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The $\alpha 1\beta 1$ integrin recognition site of the basement membrane collagen molecule $[\alpha 1(IV)]_2\alpha 2(IV)$

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Cells interact with type IV collagen mainly via the integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$. A triple helical CNBr derived fragment CB3[IV], which contains the recognition sites for both integrins, was isolated from type IV collagen. Trypsin treatment of CB3[IV] gave rise to four smaller fragments, F1-F4, of which the smallest one, F4, contained the recognition site for $\alpha 1\beta 1$. Further fragmentation of F4 by thermolysin treatment at 50°C led to fragment TL1, which represents the C-terminal half of F4, and which was no longer able to interact with $\alpha 1\beta 1$. Therefore the recognition site of $\alpha 1\beta 1$ had to be located within the N-terminal half of F4, a position which was verified by electron micrographs of a crosslinked F2-$\alpha 1\beta 1$ complex. Modification of the Arg and Asp residues, which abolished the binding activity of F4, led to the identification of Arg (461) within the $\alpha 2$(IV) and Asp (461) within the $\alpha 1$(IV) chain as essential residues for the $\alpha 1\beta 1$. The array of these two residues on the surface of the triple helix is discussed.

Key words: $\alpha 1\beta 1$ integrin/collagen type IV/recognition site

Introduction

Basement membranes are important as supports for cells of different origins. By adhering to and migrating on basement membranes, cells are influenced in their differentiation during embryonic development and organogenesis, whereas mature cells need contact with basement membranes to maintain their phenotype and function (Timpl and Dziadek, 1986). In order to get a better insight into the molecular mechanisms involved, the interaction of cells with the main constituents of basement membranes, such as collagen IV and laminin (Aumailley and Timpl, 1986; Tomaselli *et al.*, 1988; Clyman *et al.*, 1989; Languino *et al.*, 1989; Hall *et al.*, 1990; Ignatius *et al.*, 1990; Vandenberg *et al.*, 1991), has been investigated with the aims of locating the cell recognition sites on the ligand molecules and of characterizing the cell receptors involved in binding.

The macromolecular organization as well as the biomechanical stability of basement membranes are determined by a collagenous network, which is formed by the most ubiquitous isoform of collagen IV with the molecular composition $[\alpha 1(IV)]_2\alpha 2(IV)$ (Trüeb *et al.*, 1982; Vandenberg *et al.*, 1991). It contains one main binding site for cells, which is located in a 40 nm long section of the triple helical domain ~100 nm away from the N terminus of the molecule. The receptors responsible for cell binding to this area were found to be the integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (Vandenberg *et al.*, 1991; Kern *et al.*, 1993). They belong to the widespread integrin family, which exists in several subfamilies and which mediates cell matrix as well as cell-cell interaction (Albelda and Buck, 1990; Hynes, 1992). All integrins are heterodimers, consisting of α and β subunits, whereby the affiliation to subfamilies is determined by the β chains. The members of the $\beta 1$ family are mainly involved in the interaction of cells with components of the extracellular matrix. $\alpha 1\beta 1$ and $\alpha 2\beta 1$ appear to be major collagen receptors although they also interact with other extracellular matrix proteins, e.g. laminin (Tomaselli *et al.*, 1988; Kramer and Marks, 1989; Staatz *et al.*, 1989, 1990; Belkin *et al.*, 1990; Hall *et al.*, 1990; Vandenberg *et al.*, 1991; Gullberg *et al.*, 1992; Pfaff *et al.*, 1993).

In the short section of the collagen IV molecule containing the recognition site for $\alpha 1\beta 1$ and $\alpha 2\beta 1$, the triple helical domain is stabilized by disulfide bridges between the three α(IV) chains. This part has been isolated from the molecule in the form of the triple helical fragment CB3[IV] by cyanogen bromide treatment (Vandenberg *et al.*, 1991). Further digestion of this fragment with trypsin led to four shorter triple helical fragments F1-F4 (Figure 1). Their investigation revealed the presence of two recognition sites for the integrin $\alpha 2\beta 1$ and an area of ~80 amino acid residues which interacts with $\alpha 1\beta 1$ (Kern *et al.*, 1993). In order to characterize the recognition site for $\alpha 1\beta 1$ in more detail, CB3[IV] was further fragmented with thermolysin. Protein chemical, electron microscopical and immunological investigations of the breakdown products obtained resulted in the localization and characterization of the $\alpha 1\beta 1$ recognition site. Three amino acids, two aspartate residues within each $\alpha 1$(IV) chain and one arginine residue within the $\alpha 2$(IV) chain, were found to be essential for the interaction with $\alpha 1\beta 1$.

Results

Degradation of the trimeric fragment CB3[IV] with thermolysin

The central part of the triple helix of CB3[IV] is stabilized against proteolytic attack by the disulfide knots I and II (Figure 1) by which the α-chains are connected to one another. In order to cleave the area between the two disulfide knots, CB3[IV] had to be digested with thermolysin at a temperature of 50°C. The complex mixture of breakdown products was separated by gel permeation chromatography on a tandem of a TSK G3000 and a TSK G2000 column (not shown). The peak containing the disulfide bridged trimeric fragments was further chromatographed on an ion exchange Mono S column, which yielded a somewhat heterogeneous pool of trimeric fragments with an apparent molecular weight between 22 and 25 kDa. For further characterization they were reduced and carboxymethylated and the resulting monomeric $\alpha 1$(IV) and $\alpha 2$(IV) fragments separated by a reversed phase chromatography on a C18 column. Finally, the major peaks, representing >90% of the peptide mixture, were submitted to Edman degradation. The major cleavage points of thermolysin were found to be

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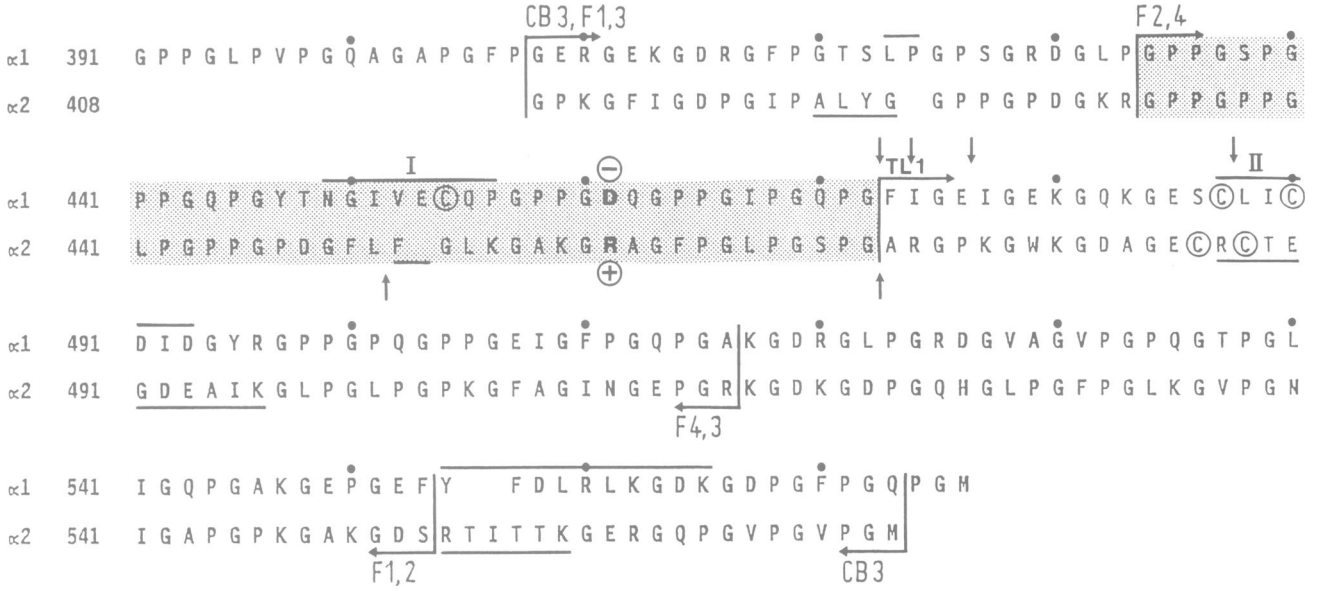

Fig. 1. Amino acid sequence of human α1(IV) and α2(IV) chains of the cyanogen bromide derived fragment CB3[IV]. The non-triple helical interruptions of the α1(IV) and the α2(IV) chains are indicated by thin lines above and below the sequences, respectively. The cysteine residues involved in interchain disulfide bonds are encircled. They are arranged in two disulfide knots, numbered I and II. The N- and C-terminal ends of the triple helix of CB3[IV] and its fragments F1−4 are designated. Vertical arrows designate the cleavage sites of thermolysin. The N-terminal half of F4, which contains the α1β1 recognition site, is shaded. The arginine binding at position 461 within α2(IV) and the aspartate residue at position 461 (both in bold) within the two α1(IV) chains are essential moieties of the α1β1 recognition site (see text). The numbers are position numbers of the aligned α1(IV) and α2(IV) chains. They do not coincide with the residue numbers of the single α-chains (Brazel *et al.*, 1988).

located at similar sites in both chains. About 80% of α1(IV) fragments started with the residue Phe473, Ile474 or Ile477 (Figure 1). The α2(IV) fragment was more homogeneous with Ala473 as the major N-terminal residue. According to these experiments, we concluded that the thermolysin digestion of CB3[IV] led to a trimeric fragment, designated in the following as TL1, which contains the C-terminal half of the tryptic fragment F4 (Figure 1).

In order to ascertain whether TL1 still contains the recognition site for α1β1, an inhibition assay was carried out. Whereas F4 was able to inhibit the binding of α1β1 to CB3[IV] coated on plastic, TL1 was found to be inactive (Figure 2).

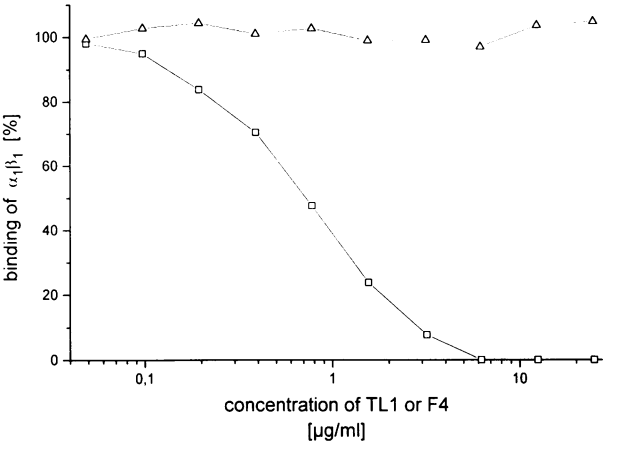

Fig. 2. Inhibition of the binding of α1β1 to CB3[IV]. α1β1 (2 μg/ml) was incubated with increasing concentration of TL1 (△) or F4 (□) for 30 min before adding to CB3[IV] coated microwell plates (2 μg/ml; 100 μl/well). The OD of α1β1 bound to adsorbed CB3[IV] in the absence of inhibitor was set to 100%. Data represent means of duplicates. On average, the OD values varied 3.5% from the mean.

The triple helical conformation of TL1

A prerequisite for the interaction of collagen IV with α1β1 is its triple helical conformation. We therefore examined the conformation of TL1 in comparison with the fragments F1 and F4 using CD spectroscopy. All three fragments exhibited a positive signal at 221 nm, a characteristic property of a triple helical structure. The melting curve of F1 recorded with CD at 221 nm (Figure 3) exhibited two different transition temperatures (T_M), which indicated triple helical segments of different stability (T_M of 38 and 50°C). The more stable segment can be ascribed to the central part of F1, represented by F4, which is stabilized by the two disulfide knots. In contrast to F1 and F4, the transition curve of TL1 was much broader, with a T_M of 31°C, a sign for a shorter and somewhat imperfectly ordered triple helical structure. Between 50 and 60°C the transition curve of TL1 indicated a small triple helical section with a stability of more than 50°C, possibly representing the triple helical area around the disulfide knot II.

There is additional independent proof for the triple helical conformation of TL1. The monoclonal antibody (mAB) CIV22 reacts with CB3[IV], F1, F4 and also with TL1 (Figure 4). Since reduced and denatured fragments do not bind mAB CIV22, this interaction is conformation dependent and a demonstration for a triple helical epitope. The mAB CIV22 was found to be unable to inhibit the interaction of F1 and F4 with α1β1, indicating different locations for the antibody binding epitope and the recognition site of α1β1. From these experiments we conclude that TL1 possesses the triple helical epitope for mAB CIV22, but has lost the recognition site for α1β1.

Electron microscopical investigation of the α1β1 – F2 complex

F4 is the smallest triple helical fragment which is able to bind α1β1. Since TL1, which comprises the C-terminal half

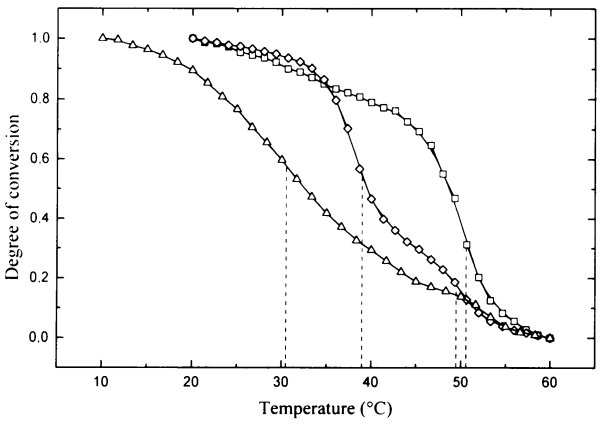

Fig. 3. Thermal melting profiles of fragments F1 (—◇—), F4 (—□—) and TL1 (—△—). Fragments were dissolved in 0.05 M sodium phosphate buffer, pH 7.4, containing 0.1 M sodium chloride and heated from 20 to 60°C with a linear temperature gradient of 20°C/h. Profiles were recorded with CD at 222 nm. The melting temperatures (T_M) are indicated by vertical dashed lines.

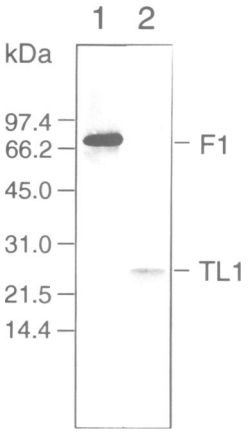

Fig. 4. Western blot of the fragments F1 (lane 1) and TL1 (lane 2) stained with the monoclonal antibody CIV22. F1 and TL1 were submitted to an SDS−PAGE in an 8−18% polyacrylamide gradient and blotted onto an Immobilon membrane (Millipore Corporation, Bedford, MA, USA). The immobilized fragments were stained with mAB CIV22 as primary antibody and an anti-mouse IgG antibody coupled to peroxidase as secondary antibody. 4-Chloronaphthol and H_2O_2 were used as substrate for the peroxidase.

of F4, has lost the capacity to interact with α1β1, the recognition site can be ascribed to the 39 amino acid long N-terminal half of F4. This conclusion, drawn from protein chemical investigations, was verified by electron microscopical investigations of a complex between α1β1 and the fragment F2. F2 has the same N-terminus as F4, but contains at the C-terminal end an additional triple helical segment of 36 residues in length which, however, does not interact with α1β1 (Figure 1). The α1β1−F2 complex was stabilized by crosslinking with bis(sulfosuccinimidyl)-suberate and subsequently purified using a two-step procedure. The surplus of the ligand F2 was removed by affinity chromatography on wheat germ agglutinin (WGA)−Sepharose, which bound the integrin (Figure 5, lane 4), whereas the integrin not crosslinked with F2 was subsequently separated by immunoadsorption on a mAB CIV22−Sepharose. The purified complex appeared in SDS−PAGE as a band of 400 kDa (Figure 5, lane 5), which was stained in Western blot analysis with anti-human CB3[IV] as well as with β1 antibodies (not

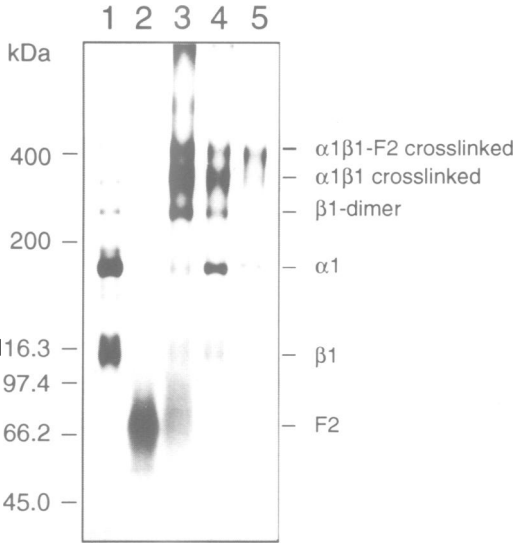

Fig. 5. Purification of the covalently crosslinked α1β1−F2 complex. Lane 1, α1β1; lane 2, fragment F2; lane 3, mixture of α1β1 and F2 (molar ratio 1:15) after crosslinking with bis(sulfosuccinimidyl)-suberate. The crosslinked α1β1−F2 complex appeared as a new band of 400 kDa. Lane 4, α1β1−F2 complex after affinity chromatography on WGA−Sepharose to remove the surplus F2; lane 5, α1β1−F2 complex after immunoadsorption chromatography on CIV22−Sepharose to remove α1β1 not covalently bound to F2. The samples were separated by SDS−PAGE in a 3−12% polyacrylamide gradient gel under non-reducing conditions and detected by silver staining. The molecular weights of globular marker proteins and the laminin A-chain (400 kDa) are indicated.

shown). After rotary shadowing the receptor α1β1 is visualized in the electron microscope as a globule with a diameter of 17 nm (Figure 6). The triple helical fragment F2 can be seen as a rod which forms a kink after having left the globule. The electron micrograph is perfectly in agreement with the protein chemical data. The receptor is attached to the N-terminal part of F2, the kink reflects the non-triple helical region comprising the disulfide knot II and the adjacent 16 nm long rod coincides with the 55 residue C-terminal triple helical section of F2, which does not interact with α1β1 (Kern *et al.*, 1993).

An aspartate and an arginine are essential amino acid residues of the α1β1 recognition site

In many ligands interacting with integrin receptors, arginine and aspartic acid are essential members of the recognition site. We therefore modified the arginine residues of F4 with the α-diketo reagent *p*-azido-phenylglyoxal and the carboxyl groups of aspartate and glutamate residues of F3 with norleucine methylester after activation with a carbodiimide derivative. The modified fragments of F4 and F3 were subsequently used to inhibit the binding of α1β1 to coated CB3[IV] (Figure 7). In both cases the inhibition capacity was greatly reduced, which indicated that the arginine as well as the aspartate or glutamate residues are essential for the interaction with α1β1, and which allowed an exact location of the recognition site. The only arginine of the N-terminal half of F4 is present in the α2(IV) chain at position 461, and at the same position the α1(IV) chain contains an aspartate residue (Figure 1). The N-terminal half contains two additional acidic amino acids, aspartate on α2(IV) in position 448 and glutamate on α1(IV) in position 453 which could be potentially modified. Both residues

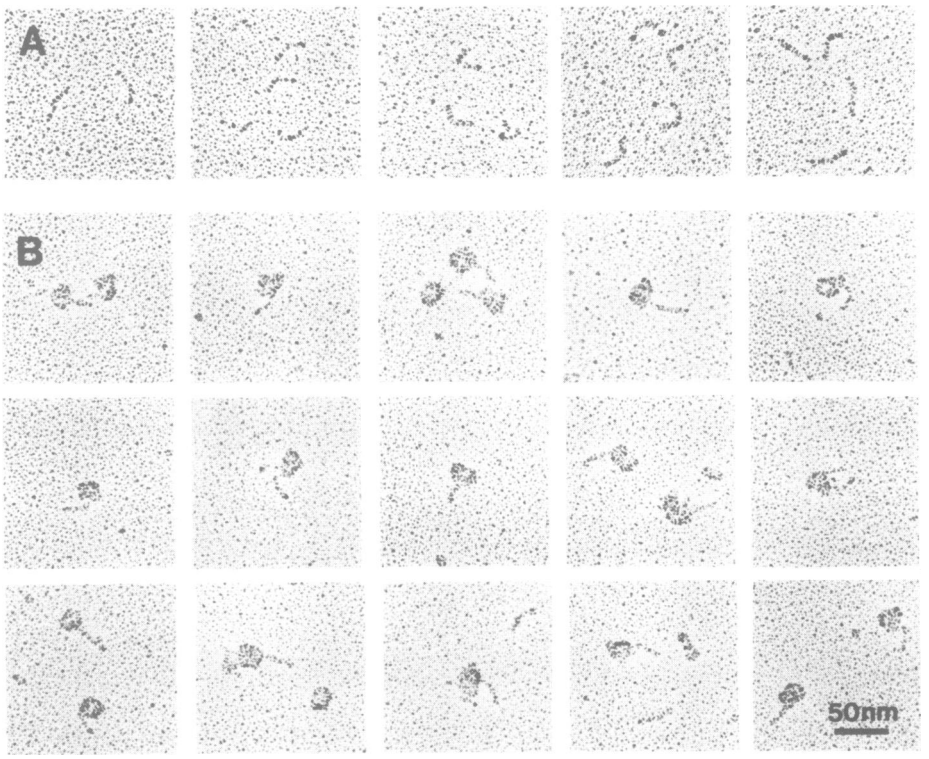

Fig. 6. Electron micrograph of the F2 fragment (**A**) and the crosslinked $\alpha1\beta1-F2$ complex (**B**). (A) The triple helical fragment F2 appears as a 32–34 nm long rod. (B) The globule with a diameter of 17 nm represents the $\alpha1\beta1$ integrin. The kink of the F2 fragment leaving $\alpha1\beta1$ reflects the interruption of the triple helix containing the disulfide knot II which is 17 nm away from the C-terminus of F2.

appear to be too far away from the essential arginine to be members of the recognition site. Another question was whether the two lysine residues at positions 456 and 459 are important for $\alpha1\beta1$ binding. Modification of the lysine residues with acetic anhydride did not reduce the inhibition capability of F4 (Figure 7). These experiments allowed the conclusion that, among the charged amino acid residues of F4, only the two residues described, arginine and aspartate in position 461, are essential for the interaction of collagen IV with $\alpha1\beta1$. In spite of the fact that they are located on three different chains, they lie close together on the surface of the triple helix (Figure 8).

In this context it was interesting to examine whether or not the binding of $\alpha1\beta1$ to CB3[IV] can be inhibited by synthetic peptides containing the sequence Arg-Gly-Asp. For this purpose we used the peptide Gly-Arg-Gly-Asp-Ser-Pro, known to inhibit the attachment of cells and platelets to various extracellular matrix proteins such as fibronectin (Pierschbacher and Ruoslahti, 1984), vitronectin (Hayman *et al.*, 1985), fibrinogen and von Willebrand factor (Plow *et al.*, 1985). In some cases cyclic peptides with restricted conformational freedom resulted in a better inhibition of cell binding to extracellular matrix proteins (Pierschbacher and Ruoslahti, 1987; Gurrath *et al.*, 1992). We therefore included in these inhibition experiments the cyclic peptide Gly-Pen-Gly-Arg-Gly-Asp-Ser-Pro-Cys-Ala. Neither the linear nor the cyclic peptide was able to inhibit the binding of $\alpha1\beta1$ integrin to coated CB3[IV]. Even at a concentration of 4 mM, which corresponded nearly to a 600 000-fold molar excess of peptides over the $\alpha1\beta1$ integrin, an inhibition of the interaction between CB3[IV] and $\alpha1\beta1$ could not be observed (not shown).

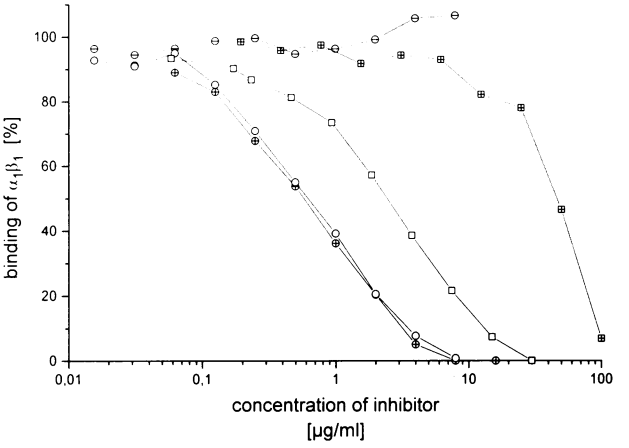

Fig. 7. Inhibition of the binding of the integrin $\alpha1\beta1$ to coated CB3[IV] by the fragments F3 and F4 before and after modification of distinct amino acid side chains. Unmodified F2 (○), F3 with 41% blocked lysine and hydroxylysine residues after treatment with acetic anhydride (⊕), F3 with completely modified carboxyl groups after treatment with norleucinemethylester in the presence of EDC (⊖), unmodifed F4 (□), F4 with *p*-azidophenylglyoxal (⊞) modified arginine residues. Inhibition ELISA was carried out as described in the legend to Figure 4. Inhibition curves of unmodified F3 and F4 did not coincide as different preparations of $\alpha1\beta1$ integrin were used.

Discussion

Recently we have shown that the trimeric cyanogen bromide fragment CB3[IV] of collagen IV contains recognition sites for the two integrins $\alpha1\beta1$ and $\alpha2\beta1$ (Vandenberg *et al.*, 1991; Kern *et al.*, 1993). After trypsin treatment of CB3[IV], four smaller fragments, F1–F4, were isolated,

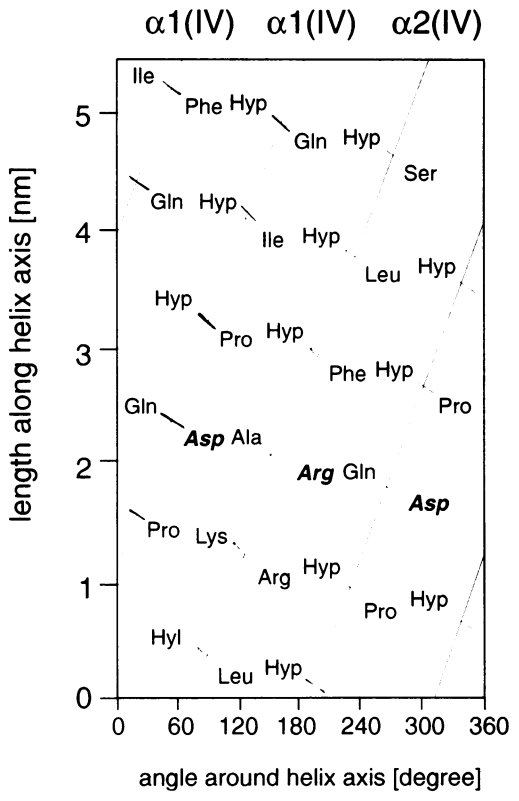

Fig. 8. Schematic representation of the recognition site of α1β1 integrin on the surface of the triple helical collagen IV molecule. The positions of the Cβ atoms on the cylinder surface with a radius of 0.5 nm are depicted. The three α-chains are arranged in the order α1(IV), α2(IV), α1(IV). Two screw-like structures can be observed: a steep right-handed screw with a pitch of ~8 nm formed by the α-chains and a flat left-handed screw with a pitch of 0.9 nm formed by the ridge of the side chains of the amino acids in X and Y position of all three α-chains, staggered against each other by one residue. The Arg and Asp residues (bold italics) essential for the recognition site of α1β1 are arranged along the flat screw. The Arg and Asp residues of the sequence Arg-Gly-Asp would be arranged along the steep screw.

of which the smallest one, F4, contained the recognition site for α1β1. The triple helix of F4 is interrupted by the two non-triple helical areas I and II in which either the two α1(IV) or all three α(IV) chains, respectively, are connected by disulfide bridges. Because of the stabilizing effect of the interchain crosslinks, F4 has a denaturation temperature of 50°C. Therefore, proteolytic enzymes such as trypsin, chymotrypsin or pepsin are not able to cleave peptide bonds in the area between the two disulfide knots. Only with thermolysin at 50°C was a proteolytic attack of this area observed. Thermolysin digestion of CB3[IV] resulted in the trimeric fragment TL1, which was characterized by Edman degradation. These studies revealed that the major part of TL1 starts in the middle between the two disulfide knots at position 473 and proceeds to the C terminus of F4, thus covering its C-terminal half (Figure 1).

Since TL1 did not interact with α1β1, it was important to determine whether TL1 is still triple helical. This was shown with two independent methods. First by CD spectroscopy, which revealed a triple helical conformation with a transition temperature (T_M) of 31°C, and secondly by the interaction of TL1 with the mAB CIV22, which interacts only with its triple helical epitope (Odermatt *et al.*, 1984). Thus the loss of binding activity cannot be explained by damage of the triple helix but must be due to the proteolytic degradation of the N-terminal half of F4, which therefore should contain the recognition site for α1β1.

This indirect conclusion drawn from protein chemical data was directly confirmed by electron microscopical investigations of a complex between α1β1 and the tryptic fragment F3. In the electron microscope, a triple helix appears as a rod and non-triple helical interruptions as kinks. Locations of kinks along the rods can be used to differentiate between N- and C-termini of a triple helical molecule. F4 was inappropriate for such experiments, since the distance of the two interruptions I and II from the N- and C-terminus of F4, respectively, is similar. We therefore used fragment F2 for this experiment, which contains as marker an additional triple helical segment at the C-terminus of F4. The electron micrograph of the complex clearly demonstrated that α1β1 interacts with the N-terminal area and that F2 protrudes from the α1β1 globule with its C-terminal region.

The recognition site for α1β1 could be located exactly because the only arginine of the N-terminal half of F4 in position 461 of α2(IV) is essential for the interaction with α1β1 and because the same is likely for aspartate at α1(IV) in position 461. Modification of these residues abolished interaction of F4 with α1β1. This is the first time that a recognition site of α1β1 has been localized and characterized. This binding site is embedded in a short triple helical segment, which is stabilized by intramolecular disulfide bridges and is therefore highly resistant to proteolytic attack. The involvement of Asp and Arg in the α1β1 binding site is also remarkable, since there are a number of other proteins which also contain the same amino acid residues in their recognition site for integrins. The best known example is fibronectin, which has a high affinity to α5β1 (Pytela *et al.*, 1985; Takada *et al.*, 1987). Other ligands with Arg-Gly-Asp (RGD) containing recognition sites are von Willebrand factor, fibrinogen, vitronectin, osteopontin and bone sialoprotein, all interacting with integrins of β1, β3 or β5 subfamilies (Ginsberg *et al.*, 1988; Lawler *et al.*, 1988; Oldberg *et al.*, 1988; McLean *et al.*, 1990). The sequence Arg-Gly-Asp occurs relatively frequently in collagens. This is because arginine prefers position Y of the tripeptide unit G-X-Y, whereas aspartate is more evenly distributed between X and Y. Nevertheless, the interaction of the native collagens, e.g. collagen I, III, IV and VI, which interact mainly with α1β1 and α2β1 is RGD independent. After denaturation, an RGD dependent binding of cells can be observed (Aumailley *et al.*, 1989; Vandenberg *et al.*, 1991; Gullberg *et al.*, 1992; Kern *et al.*, 1993; Pfaff *et al.*, 1993). Cardarelli *et al.* (1992), however, found that α2β1 binds to the cyclic peptide Cys-Gly-Arg-Gly-Asp-Ser-Pro-Cys and that α2β1 mediated attachment of MG-63 cells to collagen I can be inhibited by this cyclic peptide. In our experiments, the very similar cyclic peptide Gly-Pen-Gly-Arg-Gly-Asp-Ser-Pro-Cys-Ala was not able to inhibit the binding of α1β1 to CB3[IV].

To understand specific binding to collagen in molecular terms one has to consider not only the linear sequences of the polypeptide chains but also the array of the residues in the X and Y positions on the surface of the triple helix. Two different, screw-like structures are characteristic for the surface of a triple helix (Figure 8): a right-handed steep screw, which follows the course of the α-chains with a pitch

of 8 nm, and a flat left-handed screw, formed by the ridge of the residues at X and Y position of all three α-chains with a pitch of 0.9 nm. The sequence Asp(α1), Arg(α2), Asp(α1) at position 461 belongs to the flat left-handed screw and covers 214° around the triple helical cylinder (Figure 8). Depending on the alignment of the three α-chains, which is not known, the sequence Asp(α1), Asp(α1), Arg(α2) or Arg(α2), Asp(α1), Asp(α1) would also be possible. On the other hand, the residues Arg and Asp of the linear sequence Arg-Gly-Asp would be arranged along the steep screw formed by the α-chains. Both arrangements differ substantially in their relationship to the helix axis and should therefore comprise completely different binding structures.

The spatial combination Asp-Arg at about the same position in two different α-chains occurs only once in the entire human $[\alpha1(IV)]_2\alpha2(IV)$ collagen molecule. This agrees well with our earlier findings that the main binding sites of collagen IV for cells are located in the fragment CB3[IV] (Vandenberg *et al.*, 1991; Kern *et al.*, 1993) and with the fact that the interaction of $\alpha1\beta1$ with type IV collagen can be completely inhibited by the fragment F4 (not shown). However, the presence of minor recognition sites with lower affinity and different sequence cannot be entirely excluded. Also, other collagens, e.g. collagens I, III and VI, bind $\alpha1\beta1$, although with lower affinity than collagen IV (Kern *et al.*, 1993; Pfaff *et al.*, 1993). Inspection of the sequence of the $[\alpha1(III)]_3$ and $[\alpha1(I)]_2\alpha2(I)$ molecules revealed the absence of a similar Asp-Arg arrangement shown here to be essential for the $\alpha1\beta1$ recognition site of collagen IV. Nevertheless, binding of $\alpha1\beta1$ to collagens I and III can be inhibited by CB3[IV] (E.Schlosser-Hopstock and A.Lichy, unpublished). This indicates that collagen IV and the two fibre-forming collagens compete for the same interaction area of $\alpha1\beta1$, although the amino acid arrangement on the surface of the triple helix is different. In this context, it would be important to know to what extent the amino acids neighbouring the essential arginine and aspartate residues of F4 influence the recognition of $\alpha1\beta1$ or whether the imperfection of the triple helix in the N-terminal vicinity is involved in this interaction. A broad specificity of several integrins has been reported. A typical example is $\alpha2\beta1$ which interacts with collagen I, II, IV, VI and laminin (Tomaselli *et al.*, 1988; Staatz *et al.*, 1989; Vandenberg *et al.*, 1991; Gullberg *et al.*, 1992; Pfaff *et al.*, 1993). Although the recognition sites of a triple helical collagen and laminins should be quite different, the binding of $\alpha2\beta1$ to collagen IV can be inhibited by laminin and vice versa (Staatz *et al.*, 1991).

Materials and methods

Isolation of the receptor α1β1 and the collagen IV fragments CB3[IV], F1–F4 and TL1

The integrin $\alpha1\beta1$ was isolated as described earlier (Vandenberg *et al.*, 1991). In short, $\alpha1\beta1$ was extracted from human placenta with TBS containing 1% reduced Triton X-100 (Aldrich, Steinheim, Germany) and 1 mM $MgCl_2$. Affinity chromatography was carried out on a CB3[IV] Sepharose column in the presence of 1 mM $MnCl_2$. Bound $\alpha1\beta1$ was eluted with TBS containing 25 mM octylglucoside and 10 mM EDTA. Purity of the isolated receptor was analysed by SDS–PAGE (Laemmli, 1970), Coomassie staining and Western blot analysis (Kern *et al.*, 1993).

The trimeric fragment CB3[IV] was isolated after CNBr treatment of pepsin derived tetrameric collagen IV from human placenta. Fragments F1–F4 were isolated after limited trypsin digestion of CB3[IV]. Both procedures are described by Vandenberg *et al.* (1991) and Kern *et al.* (1993).

For the preparation of TL1, ~10 mg of CB3[IV] were dissolved in 0.1 M phosphate buffer, pH 7, and kept at 50°C for 15–20 min before adding 100 μg thermolysin. After 2 h, another 100 μg of thermolysin were added and the incubation continued for a further 2 h. Digestion was stopped with EDTA at a concentration of 10 mM and the reaction mixture was lyophilized. The first separation of the fragments occurred by gel permeation chromatography on a tandem of a TSK G3000 SWXL and a TSK G2000 SWLX column (both 7.8 × 300 nm; Tosohaas, Germany) in 0.2 M ammonium acetate containing 0.1% trifluoroacetic acid at a flow rate of 0.3 ml/min. These fractions containing trimeric fragments reducible by mercaptoethanol were pooled, lyophilized and further separated by ion exchange chromatography on a Mono S HR5/5 column (Pharmacia, Sweden) equilibrated with 0.1% trifluoroacetic acid titrated to pH 2.5 with NaOH (solvent A). After washing the column for 10 min with solvent A, bound fragments were eluted with 0.1% trifluoroacetic acid containing 2 M NaCl (solvent B), using a linear gradient from 0 to 50% B in 80 min at a flow rate of 0.5 ml/min. For further characterization by Edman degradation, TL1 was reduced and carboxymethylated as described (Vandenberg *et al.*, 1991) and the individual α1(IV) and α2(IV) fragments were separated on a C18 reversed phase column (0.46 × 25 cm; Vydac, Hesperia, CA) using 0.1% trifluoroacetic acid (A) and 70% acetonitrile in 0.1% trifluoroacetic acid (B) with a gradient of 0–60% B in 160 min at a flow rate of 0.25 ml/min.

SDS–PAGE of TL1 was done with the 12%T, 3%C gel system described by Schägger and von Jagow (1987). Amino acid sequences were determined by Edman degradation with gas–liquid phase sequencers (Applied Biosystems, models 470A and 473A) according to the manufacturer's instructions.

Modification of arginine, aspartate/glutamate and lysine/hydroxylysine residues

The arginine residues were modified according to Takahashi (1968) and Ngo (1981). In order to modify arginine, lyophilized F4 was dissolved in 100 nM sodium hydrogen carbonate buffer, pH 9.0, at a concentration of 330 μg/ml. A stock solution of *p*-azido-phenylglyoxal (Sigma, Deisenhofen, Germany) was added to a final concentration of 10 mM. After incubation for 3 h at 25°C, the reaction mixture was neutralized by addition of acetic acid to a concentration of 100 nM. The phenylglyoxal derivative was removed by dialysis against a 10 mM ammonium acetate buffer, pH 6.6, at 4°C. According to amino acid analysis (Biotronik LC 5000), 45% of the arginine residues were found to be modified. Since di(phenylglycosal)-arginine is partially cleaved during acid hydrolysis, the actual yield of modified arginine should be >45%.

The carboxyl groups of the fragment F3 were modified according to Hoare and Koshland (1967). Briefly, 400 μg of lyophilized F3 fragment were dissolved in 1.5 ml 1 M norleucinmethylester solution, pH 4.8 (Sigma). After adding 500 μl of 0.4 M 1-ethyl-3′-dimethylamino-propyl-carbodiimide (EDC; Sigma) the reaction was carried out for 200 min at 25°C and stopped by addition of acetic acid to a final concentration of 0.5 M. Reagents were removed by dialysis against 0.5 M acetic acid. The yield of modified carboxyl groups was determined by amino acid analysis (Biotronik LC 5000). According to the norleucine liberated after hydrolysis of modified F3, the carboxyl groups of the aspartate and glutamate side chains were completely blocked.

The side chains of lysine and hydroxylysine were acetylated with acetic anhydride according to Fraenkel-Conrat (1957). 600 μg of F3 were dissolved in 1 ml of cold, half-saturated sodium acetate solution. During 60 min 6 × 2 μl of acetic anhydride were added to the ice-cooled solution under stirring. Before dialysis against 0.5 M acetic acid, the reaction was allowed to proceed for a further 60 min. To determine the number of blocked lysine and hydroxylysine residues, the unmodified lysine and hydroxylysine residues were dinitrophenylated. 50 μg of modified and unmodified F3 fragment were dissolved in 200 μl of 0.5 M sodium hydrogencarbonate solution. 200 μl of a 0.5 M 2,4-dinitrofluorbenzene solution (Fluka, Buchs, Switzerland) in dioxan were added to both solutions. The reaction was allowed to proceed overnight at room temperature with vigorous stirring; it was stopped by adding 6 μl acetic acid and dialysing against 0.5 M acetic acid. During acid hydrolysis, acetyllysine and acetylhydroxylysine were cleaved to give lysine and hydroxylysine respectively, whereas the ε-*N*(2,4-dinitrobenzene) derivatives of lysine and hydroxylysine remained stable. From amino acid analysis (Biotronik LC 5000), the yield of acetylation of lysine and hydroxylysine residues was calculated to be 41%. In a control experiment without acetylation it was shown that only 87% of the free lysine and hydroxylysine residues reacted with 2,4-dinitrofluorbenzene.

Thermal denaturation

Circular dichroism (CD) was used to record unfolding and refolding of the triple helix during thermal denaturation and renaturation, respectively. F1,

F4 and TL1 (~50 μg/ml) were dissolved in 50 mM sodium phosphate buffer, pH 7.4, containing 0.1 M NaCl. Mean molar ellipticities (θ) were monitored at 221 nm in an autodichrograph Mark IV (ISA, Jobin Yvon, Division d'instruments SA) in a thermostated cell (Hellma) with a path length of 1 mm. For unfolding, the temperature was increased from 10 to 60°C with a linear gradient of 20°C/h using a thermostat (RKS20; Lauda) with an automatic programmer (PM351; Lauda). For refolding, the temperature was reduced from 60 to 10°C with the same linear gradient as above. The degree of conversion was calculated as described (Bächinger *et al.*, 1980).

Interaction of collagen IV fragments with α1β1

The binding of F4, TL1 and the modified F3 and F4 to α1β1 was determined using an inhibition assay in which binding of soluble α1β1 to immobilized CB3[IV] was inhibited by the respective ligands. To coat the 96-well ELISA plate (Greiner, Nürtingen, Germany), 100 μl of a CB3[IV] solution (2 μg/ml) in TBS (50 mM Tris−HCl, pH 7.4, 150 mM NaCl) were added to each well and incubated overnight at 4°C. Subsequently, the wells were washed three times with TBS. To block the remaining non-specific protein binding sites, the microtitre plates were treated with 100 μl of a 1% denatured bovine serum albumin (BSA; Sigma) solution in TBS for 2 h at room temperature. 2 μg/ml α1β1 in solution A (containing 1% BSA, 25 mM octylglucoside, 1 mM $MgCl_2$ and 1 mM $MnCl_2$ in TBS, pH 7.4) were preincubated for 30 min with increasing concentrations of collagenous ligands. 100 μl of each mixture were then added to wells coated with CB3[IV]. After 60 min incubation and washing the wells three times with solution A, the amount of bound receptor was determined using an ELISA described previously (Kern *et al.*, 1993). Each experiment was done in duplicate.

Interaction of CB3[IV] with α1β1 in the presence of RGD containing synthetic peptides

The linear peptides Gly-Arg-Gly-Asp-Ser-Pro and Gly-Arg-Gly-Glu-Ser-Pro (Biomol, Hamburg, Germany) and the cyclic peptide Gly-Pen-Gly-Asp-Ser-Pro-Cys-Ala (Pen = penicillamine) (GIBCO BRL, Gaithersburg, MD, USA) were used to inhibit the binding of α1β1 to coated CB3[IV]. The inhibition ELISA was performed as described above with a slight modification. To avoid a pH shift to lower values when using the acidic peptides at higher concentrations (4 mM) the Tris content was increased from 50 to 100 mM in the buffer in which the soluble α1β1 interacted with coated CB3[IV] in the presence and absence of synthetic peptides.

Isolation and electron microscopy of the crosslinked complex α1β1−F2

To a solution of 200 nM F2 in a buffer containing 20 mM triethanolamine−HCl, pH 7.8, 50 mM octylglucoside, 150 mM sodium chloride, 1 mM $MgCl_2$ and 1 mM $MnCl_2$, α1β1 dissolved in the same buffer was added to a concentration of 4 μg/ml. The α1β1−F2 complex was formed during 100 min of gentle shaking at room temperature. In order to crosslink the complex, a solution of 50 mM bis(sulfosuccinimidyl)suberate (BS3; Pierce, Rockford, USA) in deionized water was added to a final concentration of 0.5 mM BS3. After 45 min, the crosslinking reaction was terminated by adding 1/10 vol. stop buffer containing 0.5 M ethanolamine (Merck, Darmstadt, Germany) and 0.1 M EDTA (Merck) in 1 M Tris−HCl buffer, pH 7.4, and shaking at room temperature for another 2 h to destroy unreacted BS3 crosslinker.

Purification of the crosslinked complex consisted of two steps. First, the excess of F2 was removed by binding the complex to WGA−Sepharose. In the second step, the integrins not crosslinked to F2 were separated by interaction with the murine mAB CIV22 immobilized on Sepharose. Monoclonal AB CIV22 was kindly provided by Dr B.Odermatt (Universität Zürich).

For the first purification step the solution with the crosslinked products was incubated with WGA−Sepharose in WGA buffer containing 25 mM octylglucoside and 1 mM $MgCl_2$ in TBS, pH 7.4, by gentle shaking at 4°C overnight. Subsequently the WGA−Sepharose was packed into a glass column and washed with WGA buffer supplemented with 10 mM EDTA to remove non-covalently bound F2. After washing with WGA buffer, the integrins including the complex were eluted with 10% (w/v) *N*-acetylglucosamine in WGA buffer. *N*-acetylglucosamine was removed by washing the eluate with WGA buffer in a centricon 30 tube by centrifugational ultrafiltration.

For the second purification step the WGA eluate was diluted to a protein concentration of ~25 μg/ml in CIV22 buffer (20 mM octylglucoside and 1 mM $MgCl_2$ in TBS, pH 7.2) and mixed with CIV22−Sepharose (see below) equilibrated with the same buffer. After incubation at 4°C overnight, the CIV22−Sepharose was packed into a glass column, washed with CIV22 buffer containing 10 mM EDTA and finally the α1β1−F2 complex was eluted with 3 M potassium thiocyanate (Merck) in CIV22 buffer. To remove potassium thiocyanate and to concentrate the complex, the eluate was thoroughly washed with CIV22 buffer by centrifugation-ultrafiltration using a centricon 30 tube. The purity of the α1β1−F2 complex was shown by SDS−PAGE.

In order to prepare the immunoaffinity matrix, the murine monoclonal anti-F4 antibody CIV22 was purified from the hybridoma supernatant according to standard protocols (Harlow and Lane, 1988) using ammonium sulphate precipitation, gel filtration on a Sephacryl column (Pharmacia LKB; Sephacryl S 3000 H/Load 16/600) and affinity chromatography on protein G−Sepharose (Sigma). The K_d value of mAB CIV22 to CB3 or F4 was determined to be 0.6 nM and 1.6 nM in the absence and presence of 25 nM octylglucoside, respectively. The purified mAB was coupled to cyanogen bromide activated Sepharose according to the producer's intructions (Pharmacia LKB, Freiburg, Germany) with the slight variation of decreasing the sodium chloride concentration of the coupling reaction to 300 mM. About 95% of the mAB was coupled to Sepharose, resulting in an antibody capacity of 0.7 mg mAB CIV22 per ml Sepharose.

For electron microscopy, the α1β1−F2 complex in CIV22 buffer was diluted to a concentration of 45 μg/ml in 100 μl $MgCl_2$ in TBS, pH 7.2, and mixed with 100 μl of 87% glycerol (Merck). To prevent precipitation of the complex, diluting and mixing was performed immediately before spraying onto freshly cleaved mica. Rotary shadowing and observation in the electron micrograph (CM 10; Philips) was done as described (Kühn *et al.*, 1981).

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