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Chemical cross-linking leads to two high molecular mass aggregates of rat $\alpha_1\beta_1$ integrin differing in their conformation but not in their composition

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Abstract In order to detect protein interactions of the collagen/laminin receptor $\alpha_1\beta_1$ integrin, covalent chemical cross-linking was performed with the homo-bifunctional, amine reactive reagents DSS (disuccinimidylsuberate) and DSP (dithiobis(succinimidylpropionate)). After cross-linking of the 190 kDa rat α_1 integrin subunit, immunoblotting revealed two additional, immunoreactive, high molecular mass complexes (M_r 240/290 k). Generation of the 240/290 kDa aggregates depended on the presence of the intact tertiary protein structure. As shown with immunoaffinity purified proteins, the 240/290 kDa aggregates consist exclusively of α_1 and β_1 integrin subunits. No other cross-linked proteins associated with the α_1 or β_1 subunit were detected. In contrast to the non-cross-linkable $\alpha_1\beta_1$ integrin, the 240/290 kDa aggregates presumably represent active forms of the adhesion receptor, because both bound in vitro to collagen I and IV. This ability of $\alpha_1\beta_1$ integrin to cross-link and produce two additional high molecular mass forms is shared by rat $\alpha_9\beta_1$ integrin. Thus, the cross-linking approach directly indicates that β_1 integrins occur in different conformations caused by variations in the folding and/or spatial arrangement of their subunits.

Key words: Chemical cross-linking; Rat $\alpha_1\beta_1$ integrin

1. Introduction

Integrins are a family of heterodimeric plasma membrane glycoproteins which are involved in many cellular recognition, binding and adhesion processes. So far, 15 α and 8 β subunits have been characterized, which combine to form at least 20 different, non-covalently associated dimers mediating either cell–cell or cell–matrix interactions [1,2].

Beside their function as adhesion receptors, integrins also have been shown to participate in signal transduction, transferring information from the outside into the intracellular compartment (outside-in) or from the cytoplasm to the immediate extracellular environment (inside-out). As shown particularly for integrins $\alpha_{IIb}\beta_3$, $\alpha_L\beta_2$ and $\alpha_M\beta_2$, conformational changes of the molecule participate in the regulation of the binding affinity and ligand specificity (reviewed in [3,4]). Early responses to integrin-ligand binding involve intracellular changes, e.g. in tyrosine phosphorylation of selected cytoplasmic proteins, and alterations in intracellular Ca^{2+}-concentration or pH (reviewed in [2,5]).

The complex processes of integrin-mediated signal transduction events depend on the coordinated interaction of different biomolecules in close contact with the integrin receptors. These integrin-protein interactions are mostly realized by the β subunits, which directly bind via their cytoplasmic domains to α-actinin and talin [6,7]. Recently, a β_3 integrin associated transmembrane protein was described which belongs to the immunoglobulin superfamily [8] and participates in the integrin-mediated Ca^{2+} influx into the cell [9]. Most other integrin–protein interactions have been discovered by comparative analysis of the protein composition present in focal contacts [4]. However, the detailed structural and functional relevance of these integrin associations have to be determined.

We have now studied protein interactions of the rat collagen/laminin receptor, $\alpha_1\beta_1$ integrin, by using group-specific chemical cross-linkers. This integrin is expressed in almost all rat organs of the digestive, respiratory and urogenital system, as well as in lymphatic organs, but not in the brain [10]. Cross-linking of rat liver membrane proteins produced two high molecular mass protein complexes (M_r 240/290 k) of $\alpha_1\beta_1$ integrin. Biochemical analysis of these complexes revealed that both aggregates consist exclusively of α_1 and β_1 subunits in different conformations, and that they represent the ligand-binding form of $\alpha_1\beta_1$ integrin. In addition, investigation of other β_1 integrins showed that the formation of the high molecular mass complexes is a general structural feature of β_1 integrins.

2. Materials and methods

2.1. Reagents and solutions

All chemicals used in the present study were of analytical grade and obtained from Merck (Darmstadt, Germany). The ECL immunoblot detection kit was purchased from Amersham Buchler (Braunschweig, Germany), peroxidase-conjugated secondary antibodies from Sigma (Deisenhofen, Germany), nitrocellulose from Schleicher und Schüll (Dassel, Germany), DSP (dithiobis(succinimidylpropionate)) and DSS (disuccinimidylsuberate) from Pierce (Rockford, USA) and Kodak XR-5 films from Kodak (Rochester, USA).

2.2. Antibodies

mAb 33.4 with specificity for rat α_1 integrin subunit and antisera against rat α_1 and β_1 integrin subunits have been previously described [10,11]. The antiserum against rat α_9 integrin subunit was kindly provided by Dr. Staffan Johansson [12]. mAb 13.4 is specific for rat dipeptidylpeptidase IV [13].

2.3. SDS-PAGE and immunoblotting

Protein samples were submitted to SDS-PAGE [14] on a 6% separation gel with a 3.5% stacking gel under standard conditions using a Mini-Protean II system (BioRad, München, Germany), followed by

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Abbreviations: DSS, disuccinimidylsuberate; DSP, dithiobis(succinimidylpropionate); DTT, dithiothreitol; mAb, monoclonal antibody; M_r, relative molecular mass.

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silver staining [15]. For immunoblotting, the proteins were transferred to nitrocellulose membrane filters. After blocking in 5% (w/v) non-fat dry-milk powder in washing buffer (0.1% (v/v) Tween 20 in PBS, pH 7.4) the protein blots were incubated overnight at 4°C with the primary antibodies at a 1:5000 dilution and for 1 h with a 1:2000 dilution of peroxidase-conjugated secondary antibody. Unbound primary and secondary antibodies were removed by three incubations in washing buffer. Bound antibodies were detected by chemiluminescence by exposing blots to Kodak XR-5 film for several time periods varying between 3–120 s.

2.4. Protein cross-linking

2 mg of purified rat liver plasma membranes [16] were suspended in a total volume of 0.7 ml of PBS, pH 8.3, and solubilized with 0.5% (v/v) Triton X-100. After removing insoluble material by centrifugation, 0.5 ml of the detergent extract was mixed with 35 μl of a DSS or DSP solution (12.5 mg/ml in DMSO) and allowed to react for 10 min at room temperature. The reaction was stopped by adding 50 μl of 100 mmol/l Tris-HCl pH 8.0. Plasma membrane vesicles were cross-linked in the same way without the addition of detergent.

2.5. Purification of cross-linked $\alpha_1\beta_1$ integrin complexes by immunoaffinity chromatography

A crude membrane fraction prepared from the livers of four Wistar rats was solubilized for 2 h in 300 ml PBS, pH 8.3, 0.5% (v/v) Triton X-100, 1 mmol/l PMSF at 4°C. Insoluble material was removed by centrifugation for 20 min at 20,000 × *g*. The supernatant containing 2 mg/ml solubilized proteins was carefully mixed at room temperature with 160 mg DSP dissolved in 6 ml of DMSO and allowed to react for 10 min. Cross-linking was stopped by adding 50 ml of 100 mmol/l Tris-HCl, pH 8.0. After further centrifugation to remove precipitates from the sample, the supernatant was loaded onto an immunoaffinity column consisting of mAb 33.4 immobilized to Protein G-Sepharose 4B (Pharmacia, Freiburg, Germany) as described [11]. Nonspecifically adsorbed material was removed by washing with 50 ml of different washing buffers (10 mmol/l Tris-HCl, pH 8.2, 500 mmol/l NaCl; 10 mmol/l Tris-HCl, pH 7.8, 150 mmol/l NaCl, 10 mmol/l EDTA, 1% (v/v) Triton X-100; 10 mmol/l Tris-HCl, pH 7.8, 150 mmol/l NaCl). Specifically bound proteins were eluted from the column with 10 ml of 100 mmol/l citrate buffer, pH 3.5. Eluted fractions were neutralized with 1 mol/l Na_2CO_3 before analysis by SDS-PAGE and immunoblotting.

2.6. Binding of $\alpha_1\beta_1$ integrin to collagen

Collagen I/IV Sepharose was prepared by standard methods provided by the manufacturer (Pharmacia, Freiburg, Germany) and equilibrated with 50 mmol/l NaCl, 2 mmol/l $CaCl_2$, 2 mmol/l $MgCl_2$, 0.02% (w/v) NaN_3 before use. 400 μl of a rat liver plasma membrane detergent extract were incubated with 50 μl of collagen I/IV Sepharose with shaking for 1 h at room temperature. The supernatants were collected and incubated with an additional aliquot of collagen I/IV Sepharose. This procedure was repeated twice. Aliquots of membrane proteins from supernatants as well as from plasma membrane detergent extract were cross-linked with DSS as mentioned above and analysed by immunoblotting.

3. Results

3.1. Chemical cross-linking of $\alpha_1\beta_1$-integrin

Addition of the amine reactive, homo-bifunctional cross-linker, DSS, to Triton X-100 extracts of rat liver plasma membranes resulted in the formation of two additional high molecular mass bands, M_r 240 k and 290 k, reacting strongly with the α_1 integrin-subunit specific antiserum (Fig. 1). Titration with increasing concentrations of the cross-linker up to 250 μg/ml did not significantly change the generated cross-linking pattern, although a higher yield of the 290 kDa band was obtained. However, all samples still contained a non-cross-linkable fraction of the 190-kDa α_1 integrin-subunit, even when higher DSS concentrations were applied or, alternatively, when DSS was added several times.

A comparable result was obtained when unsolubilized membrane vesicles of rat liver hepatocytes were treated with DSS (Fig. 1). Formation of the 240/290 kDa aggregates, both containing the α_1 integrin-subunit, under these various conditions, suggests that their generation was not the artificial result of cross-linking of protein-detergent micelles. As an additional control, the same DSS cross-linked membrane proteins were

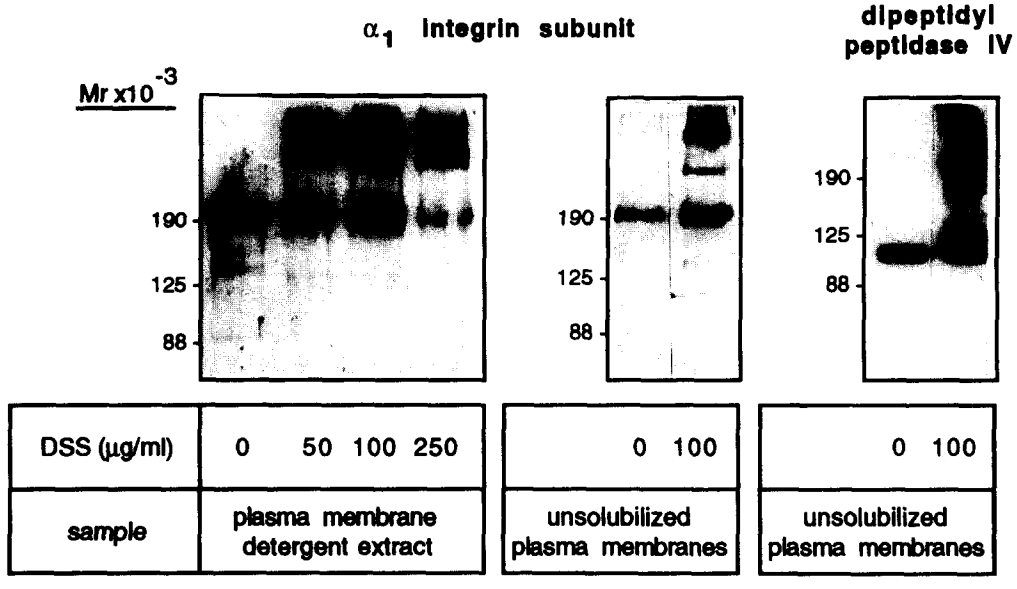

Fig. 1. DSS cross-linking of α_1 integrin-subunit of rat liver. Two high molecular mass complexes of 240/290 kDa were generated by cross-linking. Triton X-100 extracts (0.5% v/v) of rat liver plasma membranes were titrated with DSS in ascended concentrations as indicated (left). Similar complexes were produced when cross-linking was performed with unsolubilized plasma membranes (middle). Specificity of cross-linking was controlled by monitoring the distribution of dipeptidylpeptidase IV (right, band at 105 kDa) which is partially detectable under these conditions as a dimer (band at 210 kDa) in the plasma membrane sample. Protein was detected by immunoblot analysis with an antiserum against rat α_1 integrin subunit and with mAb 13.4 directed against rat dipeptidylpeptidase IV.

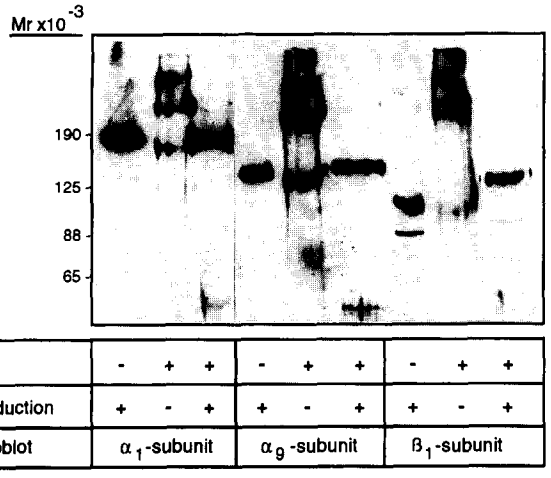

Fig. 2. High molecular mass aggregates are generated from different integrin subunits by cross-linking. A Triton X-100 extract (0.5% v/v) of rat liver plasma membrane proteins was incubated with the thiol-cleavable cross-linker DSP, subjected to electrophoresis under both reducing (DTT-reduction) and non-reducing conditions and further immunoblotted with antisera against rat α_1, α_9 and β_1 integrin subunit, respectively, as indicated.

also analysed for dipeptidylaminopeptidase IV (DPP IV), a membrane glycoprotein, which is known to be partially expressed on the rat liver cell surface in a dimeric complex consisting of two 105 kDa monomers [17]. Chemical cross-linking resulted in the detection of the 210 kDa band of the DPP IV dimer and remaining non-cross-linkable DPP IV monomers migrating at 105 kDa in SDS-PAGE (Fig. 1).

3.2. Extension of cross-linking to different integrin subunits

Immunoblotting of cross-linked membrane proteins with an antiserum specific for α_9 integrin-subunit, another prominent integrin of rat liver, detected the well-characterized 140 kDa monomer and two additional bands of 220 k and 270 k (Fig. 2). Immunoblotting of the same sample with a β_1 integrin-subunit specific antiserum revealed the 120 kDa monomer and additional bands of 220, 240, 270 and 290 kDa. The determination of the β_1, α_1 and α_9 integrin subunit containing cross-linked aggregates suggests that each is composed of at least α and β subunits. In addition, the reversibility of the cross-linking can be shown, if the thiol-cleavable cross-linker DSP [18], the homologue agent of DSS, is used. After reduction of the cross-linked fraction, only the monomers of all integrin-subunits were still detectable. These results agree with the observation that the α_V integrin-subunit of human keratinocytes could also be cross-linked with DSP into two distinct high molecular mass aggregates which were destroyed by DTT-reduction (data not shown). These findings indicate that: (i) chemical cross-linking in general yields two distinct high molecular mass integrin aggregates and is not restricted to the $\alpha_1\beta_1$ integrin; and (ii) the generation of the high molecular mass aggregates can be reversed and, therefore, is specifically derived due to cross-linking.

3.3. Structural requirements for cross-linking of $\alpha_1\beta_1$-integrin

An intact tertiary structure including disulphide bridges is necessary for $\alpha_1\beta_1$ integrin cross-linking (Fig. 3). Formation of the 240/290 kDa aggregates was completely prevented if the samples were denatured by SDS/boiling or treated with DTT prior to cross-linking. However, the success of cross-linking did not depend on the presence of divalent cations like Ca^{2+} (data not shown), which have been shown to be important for the formation of an active ligand binding site of integrins [19–21].

3.4. Composition of $\alpha_1\beta_1$-integrin complexes

Preparative cross-linking of detergent-solubilized rat liver membrane proteins was performed with DSP in combination with immunoaffinity chromatography on a mAb 33.4-immunoaffinity column. This antibody recognizes an epitope on the α_1 integrin subunit and can therefore be used to purify any protein aggregate containing the α_1 integrin subunit. As shown in Fig. 4, the 240 kDa and the 290 kDa complexes, as well as the non-cross-linkable $\alpha_1\beta_1$ integrin presented as single α_1 and β_1 integrin chains of 190 kDa and 130 kDa were quantitatively isolated by this approach. The single components of the mAb 33.4-column eluate were isolated by preparative SDS-PAGE and further subjected to analytical SDS-PAGE under non-reducing and reducing conditions. Reductive cleavage of the 240/290 kDa integrin aggregates exclusively converted both forms into bands of 190 kDa (α_1 integrin subunit) and 130 kDa (β_1 integrin subunit) with comparable intensities after silver staining, indicating similar stoichiometry. No other proteins were detected. This result strongly suggests that the 240/290 kDa aggregates are only composed of α_1 and β_1 integrin subunits.

3.5. Functional implications of the cross-linked high molecular mass $\alpha_1\beta_1$-integrin aggregates

Plasma membrane detergent extracts were depleted of collagen binding proteins by incubation with either collagen I- or collagen IV-Sepharose. They were then cross-linked with DSS and investigated for the distribution of the α_1 integrin subunit (Fig. 5). In both experiments, the ability to form 240/290 kDa aggregates was lost from the sample, whereas the non-cross-linkable 190 kDa band of the α_1 integrin subunit was still detectable in the same quantities in the supernatant. Thus, the 240/290 kDa aggregates are the ligand-binding forms of $\alpha_1\beta_1$ integrin, whereas the non-cross-linkable form is incapable of binding to collagen.

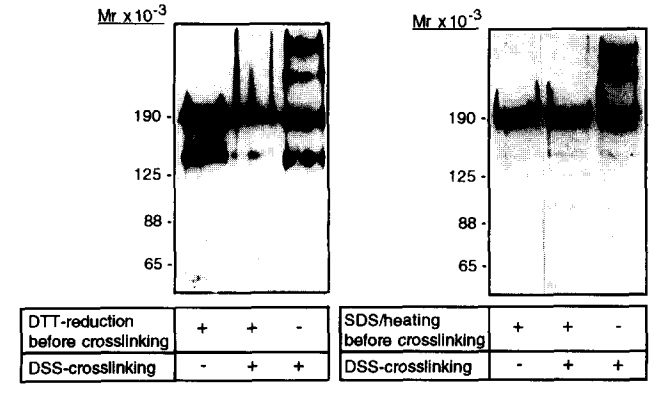

Fig. 3. Generation of cross-linked α_1 integrin subunit complexes requires intact tertiary structure. A Triton X-100 extract (0.5% v/v) of rat liver plasma membrane proteins was either reduced by incubation with 5 mM DTT for 30 min at 37°C or denatured by 1% (w/v) SDS/boiling (5 min) before DSS cross-linking. The detection of α_1 integrin subunit complexes was performed by immunoblotting with anti-rat α_1 integrin subunit-antiserum.

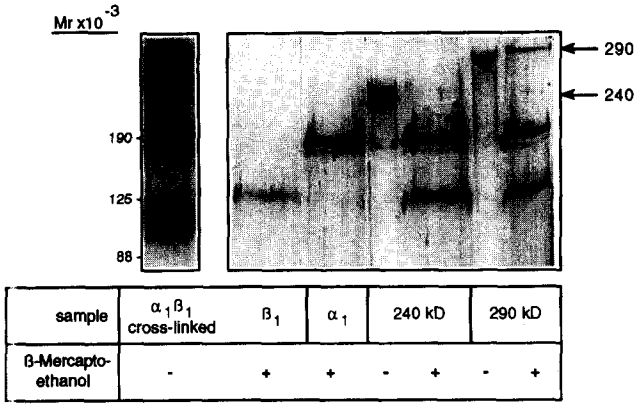

Fig. 4. Composition of cross-linked complexes containing the α_1 integrin subunit. A total preparation of solubilized membrane proteins of four rat livers was cross-linked with DSP and submitted to immunoaffinity chromatography on mAb 33.4 (anti-rat α_1 integrin subunit)-Sepharose. Eluted proteins ($\alpha_1\beta_1$ cross-linked; 120-kDa β_1 integrin subunit, 190-kDa α_1 integrin subunit, 240/290-kDa complexes containing the α_1 integrin subunit) were separated and isolated by preparative SDS-PAGE under non-reducing conditions, and re-electrophoresed either under reducing (β-mercaptoethanol) or non-reducing conditions as indicated. Proteins were visualized by silver staining.

However, integrin-collagen binding was not observed, when the same assay was performed with plasma membrane proteins which had been cross-linked with DSS before incubation with collagen-Sepharose (data not shown). The covalent modification presumably altered the physicochemical properties of the proteins and resulted in the loss of integrin binding to collagen.

4. Discussion

Protein interactions and conformational changes of the heterodimer are preliminary features of integrins associated with ligand binding, regulation and signalling. Although the molecular properties of single α or β subunits have been intensively investigated, only a few details are known about their cooperation and their interaction with other cellular counterparts.

In this report, we have investigated the collagen/laminin receptor, $\alpha_1\beta_1$ integrin, using the amine reactive, chemical cross-linkers DSS and DSP. These reagents fix inter- and intramolecular native protein associations by forming covalent links. The generated complexes are stable against denaturation and, therefore, easy to separate by SDS-PAGE from single molecules and non-cross-linked protein aggregates.

After cross-linking and SDS-PAGE, detection of the α_1 integrin subunit by immunoblotting revealed two bands of high molecular mass aggregates of 240 kDa and 290 kDa. The M_r of these aggregates was determined by comparison with the 210 kDa-dimer of DPP IV [17] and with cross-linked protein complexes separated by denaturating size exclusion chromatography [22]. In addition to the formation of these high molecular mass aggregates, some non-cross-linkable molecules of the 190 kDa α_1 integrin subunit always remained, providing evidence that three subpopulations of the α_1 integrin subunit can be distinguished by cross-linking. Furthermore, other α subunits after cross-linking (α_9 of rat hepatocytes; α_V of human keratinocytes) displayed a similar pattern, with residual non-cross-linkable α subunits and two high molecular mass forms, suggesting this might be a general characteristic of integrin cross-linking.

Initially, we assumed that the cross-linked 240 kDa form is a candidate for a protein complex consisting of one α_1 subunit and another associated protein, whereas the 290 kDa form represents the cross-linked heterodimer (α_1 and β_1 integrin subunits). This conclusion appeared to be supported by reports of an integrin associated protein with a M_r of 50 k [8,23]. However, in these investigations this protein was shown to interact with the β_3 integrin subunit, but not with any α chain. Therefore, we analysed the molecular composition of the immunopurified 240/290 kDa complexes. In all experiments, cleavage of the purified 240/290 kDa complexes resulted exclusively in the appearance of α_1 and β_1 subunits with a similar stoichiometric distribution. We always failed to detect any protein(s) associated with the α_1 (or β_1) subunit, and the subunit composition of both complexes was always the same. In conclusion, we propose that the different electrophoretic mobilities of the cross-linked high molecular mass integrin aggregates must be caused by differences in molecular size and not by a different protein composition.

Based on our results, we suggest the rat $\alpha_1\beta_1$ integrin heterodimer present in the plasma membrane can be divided by chemical cross-linking into three different subpopulations. Two subpopulations of $\alpha_1\beta_1$ integrin are accessible to cross-linking and can be converted into aggregates which migrate as bands of 240 kDa and 290 kDa in SDS-PAGE, whereas a third fraction, represented by the single α_1 and β_1 integrin chains, always remains non-cross-linkable.

One possible explanation for the appearance of these three $\alpha_1\beta_1$ integrin forms is that they differ in their subunit folding and/or subunit assembling. The subunits of the non-cross-linkable $\alpha_1\beta_1$ integrin must be arranged in such a configuration that they are presumably not in a close spatial contact and, therefore, inaccessible for molecular bridging. Alternatively, the amino groups of lysine residues of this integrin fraction might

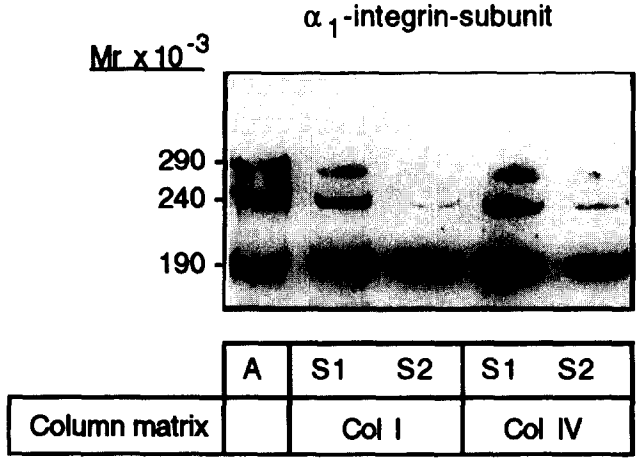

Fig. 5. The 240/290 kDa aggregates of $\alpha_1\beta_1$ integrin bind to collagen I and IV. Aliquots (A) of rat liver plasma membrane proteins were incubated with collagen I- (Col I) or collagen IV- (Col IV) Sepharose. The supernatants partially depleted of collagen binding proteins (S1) were re-incubated with collagen I- or collagen IV-Sepharose to complete the collagen binding (S2). Subsequently, all samples were cross-linked with DSS and subjected to immunoblot analysis with anti-rat α_1 integrin subunit-antiserum.

be protected or not accessible for the cross-linker. However, the two high molecular mass forms of the cross-linkable fraction of $\alpha_1\beta_1$ integrin molecules differ from each other in their individual subunit folding or subunit arrangement. The 290 kDa form might resemble a relaxed form of $\alpha_1\beta_1$ integrin, because its M_r is approximately the stoichiometric sum of its single components. In contrast, the heterodimeric 240 kDa form presumably represents a rather compact folded integrin subpopulation as compared with the 290 kDa form, since its M_r is significantly smaller than the stoichiometric sum of single α_1 and β_1 subunits. However, we do not yet know whether only one or both integrin subunits undergo this more compact folding.

A connection between integrin conformation and ligand binding activity is also confirmed by this cross-linking approach. As shown by in vitro binding assays, both the 240 kDa form and the 290 kDa form bound to collagen-Sepharose, whereas the non-cross-linkable molecules of $\alpha_1\beta_1$ integrin remained in solution. Thus, the collagen binding ability of the 240/290 kDa forms must be due to a special, near-neighboured arrangement of the α and β subunits, so that they form the ligand binding pocket. However, the assay is not suitable for investigating whether the high molecular mass forms possess different binding or activation status.

It is known from other integrins that ligand binding is regulated by conformational changes [3,4,24]. Platelet activation by agonists (inside-out) switches the fibrinogen receptor gp IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) from low affinity into a conformation with high affinity to fibrinogen [25]. A special feature of this switch consists of the expression of new, conformation-sensitive and antibody-accessible epitopes [26]. Activation from low to high affinity binding to ICAM-1 has also been described for the lymphocyte integrin LFA 1 ($\alpha_L\beta_2$) [27]. In general, the switch between different affinities has been explained by the combined movement of both subunits through the lipid bilayer under control of their cytoplasmic domains [4,28,29]. Additionally, conformational changes of integrins have also been observed after ligand binding. Upon ligand-binding, e.g. of fibrinogen, the $\alpha_{IIb}\beta_3$ integrin itself undergoes conformational changes resulting in the expression of 'ligand-induced binding sites' (LIBS) defined by newly formed, antibody-accessible epitopes [30].

However, only a few reports directly demonstrate the existence of such variable integrin conformations. It has been shown by proteolytic cleavage that the conformation of the ligand-occupied $\alpha_{IIb}\beta_3$ integrin is actually different from that of the non-binding form [31]. The α_4 integrin subunit is also known to exist in different conformations with different electrophoretic mobilities, migrating as 180 kDa/150 kDa bands in SDS-PAGE [32]. The present study therefore represents another approach to the direct investigation of changes of integrin conformation.

In summary, we have shown, that the $\alpha_1\beta_1$ integrin and some other members of the β_1 integrin family can be subdivided by chemical cross-linking in three distinct fractions with differences in their subunit conformation and ligand binding ability. Even though the molecular arrangement of these forms remains to be explored, their existence provides striking evidence that an integrin molecule undergoes conformational changes which may be important for integrin-dependent cell adhesion. Chemical cross-linking seems to be a useful technique, not only for detecting protein-protein interactions, but also for the analysis of protein structure.

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