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PROTEOLYTIC CLEAVAGES OF CYTOCHALASIN B BINDING COMPONENTS OF BAND 4.5 PROTEINS OF THE HUMAN RED BLOOD CELL MEMBRANE

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The putative hexose transport component of Band 4.5 protein of the human erythrocyte membrane was covalently photolabelled with [^{3}H]cytochalasin B. Its transmembrane topology was investigated by electrophoretically monitoring the effect of proteinases applied to intact erythrocytes, unsealed ghosts, and a reconstituted system. Band 4.5 was resistant to proteolytic digestion at the extracellular face of the membrane in intact cells at both high and low ionic strengths. Proteolysis at the cytoplasmic face of the membrane in ghosts or reconstituted vesicles resulted in cleavage of the transporter into two membrane-bound fragments, a peptide of about 30 kDa that contained its carbohydrate moiety, and a 20000 kDa nonglycosylated peptide that bore the cytochalasin B label. Because it is produced by a cleavage at the cytoplasmic face and because the carbohydrate moiety is known to be exposed to the outside, the larger fragment must cross the bilayer. It has been reported that the Band 4.5 sugar transporter may be derived from Band 3 peptides by endogenous proteolysis, but the cleavage pattern found in the present study differs markedly from that previously reported for Band 3. Minimization of endogenous proteolysis by use of fresh cells, proteinase inhibitors, immediate use of ghosts and omission of the alkaline wash resulted in no change in the incorporation of [^{3}H]cytochalasin B into Band 4.5, and no labelling of Band 3 polypeptides. These results suggest that the cytochalasin B binding component of Band 4.5 is not the product of proteolytic degradation of a Band 3 component.

Introduction

The transport of glucose across the human erythrocyte membrane has been studied extensively [1,2]. This facilitated diffusion process is inhibited by a variety of protein modifying reagents [3–5], indicating participation of an intrinsic membrane protein. The identity of the transport protein has been the subject of recent controversy. A number of studies, employing immunological methods [6], labelling techniques [7,8], the reconstitution of stereospecific glucose transport activity [9] and binding of the transport inhibitor cytochalasin B [10] have indicated that a protein with a molecular weight of approx. 50000 daltons, designated as 'Band 4.5' in the electrophoretic profile of erythrocyte membrane proteins, is involved in hexose transport. Others, however, have suggested that Band 3, the most abundant protein in the red cell membrane, is the protein responsible for glucose transport in vivo, and that Band 4.5 is a partially active product of endogenous proteolytic degradation of Band 3 that may occur during isolation of the membranes [11,12]. The major

Abbreviations: DFP, diisopropylfluorophosphate; DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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component of Band 3 is the anion transport protein which has been the subject of relatively intensive investigation [13,14].

One of the most useful procedures in elucidating the structural arrangement of the major component of Band 3 in the bilayer has involved the use of proteolytic enzymes applied to the outside or inside faces of the membrane (or both), combined with the use of covalent probes that can 'mark' specific sites in the peptide [13]. By these techniques, proteolytic fragments can be identified and proteolytic cleavage sites and binding sites (for probes) can often be concluded to be exposed at a particular membrane face. These techniques have not been extensively applied to the sugar-transporting component(s). It is clear that one or more proteolytic cleavages must occur because it has been observed that treatment with proteolytic enzymes can result in inhibition of sugar transport [15,16]. Furthermore, the inhibiting cleavage appears to be located at the cytoplasmic side of the membrane because it does not occur in cells treated with proteinases, but does occur if the enzymes are incorporated within resealed ghosts [15] or if they are applied to inside-out vesicles [16]. No particular cleavage product has, however, been reported, probably because of the difficulty of identifying fragments of the sugar-transport protein in the presence of fragments of other membrane proteins, particularly Band 3 which is considerably more abundant.

Recently, Carter-Su et al. [17] and Shanahan [18] have described a method for covalently photolabelling the Band 4.5 protein with the sugar-transport inhibitor, cytochalasin B, providing further evidence concerning the identity of the transporter. In the present paper, covalently bound [^{3}H]cytochalasin B is used to identify a particular Band 4.5 fragment produced by proteolytic cleavage of intact cells, ghosts or purified reconstituted Band 4.5. In the latter preparation, a second fragment was identified that contained the sugar attachment site (Band 4.5 contains about 15% carbohydrate [19]). The implications of the observed cleavage pattern with respect to the transmembrane topology of Band 4.5 and its purported relationship to Band 3 is discussed.

Materials and Methods

Recently outdated blood was used for most experiments but freshly drawn cells were used in particular experiments, as noted. [^{3}H]cytochalasin B (17 Ci/mmol) was obtained from Amersham; NaB^3H_4 (341 mCi/mmol) from New England Nuclear; trypsin (TPCK-treated), chymotrypsin (Type I-S), molecular weight standards (SDS-6 and SDS-7), bovine serum albumin, PMSF, DFP and SDS from Sigma; galactose oxidase from Worthington; and electrophoresis reagents from Bio-Rad. All other chemicals were reagent grade.

Membrane isolation. Human erythrocytes were separated from plasma by centrifugation and washed three times with 5 vol. phosphate-buffered saline (5 mM sodium phosphate (pH 8)/150 mM NaCl), taking care to remove the buffy coat. Ghosts were prepared by lysing washed cells in 15 vol. 5 mM sodium phosphate (pH 8) and washing the membranes in that buffer until they appeared white. In most experiments, ghosts were treated with 0.01 M NaOH/0.1 mM EDTA to remove peripheral proteins [20]. In one set of experiments, however, special precautions were undertaken to minimize endogenous proteolysis, using procedures described by Shelton and Langdon [12]. In this particular case, freshly drawn venous blood was passed through a cellulose column to remove the leukocytes and treated with the proteinase inhibitors, 0.1 mM PMSF and 0.1 mM DFP prior to lysis. Acetylcholinesterase activity of ghosts isolated from these cells was less than 0.4% of that seen with membranes isolated from untreated cells, indicating that reaction of DFP with the cells was successfully accomplished. The ghosts were used immediately, and in some experiments were not subjected to the alkaline wash, reported to enhance endogenous proteolysis [12].

Preparation of reconstituted Band 4.5. Purified, reconstituted Band 4.5 protein was prepared according to Baldwin et al. [21], with some modifications. Alkali-treated ghosts were washed with 20 vol. 50 mM Tris-HCl (pH 7.4) and resuspended in that medium at a concentration of 2 mg protein/ml. 1% β-mercaptoethanol was added. The membranes were extracted with 1% *n*-octyl glucoside, incubated for 20 min at 4°C, and centrifuged at $100\,000 \times g$ for 60 min. The supernatant fluid

(approx. 18 ml) was then applied to a column of DEAE-Sephacel (7 ml in a Bio-Rad disposable Econo-column) equilibrated with 1% octyl glucoside in 50 mM Tris-HCl (pH 7.4) and eluted with that buffer. 2.5-ml fractions were collected, and the protein content of the fractions was measured using the Bio-Rad protein assay. Protein-containing fractions (approx. 20 ml) were collected, made 150 mM NaCl by the addition of 3 M NaCl stock solution, and dialyzed against three changes of 200 vol. phosphate-buffered saline containing 0.1 mM EDTA over a 36-h period. The red cell lipids which co-eluted with the transporter allowed reconstitution of protein-containing vesicles. These were concentrated by centrifugation at $100\,000 \times g$ for 1 h, and resuspended in phosphate-buffered saline at a concentration of 1 mg protein/ml.

The preparation was compared with those previously reported [21] by assessing its reversible binding of [^{3}H]cytochalasin B, using the equilibrium dialysis technique described by Zoccoli et al. [22]. The data was corrected for D-glucose-insensitive binding and subjected to Scatchard plot analysis [23]. The correlation coefficient of the linear plot was high (0.988). The Band 4.5 preparation bound 12.0 nmol cytochalasin B/mg protein (0.55 mol ligand/mol protein of M_r 46 000) with a K_d of $1.57 \cdot 10^{-7}$ M. These values are essentially the same as those reported previously [21] (binding of 12.1 and 14.8 nmol/mg protein, and K_d values of $1.43 \cdot 10^{-7}$ and $1.64 \cdot 10^{-7}$).

Photolabelling of Band 4.5. Previously published methods for specific labelling of Band 4.5 components with [^{3}H]cytochalasin B [17,18] were modified in order to obtain increased yields of labelling (about 8% rather than 1–3%) while minimizing the photodamage which can occur during irradiation. A complete description of the modified procedure is reported separately [24]. Briefly, alkali-stripped ghosts were washed with 20 vol. 0.1 M sodium phosphate (pH 7.5) and resuspended in that buffer at a concentration of 0.5 mg protein/ml. Following addition of 0.5 μM [^{3}H]cytochalasin B, the sample was incubated in the dark for 20 min at 0 °C. The sample (1–3 ml in a quartz spectrophotometer cell) was placed in the sample chamber of an Aminco-Bowman spectrofluorometer (model No. 0223-62155). The excitation slit was removed and the excitation monochromator was set to 280 nm. During irradiation, the temperature of the sample remained below 25 °C. The ghosts were then washed four times with 50 vol. suspending buffer to remove unbound cytochalasin B. The photolabelling of the ghosts was diminished by 78% in the presence of 0.2 M D-glucose, indicating that the predominant labelled species is glucose-sensitive (is the glucose transporter).

The reconstituted Band 4.5 was labelled by similar means. Liposomes containing the 'purified' protein were diluted to 0.5 mg protein/ml with phosphate-buffered saline and 0.5 μM [^{3}H]cytochalasin B was added. Irradiation was carried out as described above. Removal of excess cytochalasin B in this case proved to be unnecessary, as the presence of cytochalasin B did not alter the pattern of cleavage products observed or produce a high level of background radioactivity in electrophoretograms of labelled samples.

Labelling of Band 4.5 carbohydrate. The carbohydrate moiety of the transporter was labelled using the galactose oxidase-$NaBH_4$ procedure described by Gorga et al. [25]. Washed erythrocytes (50% hematocrit) were incubated with galactose oxidase (5 units/ml) for 90 min at 37 °C. After the incubation was completed, the cells were washed twice with 5 vol. phosphate-buffered saline to remove the enzyme, and the oxidized carbohydrates were reduced with 2 mM NaB^3H_4 for 30 min on ice. The cells were then washed again, and the purified transporter was isolated from these cells as described above.

Proteolytic digestion. Proteolysis of the external face of intact, unlabelled erythrocytes was accomplished by incubating washed cells (25% hematocrit in phosphate-buffered saline) with 1 mg/ml trypsin, chymotrypsin or pronase for 90 min at 37 °C. Cleavages at low ionic strength were performed by replacing saline with 5 mM sodium phosphate (pH 8) in 0.3 M sucrose. Ghosts were prepared from the cleaved cells and photolabelled as described above. Labelled ghosts from uncleaved cells were suspended in 0.1 M sodium phosphate (pH 7.5) at a concentration of 0.1 mg protein/ml and incubated with 0.1 mg/ml trypsin or chymotrypsin for 90 min at 37 °C. Proteolysis was terminated by the addition of 50 μg/ml PMSF, an inhibitor of trypsin and chymotrypsin. After cleavage, the

cells or ghosts were washed twice with phosphate-buffered saline or suspending buffer, respectively, containing 0.5% bovine serum albumin in order to scavenge any remaining active proteinases, then twice with the appropriate buffer to remove the albumin.

Proteolysis of reconstituted transporter (0.5 mg protein/ml in phosphate-buffered saline) was accomplished by incubating vesicles with 3 μg/ml trypsin at 25°C for 90 min. Proteolysis was stopped by the addition of 50 μg/ml PMSF, followed by the addition of 2% SDS and 5% β-mercaptoethanol.

Other methods. Following labelling and cleavage, samples were analyzed by SDS-gel electrophoresis according to Fairbanks et al. [26] for ghosts, or Laemmli [27], in the case of reconstituted transporter. After fixing and staining with Coomassie blue, the gel lanes were cut into 3-mm slices and digested with 0.6 ml 30% H_2O_2 for 2 h at 90°C. Radioactivity of the slices was determined by liquid-scintillation counting. Periodate-Schiff's staining of the gels was performed according to Czech and Lynn [28]. Protein concentration was determined by the method of Lowry et al. [29], or with the Bio-Rad protein assay based on the method of Bradford [30] if the samples contained β-mercaptoethanol.

Results

The exterior face of erythrocyte membranes was subjected to proteolytic cleavage by incubating intact erythrocytes with several proteinases. After removal of the proteinases, ghosts were prepared that were then photolabelled with cytochalasin B. As already noted [17,18], the location of the binding component in the electrophoretic system indicates that it is about 50 kDa in size, the Band 4.5 region. As shown in Fig. 1, upper panel, digestion of the cells suspended in phosphate-buffered saline by trypsin, chymotrypsin or pronase neither substantially altered the electrophoretic mobility of the cytochalasin-B-labelled component (approx. M_r 50 kDa), nor decreased the amount of labelled protein. Hence, the cytochalasin-B-labelled component is resistant to proteolysis at the cell's external face. Exposure of cells to the same proteinases at low ionic strength, which enhances proteolytic

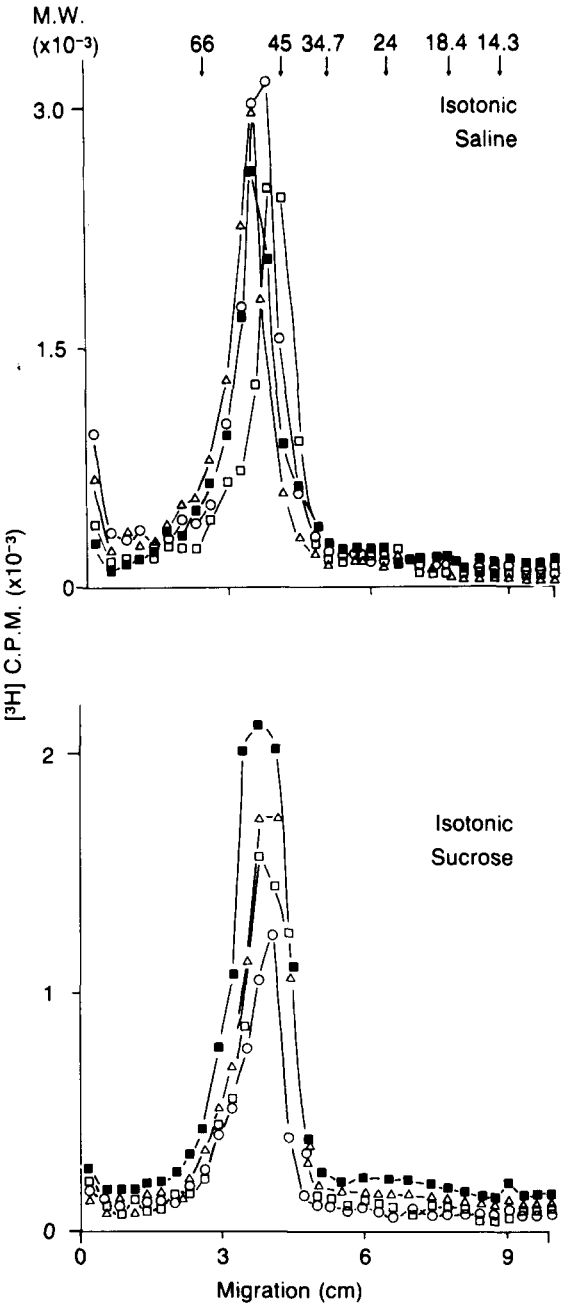

Fig. 1. Absence of proteinase effects on the [^{3}H]cytochalasin-B-labelled Band 4.5 components in intact erythrocytes. Washed erythrocytes were suspended in phosphate-buffered saline (upper panel) or isotonic sucrose (lower panel) and incubated in the absence of proteinases (○) or trypsin (△), chymotrypsin (□) or pronase (■) was described in Materials and Methods. Ghosts were then prepared, photolabelled, and subjected to electrophoresis in 7.5% acrylamide gels (Fairbanks et al. [26]). The radioactivity in 3-mm slices of the gels is represented.

activity, also had little or no effect on the labelled component (Fig. 1, lower panel).

Proteolytic digestion of alkali-stripped (unsealed) ghosts was performed in order to determine if the cytochalasin-B-labelled component was susceptible to proteolysis at the membrane's cytoplasmic face. Digestion of membranes suspended in 0.1 M sodium phosphate (pH 7.5) by either trypsin or chymotrypsin resulted in the virtually complete disappearance of the labelled 50 kDa component (Band 4.5 region) (Fig. 2), accompanied by the appearance of a labelled band at approx. 20 kDa in nearly quantitative yield (8578 cpm in the Band 4.5 region of uncleaved ghosts vs. 8013 and 9000 cpm in the 20 kDa fragment produced by cleavage by chymotrypsin and trypsin, respectively). The proteinases, therefore, cleave the transporter into at least two fragments, one of which is a 20 kDa polypeptide containing the cytochalasin B label. Other unlabelled fragment(s) of the transporter could not be visually identified on the gels, due to the presence of other more numerous peptides, especially fragments of the anion transporter, Band 3.

Purified, reconstituted Band 4.5 [21] was then studied so that the fragments generated by proteolytic cleavage of the protein could be unambiguously identified either by staining procedures or by cytochalasin B labelling. As shown in FIg. 3 (left panel), Coomassie blue-stained electrophoretograms of unproteolysed vesicles indicated the presence of a single protein in the Band 4.5 region. After treatment with trypsin, however, much of the Band 4.5 protein was no longer evident. A sharp band was, in this case, visible at 20 kDa, and a second product, a broad, poorly staining band in the 30 kDa region. This same pattern was seen with both photolabelled and unlabelled transporter, and with the proteolytic digestion carried out in the absence and presence of cytochalasin B.

The sharp 20 kDa stained band is of about the same molecular weight as the cytochalasin-B-labelled product obtained from proteolysed ghosts (Fig. 2). The reconstituted system was, therefore,

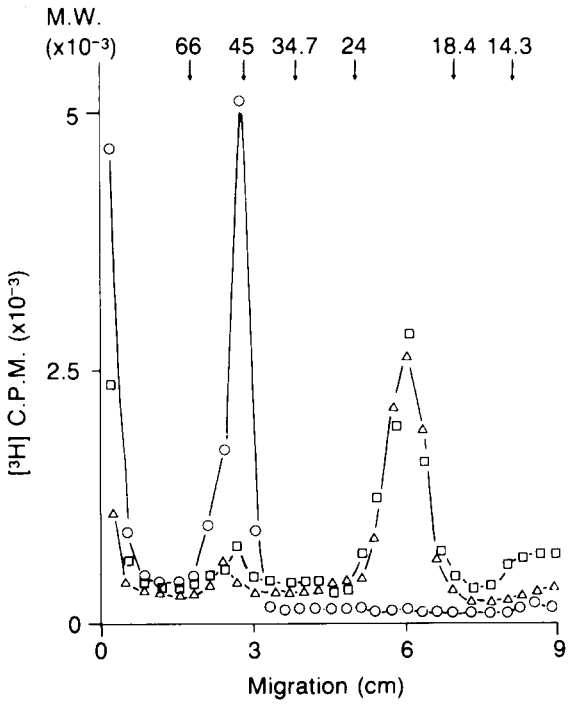

Fig. 2. Proteolytic cleavage of the [^{3}H]cytochalasin-B-labelled hexose transporter in unsealed ghosts. Photolabelled membranes were incubated without proteinases (○), with trypsin (△) or with chymotrypsin (□) as described in Materials and Methods. The samples were electrophoresed in 10% acrylamide gels (Fairbanks et al. [26]). The amount of [^{3}H]cytochalasin-B-labelled protein in each gel slice is shown.

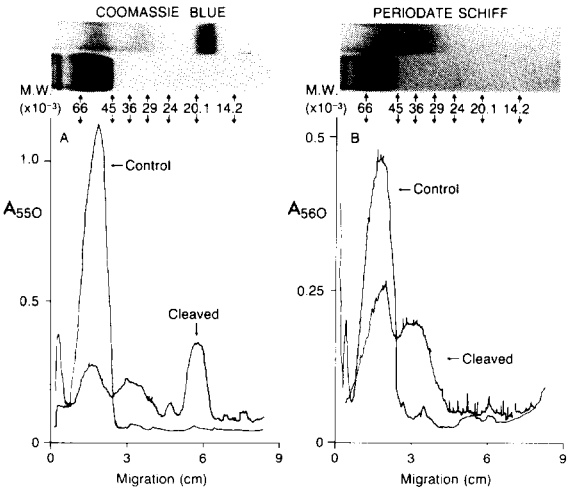

Fig. 3. Proteolysis of reconstituted Band 4.5. Reconstituted transporter was subjected to tryptic digestion (see Materials and Methods) and electrophoresed according to Laemmli [27] with a stacking gel of 3% acrylamide and a separating gel of 12.5% acrylamide. The gels were stained with Coomassie blue (left panel) and the periodate-Schiff reagent for carbohydrates (right panel). Photographs and densitometric scans of the gels are shown.

also tested using the photolabelling technique. In an unproteolysed preparation, substantial labelling was found in the 50 kDa peak (Fig. 4, upper panel). Tryptic digestion resulted in the disappearance of about 75% of the 50-kDa-labelled protein and the appearance of a 20-kDa-labelled fragment. Virtually all of the label lost from the 50 kDa protein was recovered in the 20 kDa band (3524 cpm lost vs. 3414 cpm recovered). The production of a cytochalasin-B-labelled 20 kDa peptide and the absence of label in any other proteolytic product in both ghosts and the reconstituted system suggests that the same cleavage occurs in the two preparations. In quantitative terms, however, the proteolysis is less complete in the reconstituted system, with about 25% of the label remaining in the Band 4.5 region and 75% migrating to the 20 kDa region (Fig. 4, upper panel). The reason is probably that in only 75% of the reconstituted vesicles is the orientation of the peptide inside-out, so that the cleavage sites are accessible to the enzyme.

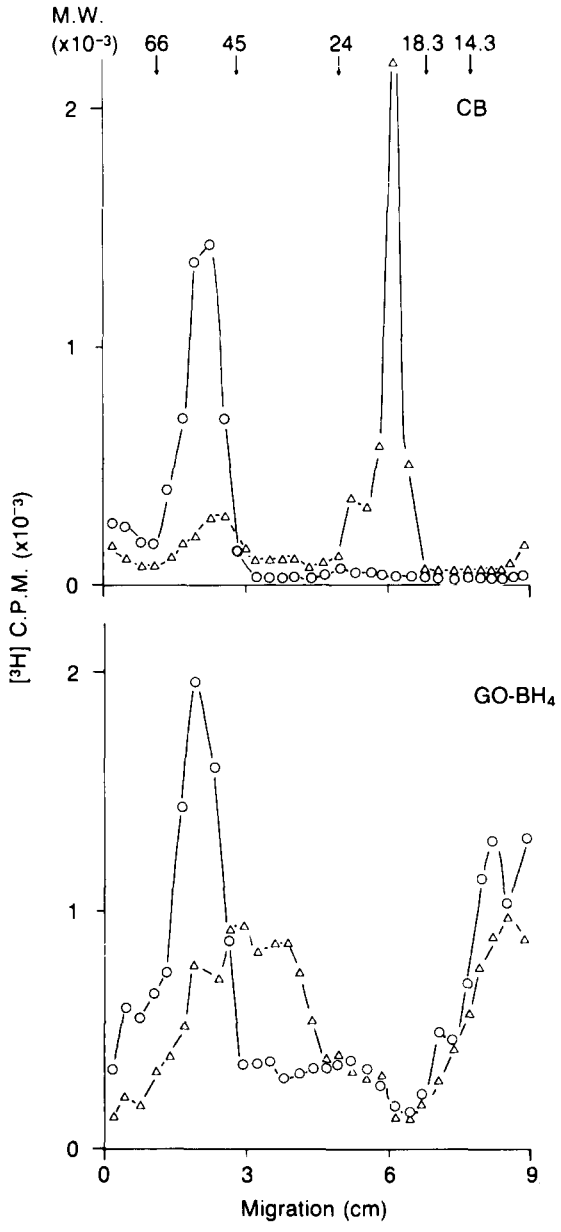

Fig. 4. Identification of the [^{3}H]cytochalasin-B-labelled and carbohydrate-bearing tryptic fragments. Reconstituted transporter labelled with [^{3}H]cytochalasin B (CB, upper panel) or having carbohydrate labelled by the galactose oxidase-borohydride method (GO-BH_4, lower panel) were subjected to electrophoresis as in Fig. 3. Control (○) and trypsin-treated samples (△) are represented.

Baldwin et al. [16] have previously demonstrated that the fraction of trypsin-sensitive transporters, as assessed by the loss of reversible cytochalasin B binding sites, corresponds to the population of molecules having an inside-out orientation. They found that reconstituted systems prepared by the removal of Triton X-100 from the membrane extracts by polystyrene beads resulted in a random orientation of Band 4.5 polypeptides in the liposomes, whereas removal of the detergent from the extracts of cholate-treated membranes by column chromatography produced liposomes in which approx. 75% of the molecules had assumed an inside-out orientation. The above results suggest that the reconstituted Band 4.5 used in the present studies resembles the latter preparation with respect to the orientation of the incorporated protein.

The poor Coomassie blue staining properties of the 30 kDa component (Fig. 3, left panel) suggested that it might be rich in carbohydrate; the breadth of the band is consistent with the known carbohydrate heterogeneity of the parent protein [25]. The location of carbohydrate before and after proteolysis was directly determined by two procedures, staining with periodate-Schiff reagent [31] (Fig. 3, right panel), and labelling with galactose oxidase-borohydride [25] (Fig. 4, bottom panel). The results, in each case, were similar. Sugar moieties are present in the uncleaved 50 kDa protein

and in the diffuse 30 kDa fragment, but not in the 20 kDa fragment (Fig. 3, right panel and Fig. 4, bottom panel), suggesting that the carbohydrate moiety of the transporter is located in the 30 kDa fragment. The accompanying densitometric or radioactive scans show approximate conservation of carbohydrate-stained or -labelled material, indicating that the 30 kDa fragment is the only glycosylated peptide produced in appreciable yield. Labelled carbohydrate species (with the galactose oxidase procedure) seen near the gel front in both control and trypsin-treated samples (Fig. 4, bottom panel) are glycolipids, previously demonstrated by Steck and Dawson [32].

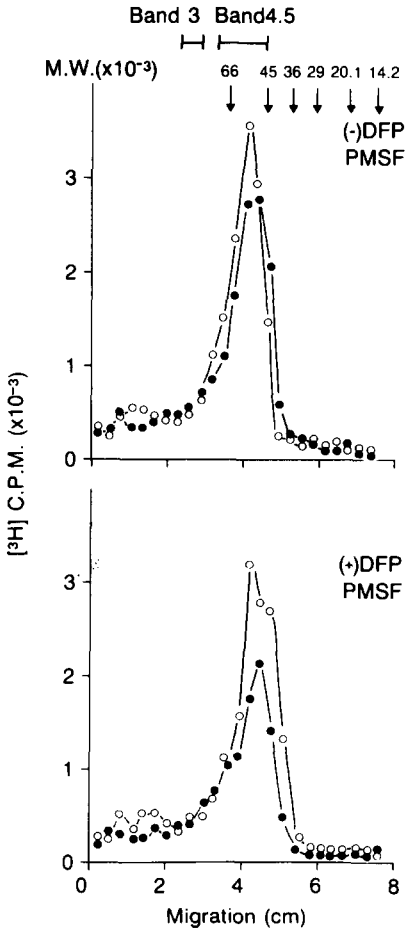

Fig. 5. Photolabelling of ghosts isolated from cells treated with proteinase inhibitors. Control samples (upper panel) and ghosts isolated from cells that had been treated with DFP and PMSF (lower panel) were photolabelled with [^{3}H]cytochalasin B and subjected to electrophoresis on 5% acrylamide gels (Fairbanks et al. [26]). Unextracted membranes (○) and ghosts that had been extracted with 0.01 μ NaOH/0.1 mM EDTA (●) prior to photolabelling are shown. The amount of radioactivity in each gel slice is shown.

It has been reported that endogenous proteolysis can result in an increase in the amount of staining in the Band 4.5 region, due to peptide fragments that are derived from the Band 3 region, and furthermore that the reaction may be enhanced by exposure of the membranes to pH 12, a procedure commonly used to extract extrinsic proteins [11,12]. In the following experiments, procedures reported to minimize endogenous proteolysis were followed as closely as possible (see Materials and Methods). The results obtained were indistinguishable from those seen with ghosts prepared without these special precautions (Fig. 5). The amount of [^{3}H]cytochalasin B incorporated into Band 4.5 of the specially treated membranes was virtually identical to that incorporated in control membranes (11 683 cpm vs. 11 216 in control and proteinase inhibitor-treated samples, respectively). No labelling of Band 3 polypeptides was observed. Similarly, omission of the pH 12 extraction of ghosts prior to photolabelling did not alter the profile of photolabelling of ghosts prepared by either procedure.

Discussion

In the present study, the proteolytic dissection of proteins involved in sugar transport were characterized in order to gain information concerning their disposition in the membrane. The agent, cytochalasin B, was used as a 'marker' for the transport protein and for products of its proteolysis, using the technique of photoactivation to covalently link it to its binding site in the protein [17,18]. Cytochalasin B is a specific inhibitor of sugar transport in red blood cells [33–35], and its inhibitory effects as well as its binding appear to involve a competitive interaction with transported sugars [35,36]. Furthermore, the particular binding sites for which D-glucose competes have been localized in the Band 4.5 region, supporting the conclusion that this labelled component is involved in cytochalasin-B-sensitive sugar transport [9,10]. Not much is presently known about the architectural arrangement of the Band 4.5 trans-

porter in the membrane. The carbohydrate attachment site [25] and a sulfhydryl group reactive with impermeant agents [8] are reported to be exposed at the outside face of the membrane, whereas inhibitory effects of proteolytic enzymes require exposure to the cytoplasmic face [15,16]. Thus, the protein must traverse the membrane, providing exposure to both sides, a general requirement for transport proteins. Based on kinetic interpretations it has also been suggested that the cytochalasin B binding site is cytoplasmic [37].

The proteolytic cleavage pattern described in this paper indicates that with the particular enzymes used (trypsin, chymotrypsin and pronase), and under the particular conditions, the Band 4.5 cytochalasin B binding component appears to be susceptible to a single cleavage at the cytoplasmic face. This conclusion is subject to some reservation inasmuch as it is based on recovery of a 30 kDa fragment (containing the carbohydrate attachment site), and a 20 kDa fragment (containing the cytochalasin B binding site) from a 50 kDa parent peptide, based on estimates of molecular weight using SDS-acrylamide gel electrophoresis. The uncertainty in the molecular weight assignments, particularly of the carbohydrate-bearing 50 kDa transporter and the 30 kDa cleavage fragment (each of which gives broad bands in the gels), prevents the ruling out of additional cleavages that might remove small peptide fragments from the protein.

One other reservation should be noted. The efficiency of photoactivated labelling by cytochalasin B is low, 1–3% by the original technique [17,18] and about 8% by the improved technique used in the present study [24]. It is therefore possible that a subpopulation is being investigated that does not represent the total population of Band 4.5 peptides defined by reversible binding of cytochalasin B. This possibility seems rather unlikely based on the observation that D-glucose affords specific protection against both the reversible and irreversible cytochalasin B binding [17,18,35,36]. Furthermore, using the reconstituted system, it is demonstrated in this paper that the cleavage pattern is the same for the total Band 4.5 component (measured by staining, Fig. 3) as it is for that fraction of Band 4.5 that is photolabelled (Fig. 4).

Thus, the covalent cytochalasin B binding component appears to be representative of the total reconstituted Band 4.5. Given that in purification and reconstitution of the Band 4.5 transport component, a substantial fraction (70–90%) of the reversible cytochalasin B binding sites are recovered [21], it can also be concluded that the photolabelled fraction is representative of the total cytochalasin B binding protein with respect to proteolytic behavior.

Other conclusions follow: (a) The cleavage reported here presumably accounts for the reported inhibition of transport and of reversible cytochalasin B binding [15,16] that results from treatment of the cytoplasmic face of the membrane with proteolytic enzymes; (b) the covalent attachment of cytochalasin B is preserved during proteolytic cleavage of Band 4.5 (Fig. 2), but, as noted above, if cleavage is carried out first, noncovalent cytochalasin B binding is no longer observed [16]. Given that this loss of binding site is not accompanied by removal of any substantial amount of peptide, it can be concluded that the cleavage results in a change in tertiary structure such that the transport site can no longer bind cytochalasin B or function in transport; (c) the two cleavage fragments (20 and 30 kDa peptides) are membrane-bound, their hydrophobic association with the bilayer perhaps accounting for the absence of additional proteolytic cleavages; (d) the 30 kDa fragment must cross the bilayer because it is produced by a cleavage at the cytoplasmic side and it also contains the sugar attachment site (Figs. 3 and 4) located at the outside [25]; (e) the 20 kDa segment must be exposed at the inside but it is not known whether it is also exposed at the outside (crosses the bilayer); (f) the cleavage pattern appears to be the same for Band 4.5 in the native membrane (unsealed ghosts) and for purified and reconstituted Band 4.5. The 20 kDa cytochalasin-B-labelled component is found in both cases, but the 30 kDa fragment cannot be visualized in the ghost preparation because of the presence of much larger amounts of a 35 kDa proteolytic fragment derived from Band 3 [13,14]. Band 4.5 is almost completely cleaved in ghosts but it is only 75% cleaved in the reconstituted system. The latter finding is consistent with a report [16] indicating that in another reconstituted system, only 75% of

the Band 4.5 is in the inside-out arrangement, with the cleavage site exposed to the enzyme; (g) although the transport component of Band 4.5 is known to be exposed at the outer surface of the membrane [8,25], no cleavages in this location are observed, even at low ionic strength which provides a better environment for proteolysis. Other exposed transport proteins of the red cell such as $(Na^+ + K^+)$-ATPase [35] and Ca^{2+}-ATPase [39] are also resistant to proteolysis from the outside. On the other hand, Band 3 [13,14] and glycophorin [40] are both cleavable from the outside.

Band 3 has also been implicated in sugar transport, based largely on the findings that maltosyl isothiocyanate, a covalent inhibitor, presumed to interact with the transport site, reacts primarily with Band 3 [11], and that 'purified' reconstituted Band 3 displays a substantial sugar-transport activity [12]. Under conditions that favor endogenous proteolysis, substantial amounts of the maltosyl isothiocyanate binding is lost from Band 3 and is recovered in the Band 4.5 region [11], supporting the premise that the sugar transporter in Band 4.5 is a proteolytic product derived from Band 3. The conclusions do not, however, accommodate the findings with cytochalasin B. This agent is reported to afford some protection against the binding of the maltosyl isothiocyanate [11], but no information is reported on possible inhibitory effects on sugar transport by reconstituted Band 3 [12]. On the other hand, cytochalasin B binding sites have been reported to be located only in Band 4.5 [9,16–18,21]. In these studies, maximum precautions were not always taken to prevent the possibility of endogenous proteolysis, but in the present studies, no difference in the amount or distribution of the cytochalasin-B-labelled components was noted even under specific conditions that minimize endogenous proteolysis of Band 3 (freshly drawn cells, freshly prepared ghosts, use of proteinase inhibitors, and omission of alkaline wash). Cytochalasin B was found only in Band 4.5 under all conditions. It has already been noted that the photolabelled component appears to be representative of the total cytochalasin B binding component. The total number of binding sites amounts to about 250 000 copies per cell [33,41] equivalent to about 3% of the total membrane protein, which represents a substantial fraction of Band 4.5. Given that cytochalasin B can inhibit essentially all (over 90%) of the specific sugar transport of the cell [33,34], it appears that cytochalasin B exerts its inhibitory effect by binding to a component of Band 4.5 and not to Band 3.

Reported differences in proteolytic cleavage patterns between Band 3 and Band 4.5 are relevant. In considering these differences, however, it must be recognized that Band 3 and Band 4.5 designate molecular weight classes separable by SDS-acrylamide gel electrophoresis, not necessarily single proteins. The major component of Band 3 after purification is, within limits of the procedures, a homogeneous peptide, which functions as the anion transport protein, and that can be readily identified by use of the covalent inhibitor DIDS that can interact quantitatively with a single specific site on each monomer [13,14]. The major component of Band 4.5, as noted, can also be purified and can be identified by its high-affinity (sugar-specific) cytochalasin B binding sites [10,21]. In the case of the major component of Band 3, proteolytic cleavage from the outside produces two membrane fragments, 35 and 60 kDa, the former containing the carbohydrate attachment site and the latter DIDS-binding sites. An inside cleavage produces fragments of 42 and 55 kDa, the former a soluble peptide and the latter containing both the carbohydrate-attachment site and the DIDS-binding site [13,14]. The 60 and 55 kDa fragments are located, after SDS-acrylamide gel electrophoresis, in the Band 4.5 region, but their properties do not match those of the cytochalasin B binding component of Band 4.5. The 60 kDa fragment contains no carbohydrate and on proteolysis is cleaved to produce a membrane-bound 17 kDa DIDS-containing fragment plus a 42 kDa segment that is released from the membrane. The 55 kDa segment on further proteolysis releases a 17 kDa DIDS-containing segment and a 35 kDa fragment containing the carbohydrate attachment site. None of these cleavages matches that reported here for the cytochalasin B binding component of Band 4.5.

The major Band 3 component is 4-times as abundant as the Band 4.5 component (about 1 000 000 DIDS-binding sites [13,14] compared to about 250 000 sugar-specific cytochalasin B binding sites per cell). Furthermore, under conditions

in which endogenous proteolysis is reported to lead to changes in Band 3 components related to sugar transport [11,12], the DIDS-binding component of Band 3 does not migrate to the Band 4.5 region [13,14]. From the above considerations, it seems quite clear that the major component of Band 3 does not give rise by proteolysis to the cytochalasin B binding component of Band 4.5.

It is possible that a minor component of the Band 3 region might be convertible by proteolysis to a peptide in the Band 4.5 region. Thus, the number of maltosyl isothiocyanate binding sites in Band 3 related to sugar transport is about 300 000 per cell [11] compared to over 1 000 000 DIDS-labelled sites [13,14]. But as noted above, none of the Band 3 peptides appears to be capable of covalent binding of cytochalasin B (by photoactivation). It is, of course, possible that cytochalasin B might bind reversibly to a Band 3 component, but that no photoactivation occurs. Clearly, at this time, a number of issues relating to the peptide components involved in sugar transport remain unresolved.

In conclusion, the cytochalasin B binding component of Band 4.5 crosses the bilayer. It is resistant to proteolytic at the outside face of the membrane, but undergoes a single cleavage at the cytoplasmic face releasing a 30 kDa transmembrane fragment that contains the carbohydrate attachment site and a 20 kDa fragment that contains the cytochalasin B binding site. The cytochalasin B binding component does not appear to be derived from Band 3 by endogenous proteolysis.

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References

1 Jung, C.Y. (1975) in The Red Blood Cell (Surgenor, D. MacN., ed.), Vol. 11, pp. 705–751, Academic Press, New York
2 Baldwin, S.G. and Lienhard, G.E. (1981) Trends Biochem. Sci. 6, 208–211
3 Krupka, R. and Devés, R. (1980) J. Biol. Chem. 255, 2051–2055
4 Smith, R.P.P. and Ellman, G.L. (1973) J. Membrane Biol. 12, 177–188
5 Bloch, R. (1974) J. Biol. Chem. 249, 1814–1822
6 Baldwin, S.A. and Lienhard, G.E. (1980) Biochem. Biophys. Res. Commun. 94, 1401–1408
7 Lienhard, G.E., Gorga, F.R., Orasky, J.E. and Zoccoli, M.A. (1977) Biochemistry 16, 4921–4926
8 Abbott, R.E. and Schachter, D. (1976) J. Biol. Chem. 251, 7176–7183
9 Kasahara, M. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7384–7390
10 Baldwin, S.A., Baldwin, J.M., Gorga, F.R. and Lienhard, G.E. (1979) Biochim. Biophys. Acta 522, 183–188
11 Mullins, R.E. and Langdon, R.G. (1980) Biochemistry 19, 1205–1212
12 Shelton, R.L. and Langdon, R.G. (1980) Biochim. Biophys. Acta 733, 25–33
13 Cabantchik, Z.I., Knauf, P.A. and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239–302
14 Knauf, P.A. (1979) Curr. Topics Membranes Trans. 12, 249–363
15 Masiak, S.J. and Lefevre, P.G. (1977) Biochim. Biophys. Acta 465, 371–377
16 Baldwin, J.M., Lienhard, G.E. and Baldwin, S.A. (1980) Biochim. Biophys. Acta 599, 699–714
17 Carter-Su, C., Pessin, J.E., Mora, R., Gitomer, W. and Czech, M.P. (1982) J. Biol. Chem. 257, 5416–5425
18 Shanahan, M.F. (1982) J. Biol. Chem. 257, 7290–7293
19 Sogin, D.C. and Hinkle, P.C. (1978) J. Supramol. Struct. 8, 447–453
20 Steck, T.L. and Yu, J. (1973) J. Supramol. Struct. 1, 220–232
21 Baldwin, S.A., Baldwin, J.M. and Lienhard, G.E. (1982) Biochemistry 21, 3836–3842
22 Zoccoli, M.A., Baldwin, S.A. and Lienhard, G.E. (1978) J. Biol. Chem. 253, 6923–6930
23 Scatchard G. (1949) Ann. N.Y. Acad. Sci. 51, 660–666
24 Deziel, M., Pegg, W., Mack, E., Rothstein, A. and Klip, A. (1984) Biochim. Biophys. Acta 722, 403–406
25 Gorga, F.R., Baldwin, S.A. and Lienhard, G.E. (1979) Biochem. Biophys. Res. Commun. 91, 955–961
26 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
27 Laemmli, U.K. (1970) Nature 227, 680–685
28 Czech, M.P. and Lynn, S.W. (1973) J. Biol. Chem. 248, 5081–5088
29 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
30 Bradford, M. (1973) Anal. Biochem. 72, 248–254
31 Zacharius, R.M., Zell, T.E., Morrison, J.H. and Woodlock, J.J. (1969) Anal. Biochem. 30, 148–152
32 Steck, T.L. and Dawson, G. (1974) J. Biol. Chem. 249, 2135–2142
33 Taverna, R.D. and Langdon, R.G. (1973) Biochim. Biophys. Acta 323, 207–219
34 Bloch, R. (1973) Biochemistry 12, 4799–4801

35 Jung, C.Y. and Rampal, A.L. (1977) J. Biol. Chem. 252, 5456–5463
36 Lin, S. and Spudich, J.A. (1974) J. Biol. Chem. 249, 5778–5783
37 Devés, R. and Krupka, R.M. (1978) Biochim. Biophys. Acta 510, 339–348
38 Knauf, P.A., Proverbio, F. and Hoffman, J.F. (1974) J. Gen. Physiol. 63, 305–323
39 Nelson, D.R. and Hanahan, D.J. (1983) Fed. Proc. 42, 1931
40 Triplett, R.B. and Carraway, K.L. (1972) Biochemistry 11, 2897–2903
41 Basketter, D.A. and Widdas, W.F. (1977) J. Physiol. 265, 39–40P.