

BBA 72188

SOLUBILISATION OF HUMAN ERYTHROCYTE BAND 4.1 PROTEIN IN THE NON-IONIC DETERGENT TWEEN 20

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(Received February 2nd, 1984)

(Revised manuscript received May 3rd, 1984)

Key words: Band 4.1; Spectrin; Tween 20; Cytoskeleton; Glycophorin; (Erythrocyte membrane)

Extraction of spectrin-depleted erythrocyte membranes with the non-ionic detergent Tween 20, in a 0.1 M glycine-NaOH buffer (pH 9.8) leads to the solubilization of band 4.1 and the sialoglycoproteins. The comigration of band 4.1 with the sialoglycoproteins in gel filtration and detergent-free electrophoresis indicated that these proteins may be associated as complexes of high molecular weight. Although treatment of intact membranes with Tween 20 under the same conditions does not lead to direct solubilization of proteins, severe disruption of the membranes was observed under phase contrast microscopy. Suspension of the treated membranes in 5 mM phosphate buffer (pH 8.0) leads to the solubilization of band 4.1, spectrin, actin and the sialoglycoproteins. High molecular weight complexes of band 4.1 and the sialoglycoproteins were isolated from these extracts, suggesting a possible interaction between band 4.1 and sialoglycoproteins which may be important for linking the cytoskeleton to the membrane.

Introduction

Low-ionic strength incubation of erythrocyte membranes releases the cytoskeletal proteins spectrin and actin [1]. In addition, approx. 20% of the total band 4.1 protein present on the red cell membrane is released by low ionic strength extraction [2] while the rest remains with the membrane residue. Band 4.1 is believed to be an essential component of the erythrocyte cytoskeleton [3–6], and has been implicated in potentiating the interaction between spectrin and actin [7]. In addition, its affinity for both spectrin and the membrane suggests a possible role for this protein in anchoring the cytoskeleton to the membrane.

Liljas et al. [8] found the non-ionic detergent, Tween 20, could bring about some degree of selective solubilisation of band 4.1 from spectrin-de-

pleted membranes. The selectivity and degree of solubilisation of membrane proteins was dependent on pH, ionic strength and detergent concentration. The sialoglycoproteins, in particular, glycophorin A, were also readily solubilised from spectrin-depleted membranes with Tween 20 [9].

The minor sialoglycoprotein, glycophorin C, has been implicated in association with the erythrocyte membrane cytoskeleton, possibly through interaction with band 4.1 protein [10,11]. Glycophorin C is a transmembrane sialoglycoprotein which migrates in the 'PAS 2' zone of SDS-polyacrylamide gels [10,12]. Incubation of erythrocyte membranes with low concentrations of Triton X-100 releases all the sialoglycoproteins except glycophorin C, which remains associated with the cytoskeleton [10].

The present study examines the effects of the non-ionic detergent Tween 20 on whole erythrocyte membranes, as well as on spectrin-depleted

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membrane vesicles, as a means of exploring the possible interactions between band 4.1 protein and the glycoporphins.

Materials and Methods

Preparation of erythrocyte membranes. Fresh human packed cells in citrate anticoagulant were obtained from the Blood Transfusion Service, Sydney. The red blood cells were washed twice by suspension in 5 mM sodium phosphate buffer, (pH 8.0) containing 0.95% NaCl, and were collected by centrifugation for 10 min at $1000 \times g$.

The washed cells were then hemolysed in 20 volumes of 5 mM sodium phosphate buffer (pH 8.0) and the membranes were collected by centrifugation at $15\,000 \times g$ for 20 min. The membranes were washed repeatedly in the phosphate buffer until free of hemoglobin.

Care was taken to remove the buffy coat during the initial washing of the cells, and the leucocyte pellet during hemolysis, in order to minimise proteolysis [1]. All stages of membrane preparation were carried out at 4°C .

Preparation of spectrin-depleted vesicles. The membranes were diluted with 10 volumes of distilled water containing 0.3 mM phenylmethylsulphonyl fluoride. The membrane suspension was then incubated in a 37°C water bath for 30 min. The water-soluble proteins were removed by centrifugation at $27\,000 \times g$ for 30 min at 4°C , leaving the spectrin-depleted membranes in the pellet fraction.

Preparation of Tween 20-extracted proteins. Essentially, the procedure of Liljas et al. [8] was used, with minor alterations. Equal volumes of spectrin-depleted membranes and cold 0.2 M glycine-NaOH buffer (pH 9.8) containing 2% Tween 20, were incubated on ice for 60 min. The solubilised proteins were removed by centrifugation at $60\,000 \times g$ for 60 min at 5°C .

A slightly different procedure was employed for the solubilisation of proteins from intact membranes using buffers containing Tween 20. Equal volumes of packed membranes and cold 0.2 M glycine-NaOH buffer (pH 9.8) containing 2% Tween 20 and 180 mM NaCl were incubated on ice for 60 min. After incubation, the membranes were collected by centrifugation at $60\,000 \times g$ for

60 min at 5°C , and were resuspended in 5 mM sodium phosphate buffer, pH 8.0. The membranes were vesiculated by passage through a 26 gauge needle, 2 or 3 times, and the vesicles were centrifuged at $120\,000 \times g$ for 120 min at 5°C to yield a clear supernatant fraction containing solubilised proteins.

Sephacrose 4-B chromatography. Proteins solubilised from spectrin-depleted membranes with Tween 20 were concentrated to approx. 5 ml by dialysis against Aquacide II and were fractionated in the cold by gel filtration on a Sepharose 4-B column (2.4×41.5 cm) equilibrated either with 10 mM sodium phosphate buffer (pH 8.0) or with a similar buffer containing 0.1 M NaCl, 5 mM EDTA and 0.1 mM dithiothreitol. The column was eluted at a flow rate of 30 ml/h, 2.3 ml fractions were collected, and the protein concentration estimated from the absorbance at 280 nm.

Ion-exchange chromatography. Protein solutions, extensively dialysed against 7.5 mM sodium phosphate buffer (pH 8.0) were applied to a column of DEAE-Sephadex A-50 (1×10 cm) equilibrated in the same buffer. The column was then eluted at a flow rate of 40 ml/h with 40 ml of the same buffer, followed by two steps of 40 ml each of the same buffer containing 100 mM NaCl and 200 mM NaCl, respectively, at a flow rate of 66 ml/h.

Con A-Sepharose 4-B affinity chromatography. Protein samples were concentrated to approx. 2 ml by dialysis against Aquacide II, dialysed against 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, and applied to a column of Con A-Sepharose 4-B (1.2×5.8 cm) in the same buffer. After washing with 20 ml of buffer to remove any unbound proteins, bound proteins were eluted at a flow rate of 20 ml/h with 20 ml of the same buffer containing 0.1 M α -methyl-D-mannoside.

Extraction of glycoporphin C. Sialoglycoproteins were removed from intact ghosts by the method of Owens et al. [10], with slight modifications. Equal volumes of packed ghosts and 15 mM sodium phosphate buffer (pH 7.4) containing 2 mM dithiothreitol and 0.2% Triton X-100 were mixed on ice and were immediately centrifuged at $27\,000 \times g$ for 30 min at 4°C . The supernatant fraction contained most the sialoglycoproteins. The pellet, enriched in glycoporphin C, was washed once with 5–10 volumes of 7.5 mM sodium phosphate buffer

(pH 7.5) containing 1 mM dithiothreitol and 0.1% Triton X-100 and twice more in the same buffer but without Triton X-100. After each wash, the membranes were collected by centrifugation at $27\,000 \times g$ for 30 min at 4°C.

The residue after Triton X-100 extraction was incubated on ice for 60 min with an equal volume of 0.2 M glycine-NaOH buffer (pH 9.8) containing 2% Tween 20. After incubation, the residue was centrifuged at $60\,000 \times g$ for 60 min at 5°C. The Tween 20-solubilised proteins containing glycoporphin C were collected in the supernatant fraction.

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed in 5.6% polyacrylamide gels containing 0.2% sodium dodecyl sulphate (SDS) as described by Fairbanks et al. [1] and modified by Steck [13]. In some experiments, electrophoresis was performed on polyacrylamide gradient slab gels (3–26%), which were equilibrated with 0.375 M Tris-HCl buffer (pH 8.8) containing 0.1% SDS [14]. Samples for electrophoresis were dialysed overnight against 0.0625 M Tris-HCl buffer (pH 6.8) in the cold. Electrophoresis was then performed at 75 V (about 25 mA) for approx. 7 h at room temperature. The electrode buffer was 0.025 M Tris, 0.192 M glycine (pH 8.3) containing 0.1% SDS, and was constantly stirred to prevent pH changes caused by electrolysis.

Disc gel electrophoresis in the absence of detergents was carried out in the cold on 4% polyacrylamide gels with the use of a discontinuous buffer system as described by Davis [15], but without the use of spacer gels. The separation gel buffer was 0.375 M Tris-HCl (pH 8.9) and the electrode buffer was 0.025 M Tris, 0.19 M glycine (pH 8.3). A few drops of mercaptoethanol were added in the upper electrode buffer.

Acrylamide gradient electrophoresis in the absence of detergents was performed in 3–26% polyacrylamide gradient slab gels [16], for a minimum of 8 h in the cold at 50 V. The electrode and gel buffer was 0.017 M sodium phosphate buffer (pH 7.5) containing 0.13 mM dithiothreitol and 2.4 mM EDTA.

On completion of electrophoresis, gels were stained with Coomassie blue R250 [1]. Staining for glycoproteins on polyacrylamide gels was performed by means of the periodic acid-Schiff stain [1].

Phase contrast microscopy. A Zeiss photomicroscope, with phase contrast illumination and a magnification of 400, was used to view the membranes.

Results

Extraction of band 4.1 from erythrocyte membranes by use of Tween 20

Band 4.1 was highly susceptible to proteolysis. All experimental work on band 4.1 was performed within a few days of its extraction from spectrin-depleted membranes and whole ghosts. Despite measures to control proteolysis, for example, addition of 0.3 mM phenylmethylsulphonyl fluoride to inhibit serine proteases, band 4.1 underwent degradation with time, with a concomitant increase in the amount of lower molecular weight proteins, especially those in the band 4.5 region.

The extraction of band 4.1 was found to be most efficient at pH values above 9, and with solutions of low ionic strength. However, these conditions also led to the solubilisation of band 3 and the sialoglycoproteins. At pH 9.8, the selectivity of band 4.1 extraction could be controlled by manipulation of ionic strength. At an ionic strength of 0.05 M, optimal extraction of band 4.1 was achieved, with good yields and with minimal contamination by other proteins. Routine extraction procedures, therefore, employed a solution of 1% Tween 20 in a 0.1 M glycine-NaOH buffer (pH 9.8). An incubation time of 60 min was found to be adequate for optimal solubilisation of band 4.1. Prolonged incubation increased yields only marginally, and led to increased proteolysis.

Extraction of whole ghosts with Tween 20

Incubation of whole ghosts with Tween-20 solutions yielded very little soluble protein. In 0.1 M glycine-NaOH (pH 9.8) traces of spectrin, band 4.1 and low molecular weight proteins were solubilized. At lower ionic strength, band 3 was also partially solubilized, while on increasing the ionic strength by addition of NaCl, protein solubilization was reduced, being almost completely abolished with 90 mM NaCl.

In spite of the lack of protein solubilization, 1% Tween 20 in 0.1 M glycine-NaOH buffer (pH 9.8) caused considerable fragmentation of the mem-

branes, as detected by means of phase contrast microscopy. The degree of fragmentation was greater at room temperature than in the cold. Fragmentation was largely prevented by the addition of 90 mM NaCl to the Tween solution. However, even in the presence of the added salt, there were obvious signs of membrane perturbation in the odd shapes of the ghosts. Over a period of 2 h, some external budding became apparent.

This behaviour suggested that band 4.1 may have been partially displaced from the membrane allowing some dissociation of the cytoskeleton and leading to areas of unstable membrane, from which budding could occur. Fragments of the cytoskeleton produced in this way were considered likely to be trapped inside the vesicles which appeared to be largely right-side-out.

In an attempt to bring about inversion of the membranes, the membrane pellet after treatment with Tween was resuspended in 5 mM sodium phosphate buffer (pH 8.0). Vesiculation of the membranes was aided by passing the suspension several times through a 26 gauge needle. Under phase contrast, Tween-treated membranes that had been diluted into the low ionic strength buffer rapidly changed from showing external budding to showing internal budding and vesicle formation. This process of fragmentation was accelerated by the syringe passage.

After centrifugation of the membrane residue, the supernatant solution was found to contain spectrin, actin and band 4.1 (Fig. 1). Approx. 50% of the spectrin, 90% of the actin, and almost all of the band 4.1 had been solubilised. In addition, the periodate-Schiff stain revealed the presence of sialoglycoproteins.

Fractionation of the supernatant protein solution on Sepharose 4-B showed three major peaks (Fig. 2). Similar profiles were obtained when Tween 20 was present in the eluting buffers. SDS-polyacrylamide gel electrophoresis of the proteins in these peaks revealed that the void volume fraction and peak 1 contained spectrin and only traces of band 4.1 while the other protein peak contained band 4.1, the sialoglycoproteins and traces of actin. The relative size of peak 1 was variable, and appeared to depend on the degree of concentration of the sample. In samples that had been concentrated extensively, the void volume peak was

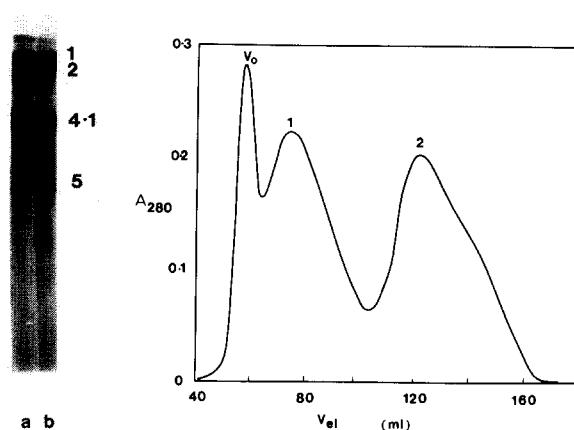


Fig. 1. SDS-polyacrylamide gel electrophoresis of the proteins solubilized after treating whole membranes with Tween 20. Membranes were incubated with 0.1 M glycine-NaOH buffer (pH 9.8)+90 mM NaCl+1% Tween 20, centrifuged at $60\,000\times g$ and resuspended in 5 mM sodium phosphate buffer (pH 8.0). The resuspended membrane residue was vesiculated and centrifuged again at $120\,000\times g$. The supernatant fraction (gel b) contained the solubilized proteins, namely spectrin, band 4.1 and actin. Gel (a) represents whole ghosts.

Fig. 2. Gel filtration on Sepharose 4-B of the proteins extracted from whole membranes by incubation with 0.1 M glycine-NaOH buffer (pH 9.8) containing 1% Tween 20, followed by centrifugation and vesiculation in 5 mM phosphate buffer (pH 8.0). Band 4.1 protein was found predominantly in peak 2.

augmented, while peak 1 was correspondingly reduced in size.

Two-dimensional electrophoresis was performed on the band 4.1-actin-sialoglycoprotein peak from the Sepharose 4-B chromatography of the proteins released after vesiculation of Tween 20-solubilised whole ghosts. First-dimensional electrophoresis in the absence of SDS on 4% polyacrylamide disc gels produced three protein bands (A-C) when stained with Coomassie blue. Each of these 3 bands gave rise to band 4.1 when electrophoresed into a 0.2% SDS, 5.6% polyacrylamide slab gel in the second dimension and stained with Coomassie blue (Fig. 3a). In addition, these bands also contained the sialoglycoproteins (Fig. 3b). Detection of actin was difficult due to the low protein concentration and the tendency for the actin to smear along the length of the first dimension. When second-dimensional electrophoresis was performed on a 3-26% polyacrylamide gradi-

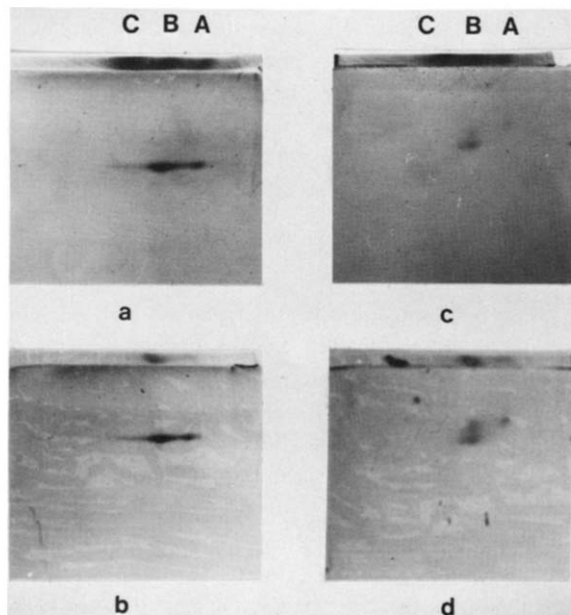


Fig. 3. Two-dimensional gel electrophoresis of peak 2 from the Sepharose 4-B profile. Electrophoresis was performed in the first dimension on 4% acrylamide gels in the absence of detergents. Migration was from right to left. The major zones are marked A, B and C. The second dimension separation was carried on acrylamide slab gels. (a) and (b). 5.6% acrylamide gels containing 0.2% SDS. Staining was with Coomassie blue (gel a), and periodate-Schiff stain (gel b). (c) and (d). 3–26% acrylamide gradient gels in the absence of detergents. Staining was with Coomassie blue (gel c), and periodate-Schiff stain (gel d).

ent slab gel, in the absence of detergent, bands A, B and C gave rise to discrete zones staining with coomassie blue and with the periodate-Schiff stain. Band A corresponded to an apparent molecular weight of $1 \cdot 10^6$ and band B corresponded to an apparent molecular weight of 500 000 when compared to ferritin.

The comigration of band 4.1 protein and the sialoglycoproteins may imply that band 4.1 and the glycoporphins were closely associated with each other, or, less likely, that the two proteins have similar electrophoretic behaviour in both the Davis system and the gradipore gel. No separation of band 4.1 from the sialoglycoproteins could be achieved by means of ion-exchange chromatography or affinity chromatography.

Extraction of band 4.1 from spectrin-depleted membranes

In contrast to the behaviour in whole membranes, proteins were extracted readily from the spectrin-depleted membranes by incubation with 1% Tween 20. Although band 4.1 was the predominant Coomassie blue-staining protein in the extracts, the periodate-Schiff stain revealed the presence of all three major sialoglycoproteins.

Chromatography of the solubilized proteins on Sepharose 4-B, in 10 mM sodium phosphate buffer (pH 8.0) yielded a major protein-containing peak. This peak corresponded to a high apparent molecular weight (approx. 500 000) and was comprised of band 4.1 and sialoglycoproteins. Traces of residual spectrin emerged at the void volume, and variable amounts of low molecular weight material, possibly proteolysis fragments of band 4.1 protein, emerged after the main peak. An additional peak of absorbance, emerging after the main protein peak and partially overlapping it, contained no protein detectable after electrophoresis and was found to be due to the presence of Tween 20. The elution profile was not altered by the inclusion of 1% Tween 20 in the eluting buffers.

Gradipore electrophoresis of both the extracts and the fraction from the Sepharose column showed a smeared Coomassie blue-staining zone of apparent molecular weight approx. 500 000, consistent with the gel filtration behaviour (Fig. 4). This zone also stained with the periodate-Schiff stain, but in addition there were traces of glycoprotein migrating ahead of the main zone.

Electrophoresis of both the extract and the gel filtration peak fraction on 4% polyacrylamide gels in the absence of dodecyl sulphate showed three Coomassie blue-staining zones, similar to those extracted from whole membranes. Second-dimension electrophoresis into a 3–26% gradient acrylamide slab again showed a series of zones of high molecular weight, each of which stained with both coomassie blue and the periodate-Schiff stain, while second-dimension electrophoresis into gels containing dodecyl sulphate revealed in each zone the presence of band 4.1 and the glycoporphins.

Ion-exchange chromatography of the Tween extracts on DEAE-Sephadex A-50, eluted with steps of 100 mM and 200 mM NaCl, showed a single protein peak eluting with 200 mM NaCl. This

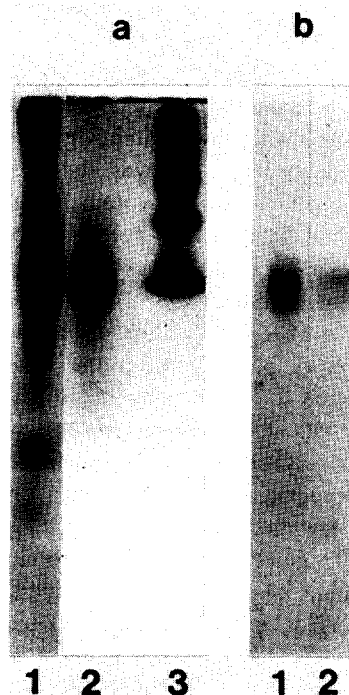


Fig. 4. Acrylamide gradient electrophoresis of: (1) Tween 20-solubilized proteins before Sepharose 4-B chromatography; (2) band 4.1 peak after chromatography; (3) ferritin oligomers, used as size markers. After electrophoresis, the proteins were stained with either: (a) Coomassie blue; or with (b) periodate-Schiff stain. The Coomassie-blue staining protein (band 4.1) and the sialoglycoproteins coelectrophoresed to the same position.

peak was shown by electrophoresis in the presence of dodecyl sulphate to contain both band 4.1 and all the sialoglycoproteins.

Affinity chromatography revealed that the bulk of the extracted protein did not bind to Con A-Sepharose. The unbound protein fraction comprised both band 4.1 and the sialoglycoproteins. A small amount of low molecular weight protein was bound to the affinity column irreversibly and no detectable protein could be eluted with α -methyl-D-mannoside.

Tween extraction of glycophorin-depleted membranes

The sialoglycoproteins, glycophorins A and B, were solubilised from erythrocyte membranes with 0.2% Triton X-100. The membrane residue after

Triton X-100 extraction contained glycophorin C and some residual glycophorin A. Solubilisation of the proteins in the membrane residue with Tween 20 released glycophorin C and some glycophorin A into the supernatant fraction. Band 4.1 was also solubilized by this procedure.

Discussion

The extraction of band 4.1 from spectrin-depleted membranes with Tween 20 was dependent on pH and ionic strength of the buffer. The present results show that Tween 20, at an alkaline pH (9.8) and at a relatively low ionic strength (0.05), could selectively extract band 4.1 from spectrin-depleted membranes. However, under all conditions we examined, sialoglycoproteins, particularly glycophorins A and C, were extracted and consistently copurified with band 4.1. Our results suggest that band 4.1 protein may interact with the glycophorins. Although there are serious difficulties in interpreting the apparent molecular weights of glycoproteins from gel electrophoresis, even with the use of gradient gels, it is clear that the complexes between band 4.1 and the glycophorins can be very large.

Band 4.1 could not be solubilised directly from whole membranes by Tween 20. However, Tween 20 did cause membrane perturbations which were visible under the phase contrast microscope. Vesiculation of Tween 20-treated ghosts, in low ionic strength buffer, resulted in the release of the cytoskeletal proteins, including band 4.1, as well as some of the sialoglycoproteins. It may be argued that the low ionic strength buffer, rather than Tween 20, was responsible for the release of the cytoskeletal proteins. However, although the cytoskeletal proteins are released by low ionic strength extraction [1,2] their solubilisation at low temperature requires prolonged incubation at an ionic strength substantially below 5 mM, while the sialoglycoproteins, which are integral proteins, remain with the membrane under these conditions [1]. The most likely interpretation is that the sialoglycoproteins were released from intact membranes as a result of the action of Tween 20 on the membranes. Presumably the cytoskeleton remains attached to the membrane via spectrin-ankyrin interactions until the ionic strength is lowered.

The appearance of spectrin and band 4.1 in the void volume fraction from the Sepharose 4-B chromatography of the proteins found after Tween 20-solubilisation and vesiculation of whole ghosts is consistent with the known *in vitro* associations between these proteins [17]. This fraction appeared to be a highly aggregated protein complex of spectrin and band 4.1, in a molar ratio of approx. 1 : 1. The absence of significant actin in this fraction is noteworthy, in that actin does not appear to be necessary to cross-link spectrin into high molecular weight complexes. This situation contrasts with the appearance of spectrin and actin in the void volume fraction from water-soluble protein extracts [18].

Non-ionic detergents, such as Tween 20, are relatively mild detergents that do not normally denature proteins [19–21]. Nonionic detergents are efficient in breaking lipid/lipid and lipid/protein interactions but are usually ineffective in breaking protein/protein interactions [22]. However, in the presence of non-ionic detergents, non-specific artifactual aggregation of proteins may occur [22]. Non-specific protein aggregation appears not to be responsible for the observed band 4.1/sialoglycoproteins complex because only band 4.1 was associated with the sialoglycoproteins. Spectrin and actin were also found in the Tween 20 extracts but no association with the sialoglycoproteins was observed. Reproducible two-dimensional gel patterns of the band 4.1/sialoglycoprotein complex from whole ghosts and from spectrin-depleted membranes were also obtained, indicating that the observed complex was specific for band 4.1.

Evidence has been presented recently [23] that band 4.1 retains its ability to bind spectrin and to potentiate the spectrin-actin interaction after solubilization by Tween 20. The direct associations observed in the present study between band 4.1 and spectrin, and band 4.1 and the sialoglycoproteins, indicated that band 4.1 may also act as an attachment protein for the cytoskeleton to the red cell membrane. The present study supports the suggestion that band 4.1 may be associated with glycophorin C which may act as an anchor for the cytoskeleton through its interaction with band 4.1 [10,11]. From two-dimensional gel elec-

trophoresis, glycophorin A also appears to be associated with band 4.1.

Since this paper was submitted for publication, Anderson and Lovrien [24] have shown by direct binding studies that band 4.1 is capable of interaction with glycophorin A. Our conclusions are in agreement with these findings.

Acknowledgement

This work was supported by a grant from the Australian Research Grant Committee.

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