Characterization of molecular aggregates of $\alpha_1\beta_1$-integrin and other rat liver membrane proteins by combination of size-exclusion chromatography and chemical cross-linking

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Abstract

Many membrane proteins display their biological activity in molecular aggregates of interacting counterparts. The analysis of these aggregates remains difficult; especially intermolecular complexes of membrane proteins tend to dissociate or artificially aggregate during detergent extraction out of membranes. Thus, the existence of protein aggregates was investigated by two approaches. First, after modest detergent extraction, the presence of three well characterized rat liver membrane proteins, $\alpha_1\beta_1$-integrin, dipeptidyl aminopeptidase IV (DPP IV) and cell-CAM 105 (CAM = cell adhesion molecule), in aggregates could be demonstrated when investigated by size-exclusion chromatography (SEC) under non-denaturing conditions. However, the applied detergents partially influenced the resolution of the separation reducing the ability to discriminate between native and artificial protein aggregates. To circumvent these problems, a second approach based on covalent cross-linking of native protein complexes by dithiobis(succinimidylpropionate) was combined with the performance of denaturating SEC. Under such optimized conditions the expression of $\alpha_1\beta_1$-integrin as heterodimer and DPP IV as homodimer was confirmed. In addition, some high-molecular-mass complexes of all model proteins consisting of unknown components could also be detected. Taken together, non-denaturing SEC and chemical cross-linking in combination with denaturating SEC represent methodological approaches for the characterization of protein aggregates.

1. Introduction

In the past years it became apparent that functional properties of proteins expressed on the cell surface are not necessarily based on their intrinsic characteristics but also depend on interactions with other components of the plasma membrane. Cell recognition and adhesion processes as well as signal transduction events need the coordinated interaction of two or more biomolecules localized on the cell surface [1–3].

Several methodological approaches have been developed to investigate molecular interactions of proteins. First, complete protein aggregates are characterized by gentle purification procedures like size-exclusion chromatography (SEC) [4], sucrose centrifugation [5] or blue native electrophoresis [6]. Second, associated proteins can be co-purified with immunopurification techniques. All approaches have some disadvantages reducing their potential: extraction of plasma membrane proteins with detergents as well as washing procedures in immunopurification, necessary for specific enrichment, tend to dissociate complexes. Moreover, size and composition

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of protein aggregates may be artificially influenced by detergent–protein interactions.

Another approach to characterize protein–protein interactions relies on the application of group-specific chemical cross-linkers [7]. These agents allow the fixing of protein complexes by covalent linking of neighbour proteins as present in vivo which cannot be destroyed even by denaturation whereas all non-cross-linked aggregates are dissociated into monomers. However, the separation and detection of these large aggregates achieved by conventional electrophoretic methods are usually not sufficient. The application of SEC in the presence of sodium dodecyl sulphate (SDS) or guanidinium hydrochloride (denaturating SEC) on inert chromatographic supports [8,9] should overcome these difficulties and enable the separation of cross-linked aggregates and monomeric proteins as well. An additional advantage of this technique is the absence of detergent influences on size and composition of protein aggregates.

In this report, we characterize aggregates of different membrane proteins from rat liver by two main experimental approaches: non-denaturating SEC and the combination of chemical cross-linking with denaturating SEC. Three different membrane proteins were investigated by these methods: $\alpha_1\beta_1$-integrin [10], a heterodimeric protein connected with proteins mediating contacts to the cytoskeleton [11,12], dipeptidyl aminopeptidase IV (DPP IV), known to be expressed as a homodimer [13] and partially associated with other membrane proteins [14], and cell-CAM 105 [15] (CAM = cell adhesion molecule), expressed in different variants and associated with unknown counterparts.

2. Experimental

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 from Pierce (Rockford, IL, USA) and Kodak XR-5 films from Kodak (Rochester, MN, USA).

2.2. Antibodies

Antisera against rat α_1- and β_1-integrin subunits [16,17] and the monoclonal antibodies 9.2 against cell-CAM 105 [18] and 13.4 against DPP IV [19] have been previously described.

2.3. High-performance liquid chromatography (HPLC)

The SE columns TSK-Gel G4000PW$_{XL}$ and TSK-Gel G5000PW (molecular mass exclusion limit $1 \cdot 10^6$ and $2.5 \cdot 10^6$ (dextran); 30 cm × 7.5 mm I.D.) were from Tosohaas (Stuttgart, Germany) and used in tandem. The HPLC system was from Gerätebau Dr.-Ing. H. Knauer (Berlin, Germany).

2.4. SDS polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis

Protein samples were submitted to SDS-PAGE (BioRad, Munich, Germany) and 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 exposing blots to Kodak XR-5 film for several time periods varying between 3 and 120 s.

2.5. Protein solubilization

A 3-mg amount of purified plasma membranes from rat liver [20,21] was suspended in PBS pH 7.2 supplemented with different detergents to a total volume of 2 ml. The final concentration of

detergents in samples prepared for SEC was 1% (w/v) n-octyl β-D-glucopyranoside (octyl-glucoside), 0.4% (w/v) 3-[(3-cholamidopropyl-ammonio)-1-propanesulphonate] (Chaps) or 1% (w/v) SDS. Phenylmethylsulfonyl fluoride (PMSF) was always added to 1 mmol/l. The solubilization of membrane proteins with Chaps and octylglucoside was performed for 2 h sub-sequently at 4°C. Samples containing SDS were boiled for 5 min. Insoluble material was removed by centrifugation for 20 min at 50 000 g.

2.6. Size-exclusion chromatography

A 250-μl volume of each sample (250–500 μg protein) was applied to the columns, eluted at room temperature at a flow-rate of 250 μl/min and monitored at 280 nm. Proteins in the elution fractions were precipitated with acetone (75%, v/v) at −20°C overnight, freeze-dried and dis-solved in 100 μl of SDS-PAGE sample buffer.

Non-denaturating chromatography

The eluent was 0.2% (w/v) octylglucoside or 0.25% (w/v) Chaps in phosphate-buffered saline (PBS), pH 7.4, supplemented with 0.02% NaN₃.

Denaturating chromatography

The eluent was 0.1% (w/v) SDS in 50 mmol/l sodium phosphate buffer, pH 6.5 [9].

Calibration of columns

The columns were calibrated using a SEC standard kit (BioRad) with thyroglobulin (dimer, relative molecular mass (M_r) 670 000), gammaglobulin (M_r 158 000), ovalbumin (M_r 44 000), myoglobin (M_r 17 000) and vitamin B₁₂ (M_r 1300). The standards were dissolved in PBS, pH 7.4, supplemented with detergents at the same concentrations as for membrane solubiliza-tion and separated using 0.2% (w/v) octyl-glucoside or 0.25% (w/v) Chaps in PBS, pH 7.4. Thyroglobulin (monomer, M_r 335 000) and oval-bumin were used as standard proteins for de-naturating chromatography. Both proteins were dissolved in PBS with 1% (w/v) SDS and boiled for 5 min prior to chromatography.

All buffers were filtered and degassed before use. The columns were thoroughly washed with five column volumes of double-distilled water between changes of solvent.

2.7. Protein cross-linking

A 2-mg amount of purified plasma membranes was suspended in a total volume of 0.7 ml with PBS, pH 8.3, and solubilized with 0.1% (w/v) Triton X-100, 0.3% (w/v) Chaps or 1% (w/v) octylglucoside. After removing insoluble materi-al, 0.5 ml of the detergent extract were mixed with 35 μl of a dithiobis(succinimdylpropionate) (DSP) solution (12.5 mg/ml in dimethylsulphox-ide) and reacted 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 with-out the addition of detergent.

3. Results

3.1. Solubilization of rat liver plasma membrane proteins

The ability of different detergents (Triton X-100, Chaps and octylglucoside) was examined to solubilize integral membrane proteins out of membrane vesicles at the lowest concentrations as possible. For these investigations the distribu-tion of the α_1-integrin subunit was analyzed in extracts and detergent-insoluble material. As seen in Fig. 1, each detergent showed an in-dividual solubilization behaviour. Octylglucoside was the weakest detergent, releasing a sufficient amount of α_1-integrin subunit only at a con-centration of 1% (w/v) whereas lower concen-trations of Triton X-100 and Chaps (0.1%, w/v and 0.3%, w/v, respectively) were needed to extract the same quantity of protein.

All fractions with insoluble material still con-tained high amounts of immunoreactive protein without regard to the detergent and the con-centrations used.
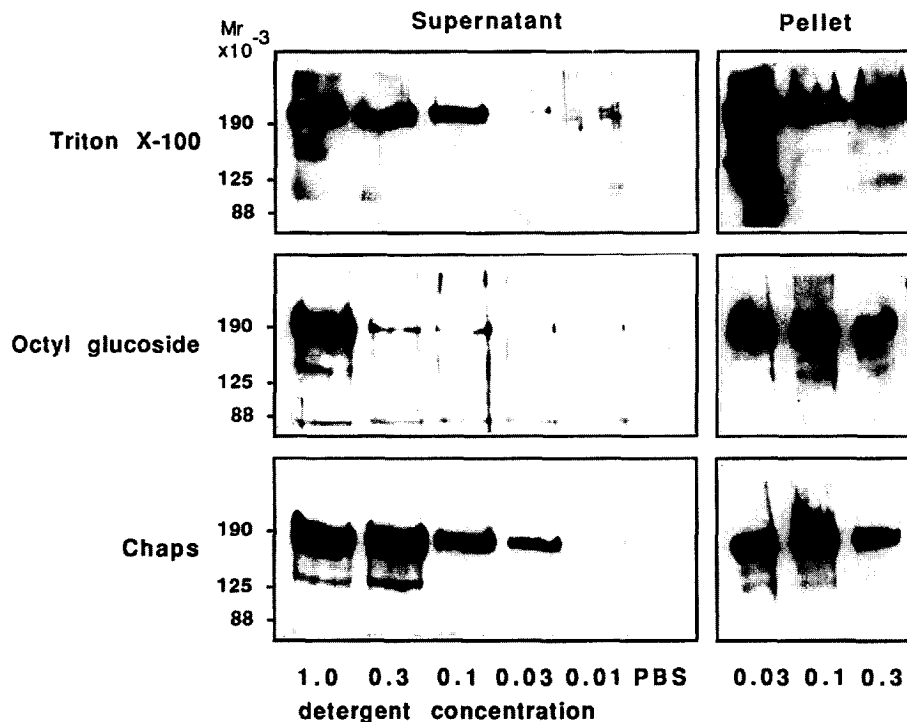
Fig. 1. Solubilization of α_1-integrin subunit. Equal aliquots of rat liver plasma membrane vesicles were extracted with detergents (Triton X-100, octylglucoside and Chaps) in varying concentrations ranging between 0.01 and 1.0% (w/v) and with PBS alone. Solubilized proteins were separated from insoluble material by centrifugation. Detergent supernatants and pellets were boiled in reducing SDS-PAGE sample buffer. A 30-μg amount of protein was applied to each lane and investigated in immunoblot analysis with anti-rat α_1-integrin subunit–antiserum.

3.2. SEC of membrane protein extracts

In order to characterize protein complexes by SEC, plasma membrane vesicles prepared from rat liver were solubilized using the lowest possible detergent concentrations of Chaps and octylglucoside, as determined in Fig. 1. Subsequent SEC was performed with the columns TSK-Gel 4000PW$_{XL}$ and TSK-Gel 5000PW in tandem which consist of polymer-modified beads resistant against detergents and chaotropic agents and separate proteins in the range of approximately 45 000–1 000 000. Aliquots of eluted fractions were investigated by immunoblotting with antibodies specific for four different membrane proteins: both subunits (α_1 and β_1) of the heterodimeric protein $\alpha_1\beta_1$-integrin (dimer, M_r 320 000; α_1 subunit, M_r 190 000; β_1 subunit, M_r 130 000), the homodimeric protein DPP IV

(dimer, M_r 210 000; monomer, M_r 105 000) and cell-CAM 105. The latter exists in different high-molecular-mass variants differing from 105 000 to 220 000.

Before sample application, columns were calibrated with standard proteins under the same detergent/buffer conditions as for membrane protein separation. Neither octylglucoside nor Chaps influenced their separation. The calibration of columns suggested a linear relationship between the logarithm of the molecular mass of standard proteins and their retention time.

Fig. 2A shows the immunoblots of fractions from SEC of octylglucoside extracts. All four investigated proteins were detectable in nearly the same fractions over a wide range (fractions 1–17) covering at least more than 600 000 M_r. This insufficient separation is confirmed by absorbance detection at 280 nm which showed a
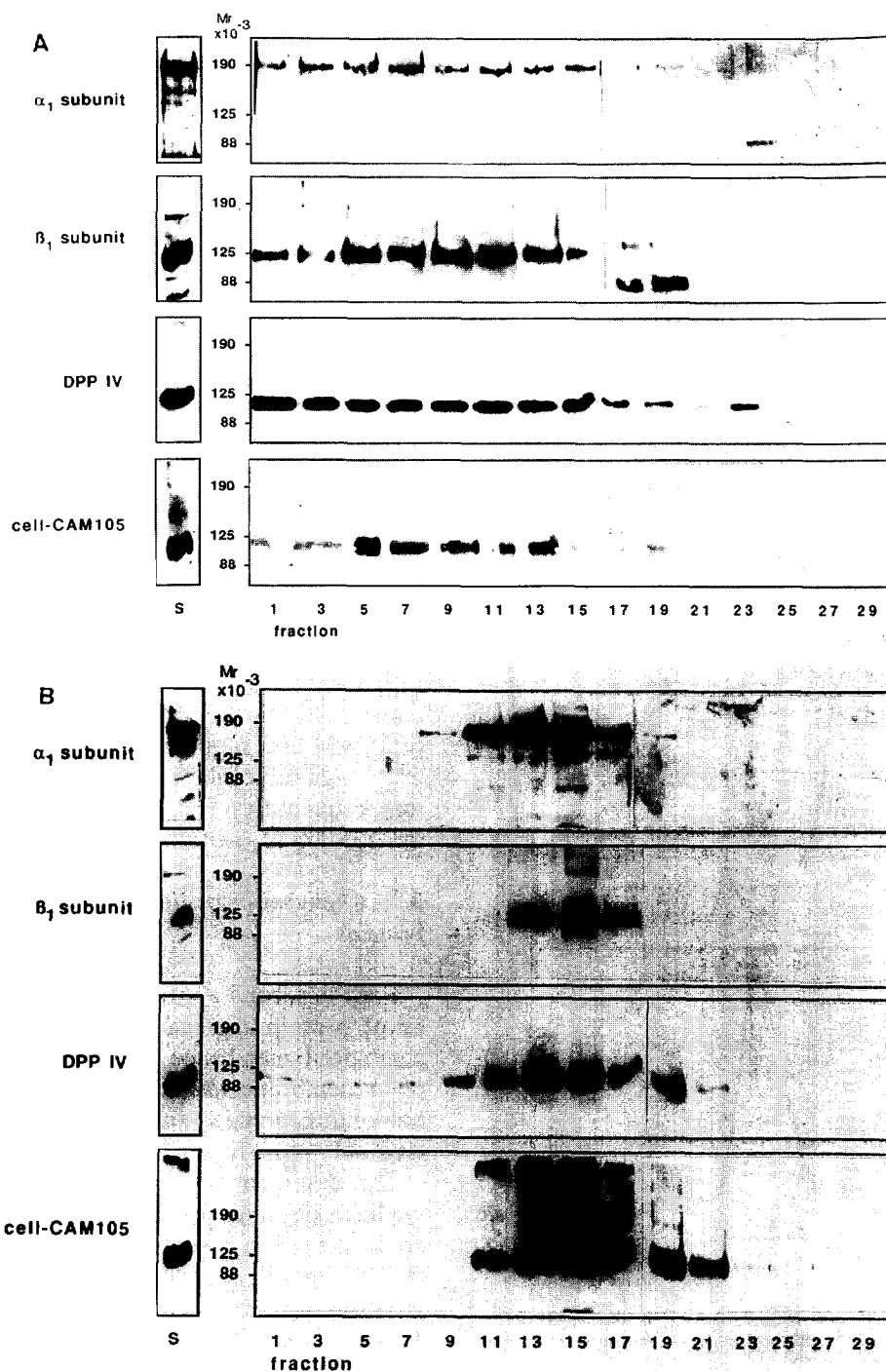
Fig. 2. Immunoblot analysis of collected fractions from non-denaturating SEC. (A) SEC with octyl glucoside, (B) SEC with Chaps. A 500-μg amount of rat liver plasma membrane proteins was solubilized (S) with 1% (w/v) octylglucoside or 0.3% (w/v) Chaps and separated on the in tandem combined SEC columns TSK-Gel G4000PW$_{XL}$ and TSK-Gel G5000PW as described in the Experimental section. Elution fractions of 370 μl were subjected to immunoblot analysis for rat α_1- and β_1-integrin subunits, DPP IV and cell-CAM 105, respectively.

broad main peak representing the exclusion volume of columns (Fig. 3) probably caused by large protein–octylglucoside micelles. Apparently, two monomeric forms of DPP IV differing by 5000 M_r could be separated by this chromatographic approach with retention times corresponding to those of the 158 000 and 44 000 M_r marker proteins.

When membrane solubilization and SEC were performed with Chaps, monitoring of absorbance at 280 nm resulted in a completely different course (Fig. 3). Although only three main peaks were obtained, both $\alpha_1\beta_1$-integrin subunits, DPP IV and cell-CAM 105 showed an individual distribution during chromatography (Fig. 2B). Both integrin subunits were mainly eluted together, but also partially separated from each other. Whereas the α_1 subunit was detect-
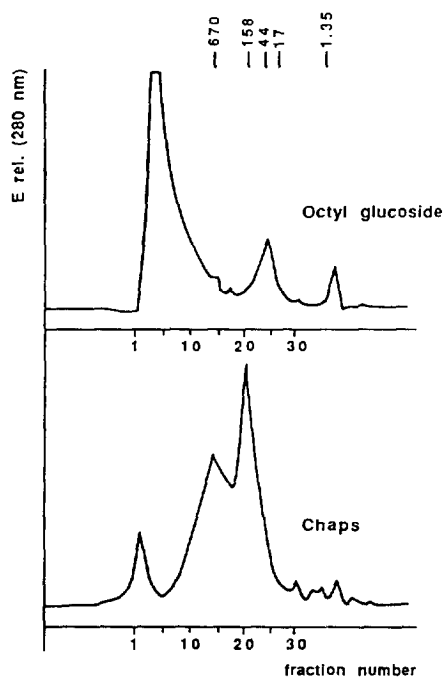
able in fractions 9–17, the appearance of the β_1 subunit was restricted to fractions 13–17, which correspond to a M_r between 200 000 and 700 000 presumably representing the heterodimeric $\alpha_1\beta_1$-integrin. The retention time of DPP IV (fractions 9–19) indicates the existence of monomeric and dimeric molecules as well as protein aggregates with M_r higher than 700 000. A similar distribution was observed in the case of cell-CAM 105 detected in fractions 11–21, which cover the M_r range 100 000–700 000.

To demonstrate that the observed protein aggregates can be converted into their monomeric components, SEC was performed with a denatured protein sample generated by the addition of SDS and subsequent boiling before separation. As shown in Fig. 4, the main portions of DPP IV, cell-CAM 105 and the β_1-integrin subunit molecules represented by bands from 105 000 to 125 000 M_r in SDS-PAGE were eluted in almost the same fractions, whereas the 190 000 M_r α_1-integrin subunit eluted four fractions earlier. Additionally, the immunoblot analysis of cell-CAM 105 supports the existence of at least four high-molecular-mass variants present in minor quantities which can be separated from the 105 000 M_r isoform by this approach.

3.3. Chemical cross-linking of membrane proteins

For the second approach to characterize membrane protein aggregates, samples of detergent-solubilized membrane proteins as well as complete membrane vesicles were incubated with the homobifunctional, amine-reactive and thiol-cleavable cross-linker DSP.

The detergents Triton X-100, Chaps and octylglucoside were examined according to their ability to retain protein complexes consisting of the 190 000 M_r α_1-integrin subunit available for subsequent cross-linking. The addition of DSP to Triton X-100, Chaps and octylglucoside extracts resulted in two additional bands with approximately 240 000 and 290 000 M_r reacting with the α_1-integrin subunit–antiserum (Fig. 5). However, all samples still contained some non cross-linkable α_1-integrin subunit molecules visuable
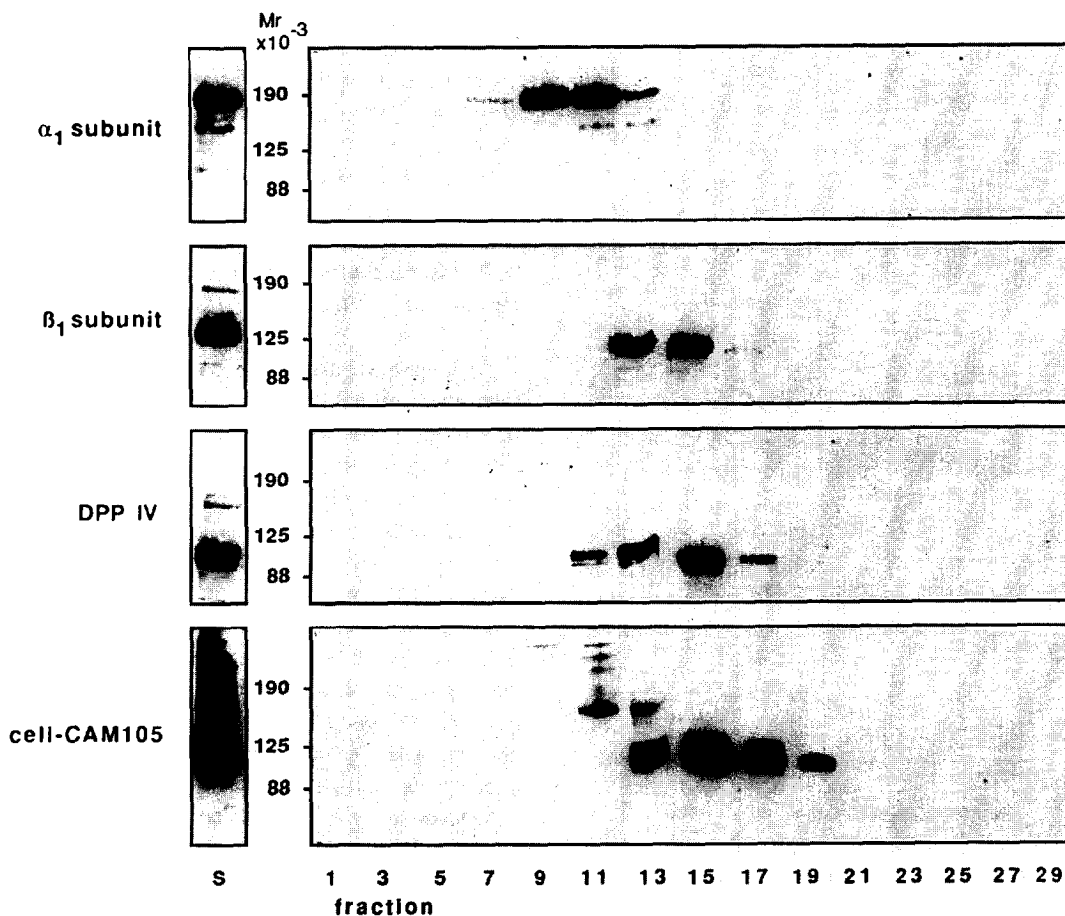

Fig. 3. Non-denaturating SEC with plasma membrane proteins solubilized and separated in the presence of octylglucoside and Chaps. A 500-μg amount of rat liver plasma membrane proteins was solubilized and separated with octylglucoside (upper graph) or Chaps (lower graph) as described in Fig. 2; detection of proteins was done by absorbance monitoring at 280 nm. The positions of marker proteins (see Experimental section) are indicated.

Fig. 4. Immunoblot analysis of fractions collected from denaturating SEC. A 500-μg amount of Chaps-solubilized rat liver plasma membrane proteins (S) was boiled for denaturation in 1% (w/v) SDS before separation. Elution fractions of 250 μl were collected and analysed as shown in Fig. 2.

as a 190 000 M_r band (Fig. 5A–C). In contrast, the α_1-integrin subunit was not accessible to DSP cross-linking after sample denaturation with SDS/boiling which destroys almost all protein–protein complexes confirming the idea that successful cross-linking needs intact protein conformations (Fig. 5A). The reversible character of the DSP cross-linkage is shown in Fig. 5B. Reduction with DTT before SDS-PAGE converted the 240 000 and 290 000 M_r bands reactive with the α_1-integrin subunit–antiserum into the monomeric 190 000 M_r α_1-integrin subunit.

To exclude the possibility that the high-molecular-mass $\alpha_1\beta_1$-integrin aggregates were generated by non-specific cross-linking of detergent-

induced artificial aggregates, a second cross-linking experiment was done with unsolubilized plasma membrane vesicles which preserve most in vivo protein associations (Fig. 5C). After reaction with DSP the membrane samples were extracted with the different detergents. To detect protein complexes that became insoluble for detergent solubilization, the detergent extracts and the pellets of insolubilized proteins were examined for the presence of the α_1-integrin subunit. As shown in Fig. 5C, this cross-linking resulted in the same 240 000 and 290 000 M_r bands visuable in Fig. 5A and B and (some) additional smeared band(s) larger than 290 000 M_r found in the detergent extracts of Triton
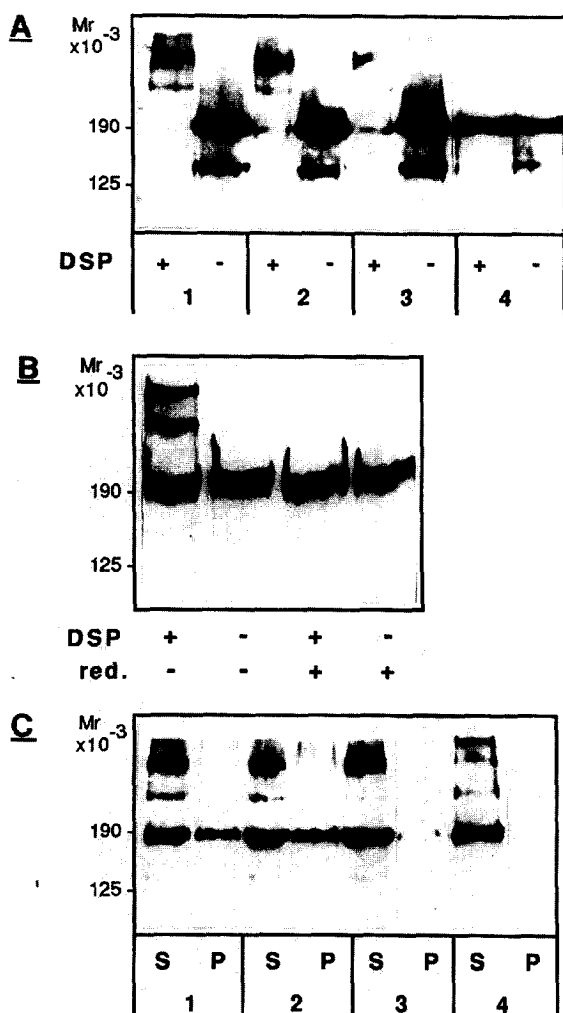
Fig. 5. Analysis of α_1-integrin subunit after cross-linking of membrane proteins. (A) Cross-linking of detergent extracts with DSP (see Experimental section). Protein extraction was done with (1) 0.1% (w/v) Triton X-100, (2) 0.3% (w/v) Chaps, (3) 1% (w/v) octylglucoside and (4) 1% (w/v) SDS/boiling. A 30-μg amount of protein was applied to each lane and investigated by immunoblot analysis with anti-rat α_1-integrin subunit–antiserum. (B) Reversibility of DSP cross-links. Membrane proteins extracted with 0.1% (w/v) Triton X-100 and cross-linked with DSP were investigated by immunoblot analysis with anti-rat α_1-integrin subunit–antiserum after sample treatment with (red.) and without DTT before SDS-PAGE. (C) Cross-linking of unsolubilized plasma membrane vesicles. After cross-linking with DSP, plasma membrane vesicles were solubilized with different detergents: (1) 0.1% (w/v) Triton X-100, (2) 0.3% (w/v) Chaps; (3) 1% (w/v) octylglucoside and (4) 1% (w/v) SDS/boiling. Aliquots of detergent supernatant (S) and pellets (P) were investigated in immunoblot analysis with anti-rat α_1-integrin subunit–antiserum after SDS-PAGE under non-reducing conditions.

X-100, Chaps and octylglucoside. All of these bands were completely dissolved after protein extraction with SDS (Fig. 5C). The detection of the α_1-integrin subunit in the pellets of detergent-insoluble components revealed only non-cross-linked protein.

3.4. Combination of chemical cross-linking with denaturating SEC

In a first approach, a membrane protein fraction was first solubilized with a low concentration of Chaps, cross-linked with DSP, denatured with SDS/boiling and at last separated by SEC. A second approach started with DSP cross-linking of intact membrane vesicles prepared from rat liver and continued with denaturation by SDS/boiling and with subsequent chromatographic analysis as in the first approach. All eluted fractions were subjected to SDS-PAGE under non-reducing and reducing conditions followed by immunoblotting. In both experiments similar results were obtained. As shown in Fig. 6A (non-reduced), denaturating SE chromatography separated single α_1 and β_1 subunits (bands at 190 000 and 130 000 M_r), cross-linked $\alpha_1\beta_1$-integrin complexes (bands at 240 000 and 290 000 M_r) and $\alpha_1\beta_1$-integrin–protein aggregates with M_r higher than 290 000 from each other as shown by SDS-PAGE/immunoblotting under non-reducing conditions. The latter bands were hardly detectable by immunoblotting because of the low resolution of SDS-PAGE for such large protein complexes. The β_1 subunit appeared in an additional, third cross-linked aggregate with M_r something lower than the 240 000 band. The chromatographic separation confirmed also the existence of monomeric and cross-linked dimeric forms of DPP IV as well as aggregates with M_r of approximately 250 000. Detection of cell-CAM 105 revealed apart from non-cross-linked molecules some aggregates with very large M_r. These large complexes could hardly be separated by SDS-PAGE under non-reducing conditions (Fig. 6A, non-reduced).

As a control, the same immunoblot analysis was performed under reducing SDS-PAGE conditions which converts the cross-linked protein

complexes into their monomers (Fig. 6B, reduced). Both integrin subunits were detectable in complexes larger than the 240 000 and 290 000 M_r aggregates. As expected, the presence of DPP IV was not restricted to the 210 000 M_r aggregate representing the cross-linked dimer but was also found in the observed complexes with M_r of 250 000 and higher. It should also be pointed out that the cross-linked complexes of cell-CAM 105 in fractions 5–9 are assembled by at least four variants, which migrate as bands higher than 150 000 M_r in reducing SDS-PAGE.
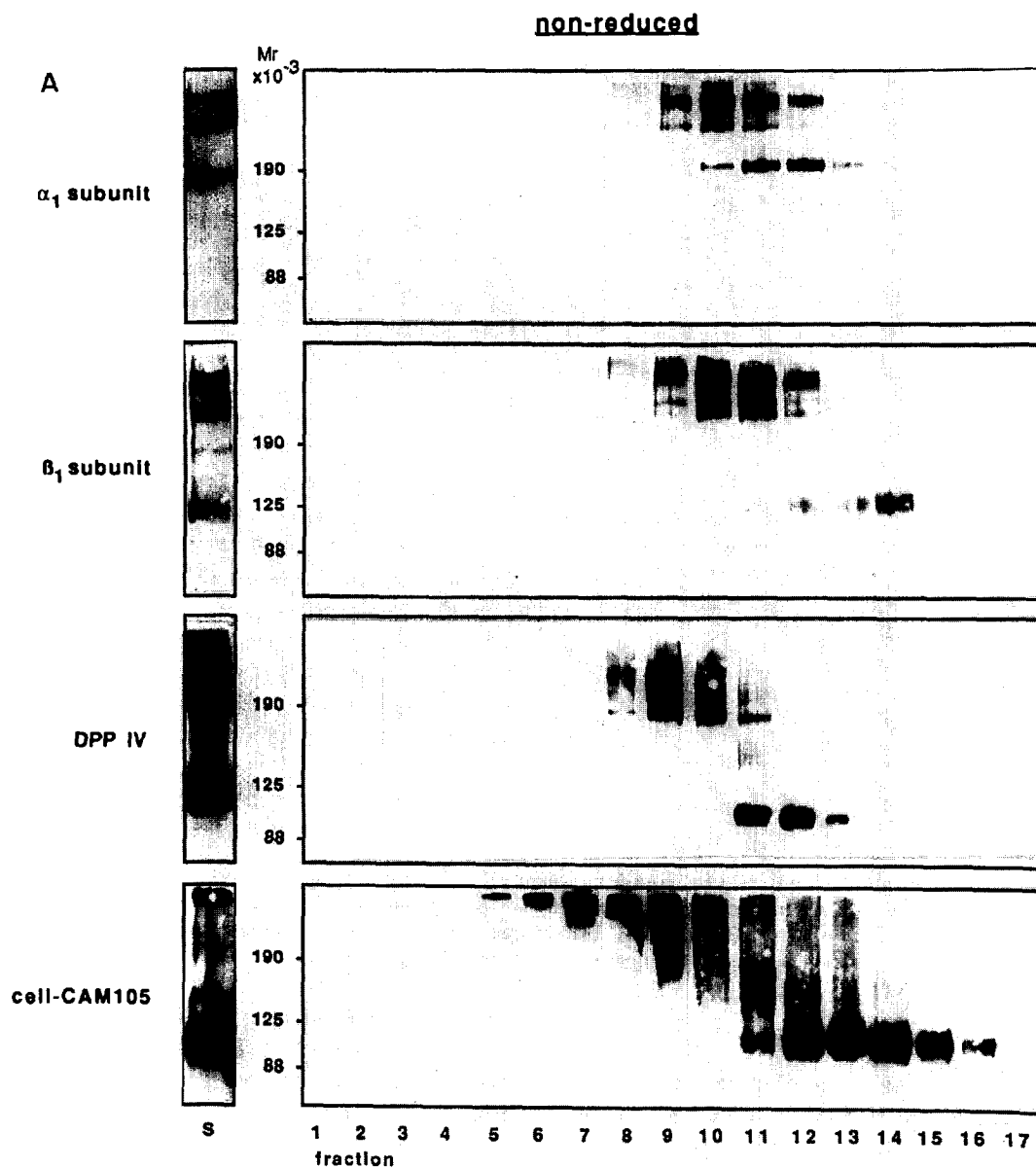
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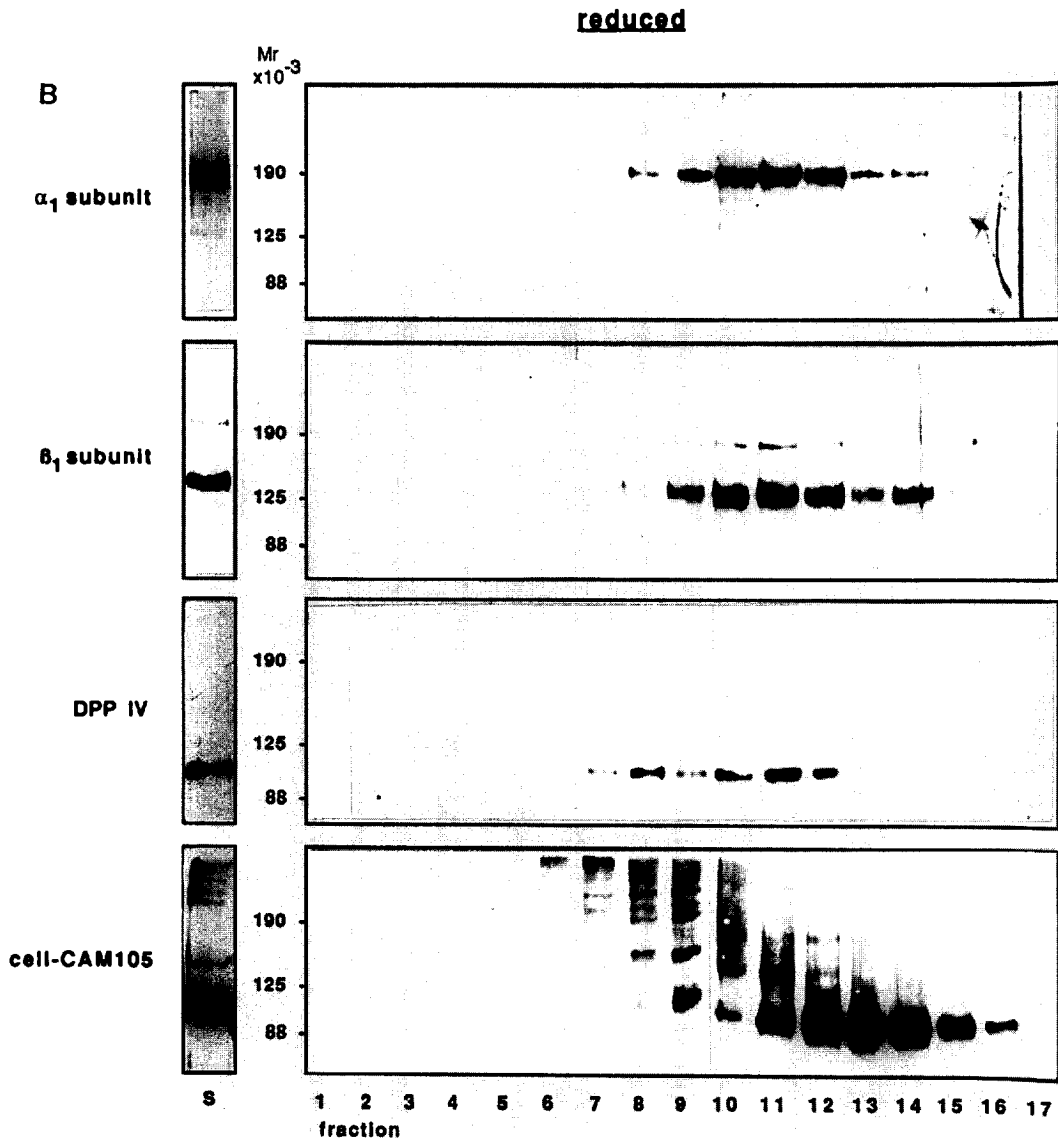

Fig. 6. (continued on next page).

Fig. 6. Immunoblot analysis of fractions collected from denaturating SEC after cross-linking with DSP. A 500-μg amount of 0.3% (w/v) Chaps-solubilized rat liver plasma membrane proteins (S) was cross-linked with DSP, denatured in 1% (w/v) SDS with boiling and separated as described under Experimental. Elution fractions of 250 μl were collected and analysed as in Fig. 2. SDS-PAGE was performed under non-reducing (A, non-reduced) and reducing (B, reduced) conditions.

4. Discussion

Membrane proteins as amphiphilic molecules are immerged with their hydrophobic portions in cellular membranes. During early steps of purification, these properties require detergents to liberate the proteins from the partially large lipid portions of membrane vesicles. Based on mild extraction methods [22], different approaches have been developed to investigate protein aggregates [4–6]. However, the applications of these methods are more or less limited by the detergents which often destroy intra- and intermolecular complexes of proteins or induce for-

mation of artificial protein aggregates interfering with the separation.

In the present investigation we characterized protein complexes of $\alpha_1\beta_1$-integrin, DPP IV and cell-CAM 105, three integral membrane proteins of rat liver as model molecules mainly by two experimental approaches: non-denaturating SEC and a combination of denaturating SEC and chemical cross-linking.

In a pilot study for non-denaturating SEC, three detergents that differ in their critical micellar concentration (CMC) and aggregation number were examined concerning their ability to solubilize the α_1-integrin subunit out of plasma membranes. This was done to minimize possible detergent influences on non-denaturating SEC. Triton X-100, a very popular non-ionic detergent with polyoxyethylene structure, showed to be the strongest detergent with a concentration of 0.1% (w/v) for sufficient $\alpha_1\beta_1$-integrin extraction. However, this value is nearly ten-fold higher than the CMC of Triton X-100 (0.012–0.056%, w/v). In addition, the micelles of Triton X-100 are approximately 63 000–97 000 M_r large, making this detergent not suitable for SEC. In contrast, the alkylglucoside octylglucoside (CMC of 0.29–0.58%, approximate M_r of micelles 24 000) and the ionic bile acid derivate Chaps (CMC of 0.37–0.62%, approximate M_r of micelles 2500–8600) proved to be more suitable detergents for SEC because of the proper ratios of the solubilization concentration and CMC.

Beside the soluble portion of $\alpha_1\beta_1$-integrin, a significant quantity of this protein remained always insoluble. This variable solubilization behaviour might be caused by strong interactions of different subpopulations of $\alpha_1\beta_1$-integrin molecules with membrane/cytoskeletal proteins which are not accessible to the applied detergents as had been shown for the β subunit of integrins which is linked to the cytoskeleton via α-actinin/talin/vinculin [11,12].

Non-denaturating SEC of solubilized membrane proteins was strongly influenced by octylglucoside. All membrane proteins investigated, $\alpha_1\beta_1$-integrin, DPP IV and cell-CAM 105, were detected in almost the same fractions covering a broad M_r range including the exclusion volume

of the SEC columns. This was surprising, because comparison of two identical runs with octylglucoside and Chaps revealed no influences on the separation of standard proteins. Presumably, the insufficient separation of membrane proteins was caused by artificial protein/detergent micelles which had been formed during protein solubilization even though octylglucoside was used in a modest concentration. Moreover, the separation of the two 5000 M_r differing DPP IV variants indicates additional (detergent-induced) interactions between protein and column support. In contrast, non-denaturing SEC with Chaps as detergent for extraction and as additive in the mobile phase enabled the resolution of monomeric and associated forms of $\alpha_1\beta_1$-integrin and DPP IV. Beside the appearance of heterodimeric $\alpha_1\beta_1$-integrin molecules, the chromatographic separation refers to α_1 subunit molecules assembled with proteins other than their common counterpart, the β_1 chain [2] which seem to be larger than the $\alpha_1\beta_1$-integrin heterodimer. The elution pattern of cell-CAM 105 also supports the existence of complexes but their composition is still unknown.

The same samples of solubilized membrane proteins used for non-denaturating SEC were also separated after denaturation with SDS/boiling and with SDS in the mobile phase [9]. This control should convert all protein aggregates into single components. Indeed, after denaturation, all model proteins could be separated into their monomers as shown by the distribution of the 190 000 M_r α_1-integrin subunit separated from the 130 000 M_r β_1-integrin subunit and the 105 000 M_r proteins DPP IV and cell-CAM 105 in SDS-PAGE. Moreover, some high-molecular-mass variants of cell-CAM 105 were separated from the major 105 000 form. Because these variants were only observed after sample denaturation, they must be tightly associated with the plasma membrane or to each other forming insoluble complexes. These results agree with other biochemical investigations [23].

To summarize, the results of non-denaturing chromatography show that this method is principally applicable to the characterization of membrane protein complexes. A careful choice of

detergents is important to minimize interference of protein–detergent complexes and interactions with the chromatographic support. Another disadvantage concerns the specific detection of proteins present as aggregates. They can only be indirectly monitored by comparison of the elution time of selected proteins and marker proteins since subsequent SDS-PAGE–immunoblotting procedures for protein detection induce the dissociation of all aggregates into their single components.

To overcome these difficulties, samples of membrane proteins were examined for the presence of protein aggregates after chemical cross-linking. This approach is based on the performance of bifunctional reagents able to fix covalently near-neighbour relationships and molecular associations present in native protein complexes after mild detergent extraction and intact membrane vesicles as well [7,24]. The generated complexes are stable against denaturation, therefore easy separable from single (covalently modified) molecules and non-cross-linked protein aggregates. They should be susceptible to separation according to their size by denaturing SEC which is not influenced by non-specific protein–protein and protein–carrier interactions [9], a main disadvantage of non-denaturing SEC. Moreover, the separation of protein aggregates during SEC can be directly monitored by the use of SDS-PAGE–immunoblotting.

Cross-linking experiments of detergent extracts with DSP generated in the case of the α_1-integrin subunit two additional high-molecular-mass aggregates. The 290 000 M_r band corresponds to the cross-linked dimer of the α_1- and β_1-integrin subunit (M_r of 190 000 and 130 000, respectively) [10], whereas the 240 000 M_r band suggests to be a cross-linked complex consisting of the α_1-integrin subunit and other protein(s) of unknown composition. Whether this component corresponds to the 50 000 M_r integrin-associated protein assembled with the $\alpha_V \beta_3$-integrin [25] remains to be answered. The formation of the 240 000 and 290 000 M_r bands during cross-linking was independent of the use of Triton X-100, Chaps or octylglucoside and, as shown by cross-linking of intact plasma membrane vesicles, not a

detergent-induced effect. Some additional bands with M_r larger than 290 000 resulted probably from cross-linking with proteins only associated with the α_1-integrin subunit in the intact plasma membrane. However, all complexes could be converted into their single components after treatment with the reducing agent dithiothreitol confirming the reversible character of DSP cross-linkings.

The application of denaturing SEC enabled the separation of cross-linked protein aggregates as well as monomers of $\alpha_1 \beta_1$-integrin, DPP IV and cell-CAM 105. All aggregates could be directly visualized by SDS-PAGE–immunoblotting. Beside the cross-linked 240 000 and 290 000 M_r forms of $\alpha_1 \beta_1$-integrin, DPP IV was cross-linked and separated as a dimer of approximately 210 000 M_r [13] and at least two aggregates with larger M_r. The cross-linked aggregates of cell-CAM 105 seem to consist of a very heterogenous composition covering a broad M_r range.

Taken together, the combination of chemical cross-linking with denaturing SEC offers a suitable approach to analyse multicomponent protein aggregates as present in vivo, not only in plasma membranes of cells, but also in organelles and cytosol. By variation of the applied protein amount and buffer conditions, a better resolution of protein aggregates should be achieved. Because denaturing SEC enables reproducible separations, the desired protein aggregates can also be prepared in larger quantities by multiple chromatographic runs. In combination with immunopurification techniques, further analysis of separated aggregates should give opportunities to characterize or isolate the single components of these protein complexes.

Acknowledgements

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