Concanavalin A binding to human erythrocytes leads to alterations in properties of the membrane skeleton

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Three properties related to the erythrocyte membrane skeleton are found to be altered after the binding of concanavalin A (Con A) to erythrocytes or their isolated membranes. (i) Con A binding to normal erythrocytes imparts resistance to heat (49 °C)-induced fragmentation of the cells. The fragmentation, due to denaturation of spectrin at 49 °C, is prevented by Con A in a dose-dependent manner, but levels off at concentrations of Con A in excess of 100 µg/ml. (ii) The binding of Con A to ghosts isolated from normal, trypsin- or Pronase-treated cells prevents (completely or substantially) the elution of the skeletal protein complex when the membranes are extracted under low-ionic-strength conditions in the cold. (iii) The Con A-agglutinated membranes of trypsin- and Pronase-treated, but not normal, cells show cross-linking of skeletal proteins and band 3 with dimethyl adipimidate, a 0.86 nm (8.6 Å)-span bifunctional reagent. The extent of cross-linking is greater in the Pronase-treated membrane than in the less-agglutinable trypsin-treated membranes. The results show that, after Con A has bound, rearrangements occur in the membrane that alter properties of the skeletal proteins. Additionally, redistribution of the skeletal proteins and the Con A receptor occurs in the lectin-agglutinated membranes.

INTRODUCTION

The binding of a ligand to its receptor on the cell surface often brings about an interaction between the receptor and cytoskeletal elements (especially microfilaments) in the cytoplasm (see refs. [1,2]). Subsequent to the interaction, the ligand–receptor complexes and the microfilaments undergo aggregation, aided by the contractile filaments. In recent years the erythrocyte-type membrane skeleton has been found in many eukaryotic cells and may constitute a universal feature of these cells [3,4]. Several instances of ligand-induced binding of the receptor to the membrane skeleton, and the co-aggregation of ligand–receptor complexes and skeletal components (spectrin- and ankyrin-type proteins) have been reported [5–9]. In erythrocytes the clustering of glycophorin and intramembranous particles together with spectrin has been known for a long time [10,11]. The binding of RCA to the erythrocyte membrane has been found to bring about rearrangement of the skeletal proteins spectrin and band 4.1 [12]. Binding of WGA or Con A to erythrocytes has been reported to confer resistance to shape changes in response to exogenous agents [13,14]. Since the membrane skeleton is generally accepted, in conjunction with phospholipids, to be responsible for the shape of the cells [15], the binding of the lectins apparently induces an alteration in the arrangement/properties of the skeleton or its components.

Vesicles free of membrane skeletal proteins isolated from normal and proteinase-treated erythrocytes failed to be agglutinated by Con A (the preceding paper [16]). This was despite the fact that (i) the proteinase-treated

cells are agglutinated by Con A, (ii) the vesicles bind the lectin to a similar extent as intact cells, and (iii) WGA and RCA are able to agglutinate the vesicles very well. The loss of Con A-agglutinability of the vesicles thus was a specific deficit of the vesicles, possibly attributable to the absence of the skeletal structure. The observation that the Con A-agglutinability of aged cellular remnants, possessing a rigid membrane skeleton, is significantly decreased supports this possibility. This would imply that the skeleton plays a positive role in the process of agglutination by Con A. The possible contractile nature [4] of the membrane skeleton could help in the aggregation of Con A-receptor molecules, in the manner of microfilaments [17].

A small fraction, 10–15%, of the band 3 population is anchored to spectrin via ankyrin [18]; the remaining molecules remain free of any interaction with skeletal proteins. If the membrane skeleton does play a role in band 3 (the Con A receptor) redistribution, as postulated above, Con A binding must first induce an interaction between the receptor and a skeletal component(s). The interaction may be direct or indirect, but as a consequence it may alter the properties of the skeletal proteins themselves. We report here that Con A binding to erythrocytes (i) confers resistance on the cells to heating at 49 °C, the denaturation temperature of spectrin, and (ii) retards the elution of skeletal proteins from the membrane under hypo-osmotic conditions; in addition (iii) the skeletal components and the receptor molecules become cross-linkable by dimethyl adipimidate, a bifunctional reagent. The last-named phenomenon, however, occurs only in the cells that are agglutinated by Con A.

Abbreviations used: Con A, concanavalin A; RCA, *Ricinus communis* (castor bean) agglutinin; WGA, wheat-germ agglutinin; PMSF, phenylmethanesulphonyl fluoride; TBS, Tris-buffered saline (0.01 M-Tris/HCl, pH 7.4, containing 0.15 M-NaCl).

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MATERIALS AND METHODS

Materials

The reagents used and their sources were listed in the preceding paper [16], except the following: *o*-phenanthroline, dimethyl pimelimidate, dimethyl suberimidate, dimethyl adipimidate, *N*-ethylmaleimide and PMSF, all of which were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Cells

The collection of blood, washing of cells, their treatment with proteinases and ghost preparation were described in the preceding paper [16].

Heat-induced vesiculation of erythrocytes

Portions (1 ml) of the cell suspension (10% haematocrit) containing different concentrations of Con A ranging from 0 to 250 $\mu\text{g/ml}$ were incubated at 37 °C for 60 min. A control, consisting of Con A (250 $\mu\text{g/ml}$) and 0.1 M-MeMan, was also included (with appropriate adjustments in the buffer to compensate for changes in the osmolarity of the solution). The suspensions were then heated for exactly 10 min at 49 °C. The tubes were immediately cooled in an ice bath and the suspensions were centrifuged at 650 g for 5 min (twice) to sediment the intact cells. The speed and time of centrifugation was chosen so as to sediment all intact cells. The supernatants were transferred to other tubes and centrifuged at 22000 g for 10 min. The pellets were then suspended in 1.0 ml of TBS and the A_{340} was measured; 340 nm was chosen because the vesicles showed maximum absorption at this wavelength.

Extraction and fractionation of membrane skeletal components

The procedure described by Gratzer [19] was followed. Membranes [10% (v/v) suspension in TBS] were treated with 100 μg Con A/ml at 37 °C for 60 min. They were washed once in the cold with 1 mM-Tris/HCl, pH 8.0, containing 0.1 mM-EDTA and 0.1 mM-PMSF. Thereafter the temperature was maintained at 4 °C or below throughout. The ghosts, without or after Con A treatment, were mixed with an equal volume of the buffer and dialysed against it for 20–24 h. The membranes were then sedimented at 150000 g for 60 min in an ultracentrifuge. The supernatants were individually fractionated on Sepharose 4B columns (38 cm $\times$ 1 cm) at a flow rate of 5 ml/h using TBS (with 0.01% NaN_3). The columns had previously been equilibrated with the same buffer. Fractions (1 ml each) were collected and monitored at 280 nm.

Cross-linking with bifunctional reagents

Ghosts prepared from normal and proteinase-treated cells, either untreated or after incubation with Con A (100 $\mu\text{g/ml}$) for 60 min at 37 °C, were treated with the following cross-linking reagents: Cu^{2+} /phenanthroline (4/20, 10/50, 20/100, 30/150 and 40/200 μM), dimethyl adipimidate (0.5 and 1.0 mg/ml), dimethyl pimelimidate (0.5 and 1.0 mg/ml) and diethyl suberimidate (0.5 and 1.0 mg/ml). Ghosts suspended in TBS (10%, v/v) were treated with the cross-linking reagent for 60 min at room temperature. The reaction was terminated with *N*-ethylmaleimide (0.4 mg/ml) (for Cu^{2+} /phenanthroline) or 0.05 M-ammonium acetate (for the imido esters). The

membranes were pelleted at 22000 g for 15 min in the cold and washed once in 0.01 M-Tris/HCl, pH 7.4. The cross-linked ghosts were taken up in 1% SDS and proteins were analysed on SDS/polyacrylamide-gel electrophoresis [20], 2.5–14% acrylamide gradient gels being used.

Protein was determined by the method of Lowry *et al.* [21], with bovine serum albumin as the standard.

RESULTS

Con A binding and resistance to heat fragmentation

Normal erythrocytes incubated with Con A (0–250 $\mu\text{g/ml}$) and subsequently heated at 49 °C, the denaturation temperature of spectrin, were found to be protected from fragmentation in a manner proportional to the concentration of Con A. However, at Con A concentrations above 100 $\mu\text{g/ml}$ the increase was marginal (Fig. 1). At a Con A concentration of 100 $\mu\text{g/ml}$ the average protection was 56%, and at 250 $\mu\text{g/ml}$ it was approx. 65%. The cells treated with Con A in the presence of MeMan were not protected, indicating that the protective effect is indeed due to Con A.

Proteinase-treated cells were also protected by Con A, but the results were complicated by the agglutination (and also some haemolysis) of cells.

Con A binding and extraction of skeletal proteins

The treatment of membranes (from normal, trypsin- or Pronase-treated cells) with 1 mM-Tris/HCl in the cold

Fig. 1. Effect of Con A binding on heat-induced fragmentation of erythrocytes

Washed normal erythrocytes were suspended in TBS at 10% haematocrit and Con A was added at indicated concentrations. A control with 250 μg of Con A and 0.1 M-MeMan ('+ α -MM') was included. After 60 min at 37 °C, the suspensions were placed at 49 °C for exactly 10 min, and then cooled. Intact cells were removed by centrifugation and the vesicles were pelleted. After washing once, the pellet was resuspended in 1.0 ml and the A_{340} was determined. Each point indicates the average result for three experiments and the vertical bars denote $\pm$ s.d.

Fig. 2. Effect of Con A binding on the elution of membrane skeletal proteins under hyperosmotic conditions

Ghosts from cells (normal, trypsin- or Pronase-digested), either untreated or after treatment with 100 μ g of Con A/ml, were washed and extracted in 1 mM-Tris/HCl, pH 8.0, containing EDTA and PMSF in the cold. After 20 h the membranes were pelleted and the supernatants were fractionated on a column of Sepharose 4B in the cold. Fractions (1 ml each) were collected and their A_{280} was measured. (a) Normal membranes; (b) membranes from Pronase-treated cells; (c) membranes from trypsin-treated cells. The continuous line represents the elution from untreated, and the broken line from Con A-treated, membranes. The small differences in the elution volume of the first peak in (a), (b) and (c) is ascribed to experimental variability.

for a prolonged period, and subsequent fractionation of the extracts on Sepharose 4B, leads to three major peaks (Figs. 2a, 2b and 2c). The peaks correspond to the spectrin-actin-band 4.1 complex, tetrameric spectrin and dimeric spectrin, respectively [22]. When extracted after Con A binding, the first peak (attributable to the complex) was not extracted at all, whereas the elution of the tetrameric and dimeric spectrins remained largely unaffected (Figs. 2a and 2b). Failure to elute the complex was observed in both normal and Pronase-treated membranes (two experiments), whereas, in another experiment, the elution of the complex was inhibited to the extent of 65% in both trypsin-treated (Fig. 2c) and normal (not shown) Con A-treated membranes.

Chemical cross-linking after Con A binding

Cu^{2+} /phenanthroline-, dimethyl pimelimidate- or dimethyl suberimidate-treated normal, trypsin- and Pronase-treated membranes showed no differences on SDS/polyacrylamide-gel electrophoresis either among themselves or when compared with their respective Con A-treated counterparts.

With dimethyl adipimidate, however, interesting differences were observed (Fig. 3). The normal, trypsin-treated and Pronase-digested cell membranes showed no evidence of cross-linking of proteins. The normal ghosts after Con A binding also failed to show any cross-linking. However, the Con A-treated ghosts from trypsin- and Pronase-treated cells revealed a decrease in the amounts of bands 1, 2, 3 (or its Pronase-cleaved fragment; Fig. 3, arrow), 4.1, 4.2, 5 and 6 (lanes d and f) compared with

Fig. 3. Cross-linking of membrane proteins by dimethyl adipimidate

Ghosts, either untreated or after treatment with 100 μ g of Con A/ml, were treated with dimethyl adipimidate (1 mg/ml) for 90 min. After washing, the membranes were dissolved in the sample buffer and exactly 100 μ g of protein was applied per lane on a 2.5–14% (w/v) polyacrylamide gradient gel. Electrophoresis was performed as described by Laemmli [20], and the gel was stained with Coomassie Blue. Lane a, normal ghosts; b, Con A-treated normal ghosts; c, ghosts from trypsin-treated cells; d, ghosts from trypsin-treated cells treated with Con A; e, ghosts from Pronase-treated cells; and f, ghosts from Pronase-digested cells treated with Con A. The band numbers on the left identify the proteins.

the corresponding membranes not treated with Con A (lanes c and e). In the case of Con A-treated membranes, the decrease in the intensities of bands was much greater in the Pronase-treated sample than in the trypsin-treated one. The cross-linking of band 4.5, however, appeared unaffected after treatment of trypsin-treated membrane with Con A. This band is superimposed on the band 3 degradation fragment in the Pronase-treated membranes.

Since in all lanes in Fig. 3 the same amount of protein was applied, the decrease in the staining intensity of bands in Con A-agglutinated (i.e. trypsin- and Pronase-treated) membranes means that larger-sized aggregates of proteins were formed which could not enter the gel (the acrylamide concentration at the top of the gel was 2.5%). Thus the molecular mass of the cross-linked product must be greater than 2 MDa [23].

DMA cross-linking has been reported to be broken by ammoniolysis [24]. However, in our experience this did not appear to be a suitable procedure, since even normal ghost proteins were degraded, altering their positions on the gel.

DISCUSSION

The binding of Con A to normal erythrocytes is found to impart resistance to heat-fragmentation of the cells. Since the fragmentation occurs at the denaturation temperature (49 °C) of spectrin [25], it is obvious that, after the binding of Con A to its receptor, an alteration occurs in the physical properties of this protein. Further, more direct, evidence of Con A-induced change in the interactions of skeletal proteins with other components of the membrane is provided by the studies involving extraction of the spectrin-actin complex from the membrane under hypo-osmotic conditions. After the interaction between the membrane (of normal, trypsin- or Pronase-treated cells) and Con A, the high-molecular-mass complex is not eluted (or is eluted in much smaller amounts) by hypo-osmotic extraction. Either the linkage between spectrin and ankyrin [4] is no longer susceptible or the partial unfolding of spectrin [26] does not occur in the hypo-osmotic buffer; moreover, new linkages may be established such as to prevent the release of the complex from the membrane. The elution of tetrameric and dimeric spectrins is not affected by Con A binding. (For reasons that are not clear, we always obtain larger amounts of spectrin dimers than tetramers.) We have not specifically looked for free actin in the column effluents. The results provide strong evidence for a rearrangement in membrane components after Con A binding.

Cross-linking of erythrocyte membrane proteins with dimethyl adipimidate, but not with the other reagents used, produces high-molecular-mass complexes in Con A-treated trypsin- and Pronase-treated ghosts. The decreased amounts of bands 1, 2, 3 (or its degradation product in Pronase-digested cells), 4.1, 4.2, 5 and 6 (but apparently not 4.5; cf. lanes c and d, Fig. 3), indicate that the skeletal proteins, the Con A receptor and the proteins associated with it (bands 4.2 and 6), are cross-linked. Most significantly, no cross-linking of proteins occurred in the case of membranes from Con A-treated normal cells. Thus the cross-linking of proteins occurs only in the Con A-agglutinable membranes. Also, the extent of cross-linking is apparently less in trypsin-treated as compared with Pronase-treated

membranes. Thus the dimethyl adipimidate-cross-linking of membrane proteins appears to follow the same pattern as the Con A-agglutination of cells. This indicates that, in the membranes of the agglutinated cells, the skeletal proteins and the receptor undergo a spatial redistribution so that they become cross-linkable by a bifunctional reagent with a span of 0.86 nm (8.6 Å). This is suggestive of aggregation of the skeletal proteins and the receptor after Con A binding to the proteinase-treated cells.

The cross-linking of spectrin and band 3 fragment by dimethyl adipimidate in Con A- and Pronase-treated erythrocyte membrane, and the lack of it in Con A-treated normal membrane, was previously reported by Wise *et al.* [27]. Our results thus confirm their observations and extend them by the finding of intermediate cross-linking in the lesser-agglutinable trypsin-treated cells.

Since Con A binding to normal erythrocytes leads to resistance to heat denaturation and to the lack of elution of the skeletal components under hypo-osmotic conditions, the lectin-induced, receptor-mediated, effects appear to be a normal consequence in the erythrocyte. Aggregation follows only in proteinase-treated cells. It is likely that the receptor-associated glycophorin may prevent the aggregation (detected by dimethyl adipimidate-cross-linking) of the receptor molecules and (the directly or indirectly linked) skeletal proteins due to charge-charge repulsion. The degradation of glycophorin by proteinases would remove this hindrance, permitting the aggregation of the lectin-receptor complexes. The formation of aggregates would then permit the binding of cells through multiple cross-bridges, leading to shear-resistant agglutination [28].

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