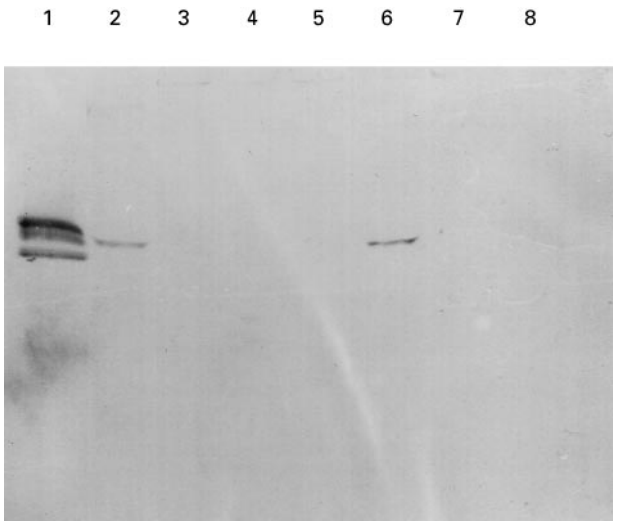


Fig. 4. Western blots showing binding of human autoantibodies from six patients to chimeric α3/α1(IV)NC1 domains. COS supernatants from transfected cells were separated on 12·5% SDS–PAGE gels and electroblotted to nitrocellulose. Recombinant chimeric human α1(IV)/α3(IV)NC1 domains were detected with sera from Goodpasture patients A–F. Lane 1 contained collagenase-solubilized (cs) human glomerular basement membrane (GBM) as a positive control. The recombinant protein in the other lanes was: lane 2, human α3(IV)NC1 (α333); lane 3, α113; lane 4, α131; lane 5, α311; lane 6, α331; lane 7, α313; lane 8, α133.



C30 columns (Amicon). By ELISA, the purified fraction of α331 showed a comparable degree of reactivity, with sera from patients with Goodpasture's disease, to that of recombinant α3(IV)NC1 and of native GBM (Fig. 6). Furthermore, α331 had the ability to inhibit the binding of Goodpasture autoantibodies to native human GBM, which was analogous to that of the recombinant antigen, α3(IV)NC1 (Fig. 7).

Characterization of chimeric human/rat α3(IV)NC1 domains

Since human autoantibodies recognize human α3(IV)NC1 considerably more strongly than rat α3(IV)NC1, it was reasoned that additional information may be obtained by studying chimeric

Fig. 5. Western blot showing binding of MoAb 17 to chimeric α3/α1(IV)NC1 domains. COS supernatants from transfected cells were separated on 12·5% SDS–PAGE gels and electroblotted to nitrocellulose. Recombinant chimeric human α3/α1(IV)NC1 domains were detected with 1:100 dilution of MoAb W17. Lane 1 contained collagenase-solubilized (cs) human glomerular basement membrane (GBM) as a positive control. The recombinant protein in the other lanes was: lane 2, human α3(IV)NC1 (α333); lane 3, α113; lane 4, α131; lane 5, α311; lane 6, α331; lane 7, α313; lane 8, α133.

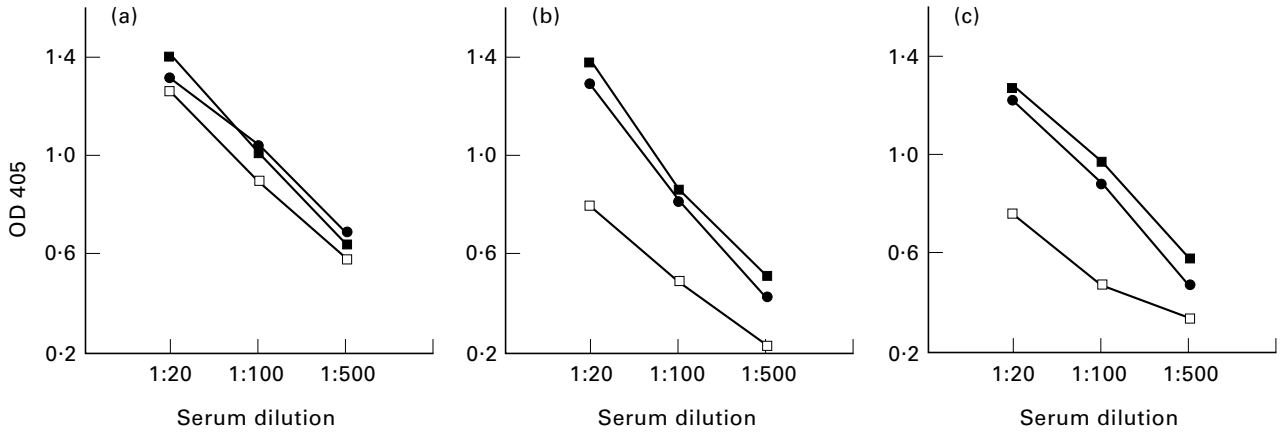


Fig. 6. ELISAs comparing binding of sera from patients with Goodpasture's disease to human collagenase-solubilized (cs) glomerular basement membrane (GBM), recombinant α3(IV)NC1 and recombinant α331. Three patients' sera are shown in assays performed in parallel on ELISA plates coated with (a) 4 μg/ml of cs-human GBM, (b) 1:2 dilution of purified recombinant antigen, or (c) purified recombinant α331. Antigen preparations were diluted in carbonate buffer pH 7·5.

forms of human/rat α3(IV)NC1. Rat α3(IV)NC1 has a 91% sequence homology with human α3(IV)NC1, compared with 75% for human α1(IV)NC1. Therefore, chimeras of human/rat α3(IV)NC1 domains were more likely to be conformationally similar to the intact recombinant human α3(IV)NC1. Thus, using an identical cloning strategy to that described above, the amino, central and carboxyl regions of human α3(IV)NC1 were sequentially substituted by the similar regions of rat α3(IV)NC1. The constructs were described according to the origins of their amino-terminal, middle and carboxyl-terminal regions, i.e. αHHR was derived from the amino-terminal and central regions of human α3(IV)NC1 and the carboxyl-terminal region of rat α3(IV)NC1. All recombinant chimeras bound human autoantibodies to some extent, but binding was strongest to chimeras containing the human α3(IV)NC1 amino region (Fig. 8). In contrast, all three chimeras with a rat α3(IV)NC1 amino region were bound weakly, irrespective of their central and carboxyl region composition. These results from the chimeric human/rat α3(IV)NC1 domain were entirely consistent with those from the chimeric human α3/α1(IV)NC1 domains. Both sets of data suggest that the amino terminal region of human α3(IV)NC1 is most important for the binding of autoantibodies. All the above experiments were repeated at least three times and consistently gave similar results.

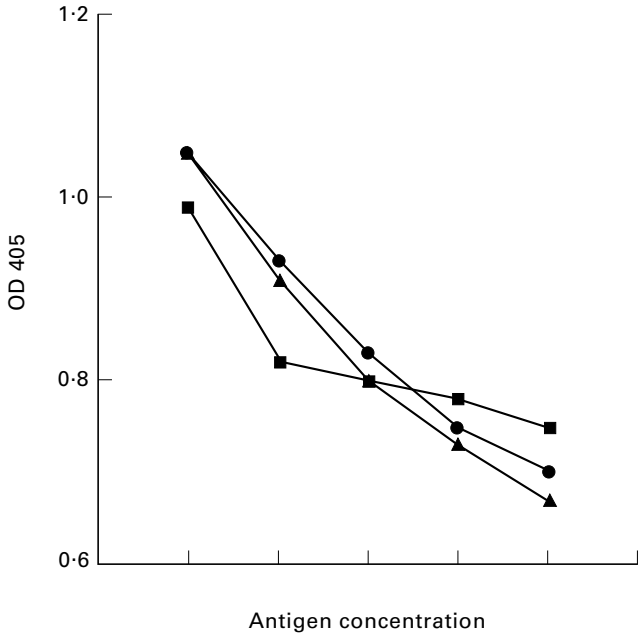


Fig. 7. ELISA showing the inhibition of human autoantibodies binding to human glomerular basement membrane (GBM) by human GBM or recombinant α3(IV)NC1 or recombinant α331. Doubling amounts (arbitrary units) of human or purified human recombinant antigen or recombinant α331 were preincubated with patients' autoantibodies. These were then allowed to bind to collagenase-solubilized (cs) human GBM on an ELISA plate. ■, Human cs-GBM; ●, recombinant antigen; ▲, recombinant α331.

Quantification of chimeric α(IV)NC1 domains by ^{35}S-radiolabelling

Since the observed differences in the reactivities of the chimeras with human autoantibodies could be due to quantitative differences in levels of expression, rather than qualitative differences in the immunoreactivity, the protein expression levels for all chimeras were compared. Seventy-two hours after transfection with the various chimeric constructs, COS-7 cell supernatants were replaced with cysteine- and methionine-free DMEM (−FCS) containing Promix (radiolabelled cysteine and methionine). The COS-7 cells were incubated for 6 h and supernatants were collected and run on SDS–PAGE gels. Immunoreactivities of chimeric proteins were assessed by Western blotting, and radioactive protein was detected by exposure of autoradiograph film to dried gels.

The assessment of expression levels for chimeric human α3(IV)/α1(IV) NC1 domains is shown in Fig. 9. The protein levels for four of the chimeras, and for recombinant α1(IV)NC1 and α3(IV)NC1, were comparable. However, there was no detectable expression of α131 and α133. The constructs for these chimeras were recloned and expressed, but a similar result was obtained. The assessment of expression levels for human/rat α3(IV)NC1 chimeras is shown in Fig. 10. For these, all six chimeras showed comparable levels of expression. The expression levels for rat α3(IV)NC1 and human α3(IV)NC1 were also comparable.

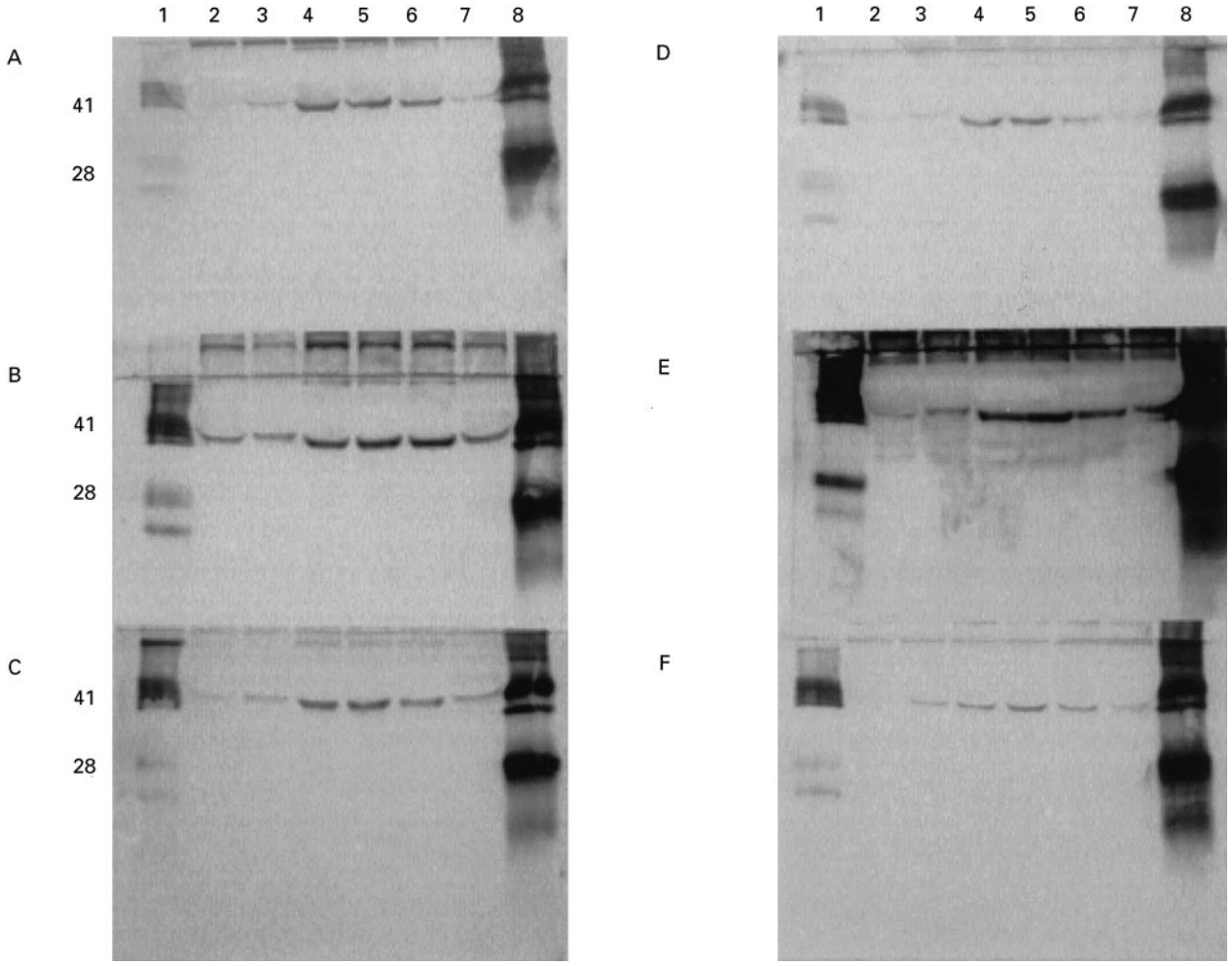


Fig. 8. Western blots showing binding of human autoantibodies from six patients to chimeric human/rat α3(IV)NC1 domains. COS supernatants from transfected cells were separated on 12·5% SDS–PAGE gels and electroblotted to nitrocellulose. Recombinant chimeric human/rat α3(IV)NC1 domains were detected with sera from Goodpasture patients A–F. Lanes 1 and 8 contained, respectively, collagenase-solubilized (cs) rat glomerular basement membrane (GBM) and human GBM as positive controls. The recombinant protein in the other lanes was: lane 2, αRRH; lane 3, αRHR; lane 4, αHRR; lane 5, αHHR; lane 6, αHRH; lane 7, αRHH.

DISCUSSION

In this study we have produced strong evidence that the amino region of the Goodpasture antigen has a critical role in the binding of autoantibodies. We have used molecules related to human α3(IV)NC1 to create stable chimeric forms of the antigen. Recombinant human α1(IV)NC1, which has 74% amino acid sequence homology with human α3(IV)NC1, does not bind autoantibodies. Recombinant rat α3(IV)NC1, which has 91% homology, binds autoantibodies poorly. Our hypothesis was that substitution of regions of human α1(IV)NC1 and rat α3(IV)NC1 for corresponding regions in human α3(IV)NC1 would provide a means of determining the dominant regions of antigenicity within the Goodpasture antigen and thus map the major B cell epitope.

The COS cell transient expression system proved a very reliable and robust method for producing recombinant antigen for these studies. The system provided a means of rapidly expressing recombinant proteins at levels that were readily detectable in the COS supernatant. The results show that the construct previously used to produce conformationally correct antigen in insect cells [32] can be effective in other cell types. Most conformational epitopes are complex and are believed to contain five or six critical residues distributed across the molecule, or at least over a large region of it, which will only form the epitope 'groove' in the correctly folded protein [46]. Thus it is important to use experimental approaches which preserve the tertiary structure of the subject protein.

Other studies aimed at identifying the Goodpasture epitope have used two approaches. First, those using short synthetic peptides have produced conflicting results. One study claimed to have localized the major epitope by showing that synthetic peptides corresponding to sequences in the carboxyl region of the antigen were able to inhibit the binding of human autoantibodies to bovine α3(IV)NC1 monomer [28]. Our own studies, which examined the binding of Goodpasture antibodies to a panel of membrane-bound peptides spanning the entire antigen, failed to identify this region [29]. However, linear sequences identified by autoantibodies may contribute towards binding in a conformational epitope. It is also possible that antibodies binding to peptides in

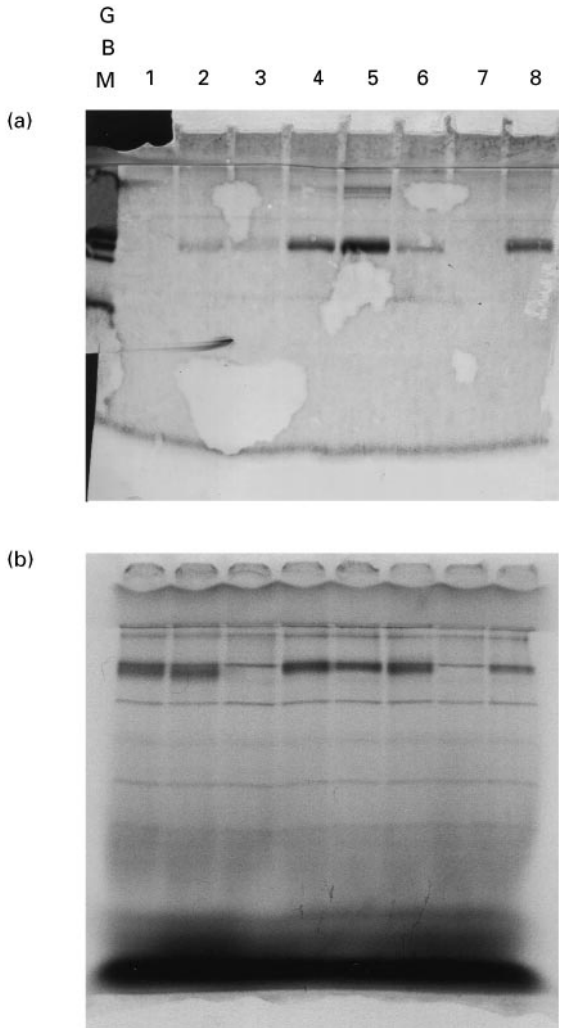


Fig. 9. Western blot and autoradiograph of radiolabelled chimeric human α3(IV)/α1(IV)NC1 domains. COS supernatants from transfected cells were separated on 12·5% SDS–PAGE gels, which were then electroblotted to nitrocellulose or dried for exposure to autoradiograph film. (a) Western blot showing binding of Goodpasture autoantibodies to radiolabelled chimeric α3/α1(IV)NC1 domains. (b) Autoradiograph showing the expression levels of chimeric human α3(IV)/α1(IV)NC1 domains. The radiolabelled recombinant protein in each lane was: lane 1, human α1(IV)NC1 (α111); lane 2, α113; lane 3, α131; lane 4, α311; lane 5, α331; lane 6, α313; lane 7, α133; lane 8, human α3(IV)NC1 (α333).

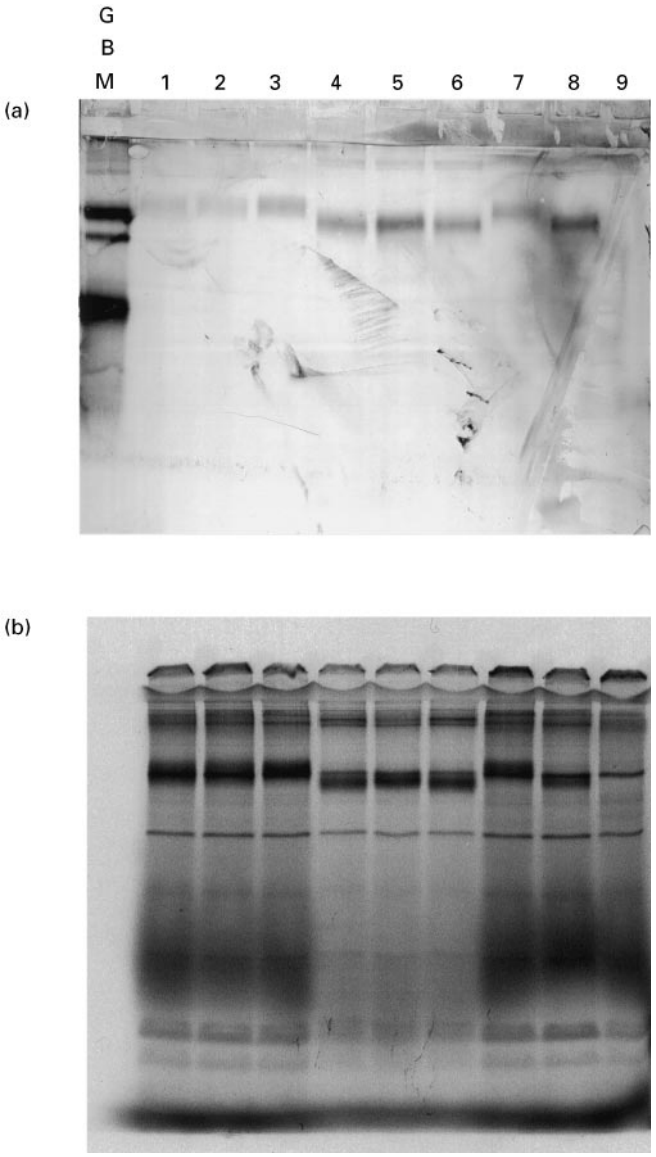


Fig. 10. Western blot and autoradiograph of radiolabelled chimeric human/rat α3(IV)NC1 domains. COS supernatants from transfected cells were separated on 12·5% SDS–PAGE gels, which were then electroblotted to nitrocellulose or dried for exposure to autoradiograph film. (a) Western blot showing binding of Goodpasture autoantibodies to radiolabelled chimeric human/rat α3(IV)NC1 domains. (b) Autoradiograph showing the expression levels of chimeric human/rat α3(IV)NC1 domains. The radiolabelled recombinant protein in each lane was: lane 1, rat α3(IV)NC1 (α333); lane 2, αRRH; lane 3, αRHR; lane 4, αHRR; lane 5, αHHR; lane 6, αHRH; lane 7, αRHH; lane 8, human α3(IV)NC1; lane 9, negative control.

such studies were originally elicited by denatured protein, as a secondary response during the progress of the disease [46]. Second, a splicing variant of α3(IV)NC1, expressed in *Escherichia coli*, that retained the amino end of the molecule but lacked a large part of the carboxyl region, still bound strongly to human autoantibodies [30]. These observations are supported by a recent publication which suggested that the amino collagenous/NC1 region of α3(IV), together with the carboxyl region, played an important role in a major conformational epitope [47].

The production of recombinant chimeric molecules has proved to be a valuable tool in determining the regions of human α3(IV)NC1 critical for binding of human autoantibodies [48,49]. Results in the present study, using two sets of chimeras, were internally very consistent, in that the pattern of autoantibody binding to each set of six chimeras was similar for all six patients' sera examined. The MoAbs P1 and W17 demonstrated a binding pattern to the chimeras similar to that of patients' antibodies. It is interesting and important that both of these antibodies have previously been shown to be capable of blocking the binding of patients' autoantibodies to the Goodpasture antigen [35,45,50,51]. These observations demonstrate that the amino region of the α3(IV)NC1 domain is crucial for antibody recognition. One of the above mentioned studies also suggested that lysine residues in the carboxyl region were crucial for antibody recognition [28].

However, it would appear from our study that the absence of these 'critical lysines' from the carboxyl region of α1(IV)NC1 does not significantly alter the binding of autoantibodies to 'conformationally correct' recombinant α331 (Figs 6 and 7).

The assessment of the expression levels of the chimeric NC1 domains is interesting, and overall supports our interpretation of the Western blotting studies. It is difficult to say why there was no detectable expression of α131 and α133. It is interesting that the juxtaposition of the amino region of α1(IV)NC1 with the central region of α3(IV)NC1 is common to both chimeras. It could be speculated that this arrangement may result in some failure at the transcriptional or translational level, or in failure of secreting. Nevertheless, our results show that the central region of α3(IV)NC1 contributes very little to the immunoreactivity of the whole molecule; e.g. α313 and αHRH bind autoantibodies very well. It is also clear from these experiments that α113 has comparable expression levels to α333 or α331, but has a poor reactivity with autoantibodies. In addition, the counterparts of α131 and α133 in the experiment involving human/rat chimeras, namely αRHR and αRHH, have relatively weak reactivities with human autoantibodies. The overall interpretation of these studies is that the α3(IV)NC1 amino region is crucial for antibody reactivity, whereas the contribution of sequences in the carboxyl region is less important than had previously been thought. However, it is possible that amino acid residues from several parts of the molecule may contribute to antibody binding.

The information derived from this study of the properties of human α3(IV)/α1(IV)NC1 and human/rat α3(IV)NC1 chimeric molecules is consistent and has increased our understanding of the immunological properties of the autoantigen. The results should lay the foundations for further work on the relationship between B and T cell epitopes, and may be of value in the designing of new approaches to immunotherapy.

ACKNOWLEDGMENTS

This work was supported by grants from the Medical Research Council, the National Kidney Research Fund and the Miranda Saunders Research Fund. N.T. was a NKRF Senior Research Fellow.

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