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Immunological evidence that band 3 is the major glucose transporter of the human erythrocyte membrane

Robert G. Langdon and Virginia P. Holman

University of Virginia School of Medicine, Department of Biochemistry, Charlottesville, VA (U.S.A.)

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We have previously reported that human erythrocyte band 3 contains 90–95% of the reconstitutable glucose transport activity of the erythrocyte membrane (Shelton, R.L. and Langdon, R.G. (1983) Biochim. Biophys. Acta 733, 25–33). We have now found that monoclonal and polyclonal antibodies to epitopes on band 3 specifically removed band 3 and more than 90% of the reconstitutable glucose transport activity from unfractionated octylglucoside extracts of erythrocyte membranes; nonimmune serum removed neither. Western blots of whole membrane extracts revealed that the polyclonal antibody to band 4.5 used to isolate cDNA clones presumed to code for the transporter (Mueckler, M., Caruso, C., Baldwin, C.A., Pancio, M., Blench, J., Morris, H.B., Allard, W.J., Lienhard, G.E. and Lodish, H.F. (1985) Science 229, 941–945) reacts strongly with six discrete bands in the 4.5 region. A monoclonal antibody to band 3 also reacts with a M_r 55000 component of band 4.5. We conclude that band 3 contains the major glucose transporter of human erythrocytes, and that the transport activity in band 4.5 might be attributable to a band 3 fragment. Band 3 is probably a multifunctional transport protein responsible for transport of glucose, anions, and water.

Introduction

In previous publications we have presented evidence that the major glucose transporter of human erythrocyte membranes is a protein of approximate M_r 100000 which is present in band 3. This conclusion was based upon covalent affinity labelling with glucosyl [1] and maltosyl [2,3] isothiocyanates, as well as upon the demonstration that after purification and reconstitution of band 3 into large, unilamellar phospholipid vesicles essentially all of the glucose transport activity of the erythrocyte membrane could be accounted for by the activity incorporated into these vesicles [4]. Similar evidence was obtained for the glucose transporter of rat adipocyte membranes [5]. On the other hand, several groups have proposed that erythrocyte band 4.5, average M_r 55000, which binds cytochalasin B in a glucose-competitive manner [6–9] and into which radioactive cytochalasin B is incorporated when membranes are irradiated with ultraviolet light [10–14], is identical with the glucose transporter. Band 4.5 has also been found to have glucose transport activity when incorporated into phospholipid vesicles [15–18], but it was much less active than band 3 and was present in such small quantities in fresh, carefully prepared membranes that it could account for only a small fraction of the transport activity of native erythrocyte membranes [4]. Both polyclonal [19,20] and monoclonal [21] antibodies have been

Correspondence: R.G. Langdon, Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, VA 22908, U.S.A.

raised against band 4.5. These antibodies were reported to react with a broad, diffuse band, average M_r 55000, on Western blots [19,21] and to remove band 4.5 from detergent solutions of the purified material; concomitantly, the reconstituable glucose transport and cytochalasin B binding activities were removed. However, no estimate was reported of the reconstitutable glucose transport activity present in unfractionated membrane extracts which could be removed by these antibodies. In a recent publication by Mueckler et al. [22] an ovine polyclonal antiserum raised against human erythrocyte band 4.5 [19] was used to screen a library of cDNA clones from a human hepatoma line; the nucleotide sequences of DNA from positive clones was obtained, and it was deduced that they coded for a 46000 Da protein which was assumed to be the glucose transporter. However, it has not been reported whether expression of this DNA results in a protein having glucose transport activity.

We now report that both ovine polyclonal and murine monoclonal antibodies against human erythrocyte band 3, which contains the 100000-M_r glucose transporter, specifically remove band 3 and approx. 95% of the reconstitutable glucose transport activity from unfractionated octylglucoside extracts of native or alkaline 'stripped' erythrocyte membranes. Furthermore, the polyclonal antibody preparation used by Mueckler and co-workers to select cDNA clones has been found not to be monospecific; it has been found to react on Western blots with six discrete bands in the 4.5 region. Both polyclonal and monoclonal antibodies to band 3 react with one of these bands, M_r approx. 55000.

Materials and Methods

All chemicals not otherwise specified were of the highest purity obtainable from commercial sources or have been described in detail in previous publications [4,5,23]. Prestained molecular weight markers for electrophoresis were obtained from Bethesda Research Laboratories; the contained proteins were myosin (M_r 200000), phosphorylase *B* (M_r 97400), bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), α-chymotrypsinogen (M_r 25700), β-lactoglobulin (M_r 18400) and lysozyme (M_r 14300). Nitrocellulose sheets (0.2 micron) for Western blotting were obtained from Hoeffer Scientific Instruments or from Pierce Chemical Co. Protein A-Sepharose CL-4B was from Pharmacia. Sigma Chemical Co. was the source of 4-chloro-1-naphthol. Affinity-purified, horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG antibodies were obtained from Bio-Rad. Iodobeads were obtained from Pierce Chemical Co., and sodium [^{125}I]iodide, 17.4 Ci/mg, was from NEN Products; *sn*-1-Palmitoyl-2-oleoylphosphatidylcholine was purchased from Avanti Polar Lipids. Ovine antiserum against human erythrocyte band 4.5 was a generous gift of Dr. Michael J. Weber; this was the same antiserum described in previous publications [19,20] including that by Mueckler et al. [22].

Erythrocyte membranes from freshly drawn human blood were obtained as previously described [4,23]; alkaline stripping (pH 12) was also performed as earlier described [4]. When not used immediately membranes were kept frozen in liquid nitrogen. Band 3 for immunization of rabbits was prepared as follows. Erythrocyte ghosts, 10 ml containing 40 mg protein, in 10 mM sodium phosphate buffer were solubilized by making the solution 2% (w/v) in sodium dodecyl sulfate, 5 mM in dithiothreitol and heating for 1 min in a steam bath. A 100 µl sample was removed prior to addition of dithiothreitol and dansylated [24]; after reaction, addition of dithiothreitol, and heating, the dansylate aliquot was recombined with the remainder of the solution. Proteins in the preparation were separated electrophoretically by applying aliquots of 2 ml to 3% stacking gels overlaying 2.2 × 18 cm cylindrical 7.5% polyacrylamide running gels using the Laemmli [25] formulation. During electrophoresis the fluorescent protein bands were visualized easily by ultraviolet illumination. After development, segments of gel containing band 3 were sliced from the gel cylinders, and their contained protein was eluted electrohoretically into dialysis sacs. On analytical gel electrophoresis [25] the band 3 preparation appeared to be homogeneous. New Zealand white rabbits were immunized by primary subcutaneous injection of 1 ml of band 3 solution (1 mg protein) plus 1 ml of Freund's complete adjuvant divided among four injections sites. Booster injections

consisted of 1 ml of band 3 solution plus 1 ml of incomplete Freund's adjuvant administered subcutaneously at intervals of 4 weeks. Animals were bled 1 week after each booster injection. The antisera were stored at −70°C in small aliquots until used. Antiserum from rabbit No. 2, designated Ab-2, had a high affinity for band 3 as determined by Western blotting and was used in those experiments employing a polyclonal antiserum to band 3. We received monoclonal antibody MAb-430 from Dr. Charles Rubin, who prepared it as follows. Erythrocyte membranes prepared by the Fairbanks method [26] were extracted with 50 mM Hepes buffer (pH 7.4) containing 2% (v/v) Triton X-100 for 60 min at 4°C. After centrifugation at 130000 × *g* for 60 min at 4°C the supernatant fraction was collected and the contained glycoproteins were partially purified by affinity chromatography on a column of wheat germ agglutinin-agarose. The glycoproteins eluted with 20 mM Hepes buffer (pH 7.4) containing 0.3 M *N*-acetylglucosamine, 5 mM EDTA, and 0.1% octylglucoside were used to immunize SJL mice (Jackson Laboratory). Animals were injected subcutaneously with 0.5 mg protein emulsified with adjuvant at 14 day intervals over a period of 8 weeks. Two weeks after the last injection the mice were killed. Isolated spleen lymphocytes were injected intravenously into lethally irradiated recipient mice. Subsequently, recipient mice were given a single booster injection and 3 days later their spleen lymphocytes were isolated and fused with NSO myeloma cells. Samples of culture medium from the resulting hydribomas were screened for antibodies by their ability to complex with human erythrocyte membrane proteins that were resolved by polyacrylamide gel electrophoresis. Positive hybridoma cells were subcloned in soft agar. An IgG with high affinity for band 3, designated Mab-430, was selected for studies described in this paper. Hybridoma cells secreting Mab-430 were established as ascites tumors by injecting $2 \cdot 10^7$ cells into the peritoneal cavities of pristine-primed SJL/Balb-c F_1 hybrid mice. Ascites fluid was collected from 7 to 20 days after injection of the cells; it contained 1–2 mg/ml of anti-band 3 IgG.

All analytical polacrylamide gel electrophoresis was performed by the Laemmli method [25] as previously described [4] and silver stained as described by Morrissey [27]. Samples to be used for Western blotting [28] were electrophoresed in 9 × 8 × 0.075 cm 10% polyacrylamide slab gels using the Laemmli formulation; prestained molecular weight standards were electrophoresed in one lane of each gel. Transfer of proteins, including the molecular weight markers, from polyacrylamide gels to nitrocellulose membranes was performed as described by Towbin et al [29] using a Hoeffer Te 42 Transphor apparatus equipped with a cooling coil. After transfer, each polyacrylamide gel was stained with Coomassie blue to ensure that transfer had been complete. Complete transfer had occurred after 1 h at 1–1.2 amperes current. When a transfer buffer containing no methanol, described by Allard and Lienhard [21], was used it was found that band 3 was removed from the polyacrylamide gel but was not retained by the nitrocellulose sheet. The nitrocellulose sheets were shaken for 30 min in 150 mM NaCl/20 mM Tris-HCl (pH 7.4) (buffer A), containing 5% (w/v) nonfat dried milk solids. They were transferred to 50 ml of the same solution containing 50 μl of the antiserum or ascitic fluid containing antibodies to the antigen of interest on the nitrocellulose and were shaken for 90 min. The nitrocellulose sheets were then washed sequentially in 200 ml of buffer A (10 min), 200 ml of buffer A containing 0.05% (w/v) Tween-20 (twice for 10 min), and in 200 ml of buffer A (twice for 10 min). They were transferred to 50 ml of buffer A containing the appropriate peroxidase-conjugated anti-ovine or anti-murine IgG and shaken for 30 min. The sheets were then washed in 200 ml buffer A/0.05% Tween-20 (10 min) and 200 ml of buffer A (10 min). The immunologically reactive proteins were rendered visible by transferring the nitrocellulose sheets into 50 ml of buffer A and adding 20 μl of a 3% (w/v) solution of 4-chloro-1-naphthol and 30 μl of 30% hydrogen peroxide. After color development, the sheets were washed twice in 200 ml of water and dried at room temperature.

Protein A-Sepharose CL-4B columns with bound immune IgG were prepared and used as follows. 100 mg portions of protein A-Sepharose were swollen in 10 mM sodium phosphate buffer (pH 8.0) and the slurry was transferred to a 1 ml tuberculin syringe barrel containing a sintered polyethylene filter disc; the packed volume was

approx. 0.3 ml. The column was washed with 20 ml of 100 mM sodium phosphate buffer (pH 8.0) at room temperature. Nonimmune rabbit serum (0.5 ml), immune rabbit serum (0.5 ml), or murine ascitic fluid (0.5 ml) containing a monoclonal antibody to band 3 was diluted with 0.5 ml of 100 mM sodium phosphate buffer (pH 8.0) and allowed to pass slowly through the column. After washing each column with 5 ml of 100 mM sodium phosphate buffer (pH 8.0) and 2 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 34 mM octylglucoside, the columns were transferred to the cold room (4°C) and allowed to achieve thermal equilibrium. To each column was slowly added 0.5 ml of an octylglucoside extract of human erythrocyte membranes, and the effluents were saved. Each column was washed with 0.3 ml of 50 mM Tris-HCl (pH 8.0) and the effluent was combined with the preceding one. The combined effluents from each column were reconstituted into the membranes of large, unilamellar *sn*-1-palmitoyl-2-oleoylphosphatidylcholine vesicles containing glucose oxidase and catalase as previously described [4,22] and their rates of glucose transport were compared with each other and with that of vesicles reconstituted at the same time with the same octylglucoside extract which had not been passed through the antibody column. For identification of the protein components remaining bound to the gel matrix after the effluent fractions had passed through the columns, the gel matrix material was transferred to 1 ml centrifuge tubes and extracted with 0.5 ml of 2% sodium dodecyl sulfate/10 mM phosphate buffer (pH 8.0)/1 mM diethiothreitol for 1 min in a steam bath. After centrifugation, the supernatant fluid was removed, its protein components were separated by polyacrylamide gel electrophoresis, and the gels were stained with silver (Figs. 4 and 5).

For other experiments (Figs. 6 and 7) designed to identify the membrane proteins retained by immunoaffinity gels, the proteins in octylglucoside extracts of erythrocyte membranes were labelled with ^{125}I. To a reaction vial containing 2 mCi sodium [^{125}I]iodide in 0.4 ml of 0.1 M sodium phosphate buffer (pH 7.0)/34 mM octylglucoside were added four Iodobeads, and the mixture was agitated gently at room temperature for 5 min. A 30 μl aliquot of an ice-cold octylglucoside extract of erythrocyte membranes containing approx. 120 μg of protein was added. After the mixture had stood at room temperature for 10 min and at 0°C for 5 min, the Iodobeads were removed. The labelled proteins were separated from unreacted radioisotope by gel filtration chromatography at 4°C on Sephadex G-20 which had been equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 34 mM octylglucoside. For immunoadsorption assay, an aliquot of the ^{125}I-labelled solution was added to unlabelled octylglucoside extract, and aliquots were passed through protein A-Sepharose columns with bound IgG as described in the previous paragraph. The gel pellet was extracted with 2% sodium dodecyl sulfate/10 mM phosphate buffer (pH 8.0)/1 mM dithiothreitol for 1 min in a steam bath; after centrifugation, the supernatant solution, which contained those components which had been bound to the gel, was collected and saved for electrophoretic analysis, radioautography, and assay of radioactivity by gel slicing as described in the Results section (Figs. 6 and 7).

Results

The reactions of a polyclonal antiserum (Ab-2) to band 3, a monoclonal antibody (MAb-430) to band 3, and a polyclonal antiserum to band 4.5 [19] were examined on Western blots of unfractionated erythrocyte membrane extracts which had been subjected to polyacrylamide gel electrophoresis. In Fig. 1 reactions of MAb-430 with components of alkaline stripped (lane 1) and unstripped (lane 2) membrane from freshly drawn erythrocyte [4,23] are shown; also shown is the reaction of a polyclonal antibody [19] to band 4.5 with unstripped (lane 3) and alkaline-stripped (lane 4) membrane components. Several things are apparent from this figure. It is evident that the anti-band 4.5 serum reacted strongly with six discrete components in the 4.5 region of unstripped membranes which are also visible as discrete components in silver-stained gels. Although alkaline stripping resulted in smearing of the components in the 4.5 region, as we have previously reported [4], multiple reactive components are still discernible. MAb-430 also reacted with several components in addition to a strong reaction with band 3.

The component of M_r approx. 200000 is presumably a dimer of band 3, while the components of M_r less than 97000 are assumed to be fragments of band 3. It is apparent that a fragment, M_r approx. 55000, corresponds closely to one of the components in the 4.5 region which reacts with anti-band 4.5 serum. In Fig. 2 are shown the the reactions of polyclonal antibodies to band 3 (lanes 1 and 2) and to band 4.5 (lanes 4 and 5) with membranes derived from freshly outdated bank blood before (lanes 2 and 5) and after (lanes 1 and 4) alkaline stripping. Lanes 3 and 6 show the reactions with purified band 3 of antibodies to band 3 and band 4.5 respectively. As in Fig. 1 it is evident that the antibody to band 4.5 reacts with multiple components in the 4.5 region and that

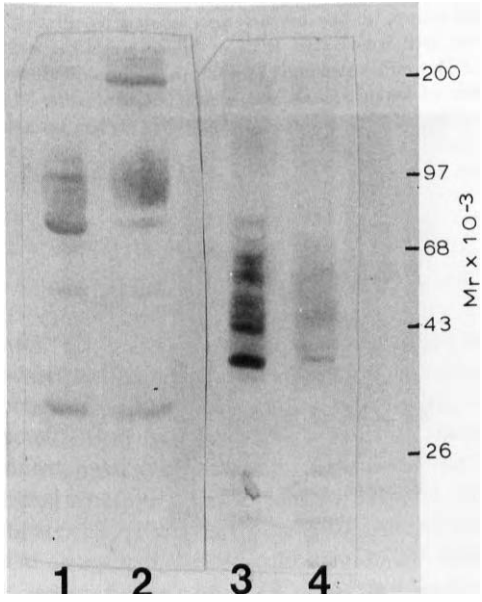

Fig. 1. Western blot of erythrocyte membranes from fresh blood reacted with a monoclonal antibody to band 3 and a polyclonal antibody to band 4.5. Lane 1: alkaline-stripped membranes reacted with monoclonal antibody MAb-430. Lane 2: membranes prior to stripping reacted with MAb-430. The secondary antibody used in lanes 1 and 2 was peroxidase-conjugated rabbit anti-mouse IgG. Lane 3: membranes prior to stripping reacted with polyclonal antibody to band 4.5. Lane 4: alkaline-stripped membranes reacted with polyclonal antibody to band 4.5 [19]. The secondary antibody used in lanes 3 and 4 was peroxidase-conjugated goat anti-rabbit IgG. The M_r values on the right ordinate are the positions of the prestained molecular weight markers which were electrophoresed on the same gel and transferred to the same strip of nitrocellulose.

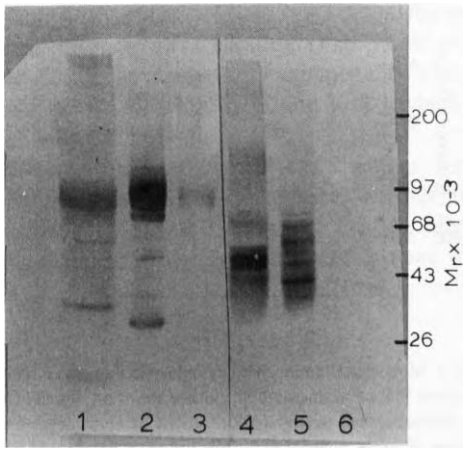

Fig. 2. Western blots of erythrocyte membranes from outdated bank blood reacted with polyclonal antibodies to band 3 and to band 4.5. Lane 1: alkaline-stripped membranes reacted with ovine anti-band 3 polyclonal antibody AB-2. Lane 2: membranes prior to stripping reacted with Ab-2. Lane 3: purified band 3 reacted with Ab-2. Lane 4: alkaline-stripped membranes reacted with ovine anti-band 4.5 polyclonal antibody [19]. Lane 5: membranes prior to stripping reacted with the same anti-band 4.5 antibody. Lane 6: purified band 3 reacted with anti-band 4.5 antibody. The secondary antibody in all lanes was peroxidase-conjugated, affinity-purified goat anti-rabbit IgG.

treatment of membranes at pH 12 results in smearing of the electrophoretic patterns. It is also apparent that diffuse background staining in the 4.5 region is more prominent than in membranes derived from freshly erythrocytes.

Previous reports indicated that polyclonal and monoclonal antibodies [21] to band 4.5 removed the reconstitutable glucose transport activity from detergent solutions of purified band 4.5, against which the antibodies had been raised. However, no evidence was presented concerning the fractions of reconstitutable transport activity which these antibodies would remove from unfractionated membrane extracts; it was therefore not possible to determine whether this was a large or small amount of the total transport activity of the membrane. Since our previous work [1–4] had indicated that most of the transport activity resides in band 3, we prepared protein A-Sepharose columns to which either monoclonal or polyclonal antibodies to band 3 or nonimmune IgG had been bound as described in the Materials and Methods

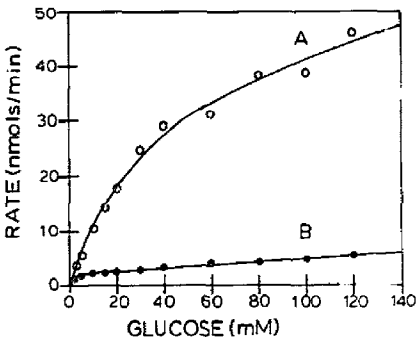

Fig. 3. Monoclonal antibody to cytoplasmic domain of band 3 removes 95% of reconstitutable glucose transport activity from an unfractionated octylglucoside extract of erythrocyte membranes. Protein A-Sepharose columns with bound MAb-430 (B) or nonimmune rabbit IgG (A) were prepared as described in the Materials and Methods section. Through each 0.3 ml column was slowly passed 0.5 ml of unfractionated erythrocyte membrane extract in 300 mM octylglucoside/10 mM phosphate buffer (pH 8.0) followed by 0.3 ml of 34 mM octylglucoside/50 mM Tris-HCl buffer (pH 8.0). The column effluents from each column were combined and were reconstituted into large, unilamellar phosphatidylcholine vesicles containing glucose oxidase and catalase. The rates of D-glucose entry into these vesicles was measured as previously described [4,23]. A: (○) vesicles reconstituted with the effluent from the nonimmune column. B: (●) vesicles reconstituted with the effluent from the MAb-430 column.

section. Unfractionated octylglucoside extracts were passed through these columns, and the effluents were reconstituted into large, unilamellar phosphatidylcholine vesicles. The transport activities of vesicles reconstituted with effluents from a monoclonal antibody column (MAb-430) and a nonimmune column are shown in Fig. 3. The activity reconstituted with effluent from the nonimmune column was indistinguishable from that reconstituted from membrane extract which had not been passed through a column. It is apparent that over 95% of the transport activity was removed by the anti-band 3 antibody column. This experiment was repeated three times with almost identical results. In five experiments using polyclonal antibodies to band 3 (data not shown) the results were also the same as those with the monoclonal antibody.

It was of importance to determine which membrane components were retained by the antibody columns and which appeared in the effluents. The gel medium was removed from each column and eluted with hot sodium dodecyl sulfate (retentate fraction). Samples of the unretained column effluents and the retentate fractions were analyzed by polyacrylamide gel electrophoresis followed by silver staining, and the results are shown in Figs. 4 and 5. It is apparent that band 3 was retained by the MAb-430 column (Fig. 4, lane 4) and could be eluted from the immunoaffinity gel with hot sodium dodecyl sulfate (Fig. 5, lane 3). The nonimmune column appeared to remove no component (Fig. 4, lane 3). Although it appeared that band 4.5 was present in the effluent fractions of both columns, the presence of IgG heavy chains in the retentate fractions precluded precise analysis of this area of the gels. Therefore, an octylglucoside extract of erythrocyte membranes was labelled

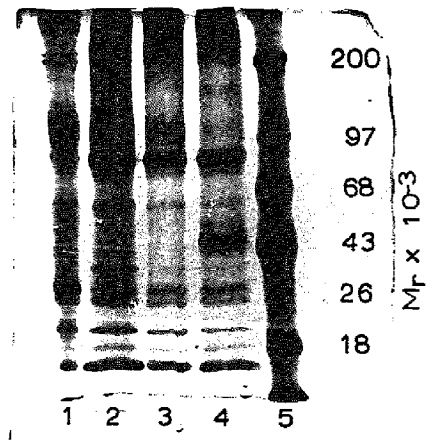

Fig. 4. Removal of band 3 from the effluent of MAb-430 immunoaffinity column as revealed by silver staining. Samples of initial membranes, octylglucoside extract, and column effluents from the experiment depicted in Fig. 3 were retained for polyacrylamide gel electrophoretic analysis, and the results are depicted here. In addition, the components retained by the protein A-Sepharose gels but eluted from the gels with hot 2% sodium dodecyl sulfate (retentates) were obtained and electrophoresed on a separate gel (Fig. 5). Following electrophoresis, the polyacrylamide gel was stained with silver [27]. Lane 1: octylglucoside extract. Lane 2: erythrocyte membranes. Lane 3: effluent from nonimmune column. Lane 4: effluent from MAb-430 column. Lane 5: molecular weight standards; the numbers on the right ordinate are the M_r values for these standards.

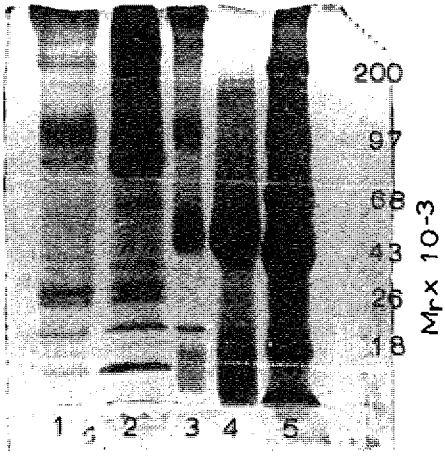

Fig. 5. Band 3 is retained by Mab-430 immunoaffinity column. After the effluent fractions had passed through the columns of Fig. 3, the gel matrices were removed, and the protein components remaining bound to them were eluted with hot 2% sodium dodecyl sulfate/10 mM phosphate buffer (pH 8.0)/1 mM dithiothreitol. These eluates were analyzed by electrophoresis, and the gels were stained with silver as in Fig. 4. Lane 1: octylglucoside extract. Lane 2: erythrocyte membranes. Lane 3: eluate from MAb-430 column. Lane 4: eluate from nonimmune column. Lane 5: molecular weight standards.

with ^{125}I, and equal aliquots of the extract were adsorbed with protein A-Sepharose to which was bound either MAb-430 or nonimmune IgG. The nonadsorbed fractions and the adsorbed fractions eluted by hot sodium dodecyl sulfate were subjected to electrophoresis prior to analysis for radioactivity by radioautography and by gel slicing and counting. Densitometric scans of the lanes of a radioautograph of a gel are shown in Fig. 6. From this figure it is evident that the nonimmune column removed no components; the MAb-430 column removed almost all of band 3, but only minor amounts of band 4.5. More quantitative results were obtained by slicing the gels and analyzing the slices for radioactivity in a scintillation counter. Shown in Fig. 7 are the results of this procedure when applied to an unfractionated octylglucoside extract and the retentates of a MAb-430 gel and a nonimmune IgG gel. Again it is apparent that, while the nonimmunme gel retained almost no detectable radioactive material, MAb-430 gel retained band 3. As would be expected from Western blotting (Fig. 1), it also retained smaller amounts of components of approximate M_r 200 000, 55 000, and 30 000, which is less evident from Fig. 6.

Western blots of the column fractions were performed routinely to determine whether the material migrating at the tops of the gels might contain abnormally migrating band 4.5; none was

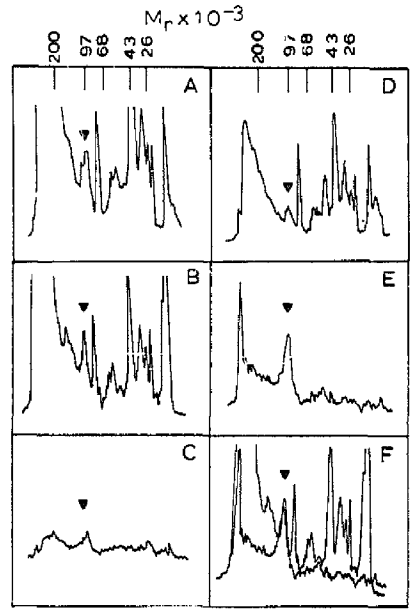

Fig. 6. Retention of ^{125}I-labelled band 3 by MAb-430 column. Densitometric scans of radioautograph. An unfractionated octylglucoside extract of erythrocyte membranes was labelled with ^{125}I and passed through a protein A-Sepharose column containing either nonimmune IgG or MAb-430 as in Fig. 3. The effluent were saved and the gel was extracted as in Fig. 5. The octylglucoside extract and each column fraction were subjected to electrophoresis on duplicate slab gels. One gel, illustrated in this figure, was analyzed by radioautography and densitometric scanning of the radiographic image, while each lane from the other was sliced into 2 mm segments which were analyzed for radioactivity in a scintillation counter as shown in Fig. 7. Panel A: octylglucoside extract. Panel B: nonimmune column effluent. Panel C: nonimmune column retentate. Panel D: MAb-430 column effluent. Panel E: MAb-430 column retentate. Panel F: MAb-430 column retentate trace superimposed on the trace of the nonimmune column effluent. In each panel the position of band 3 is indicated by a triangle (▼).

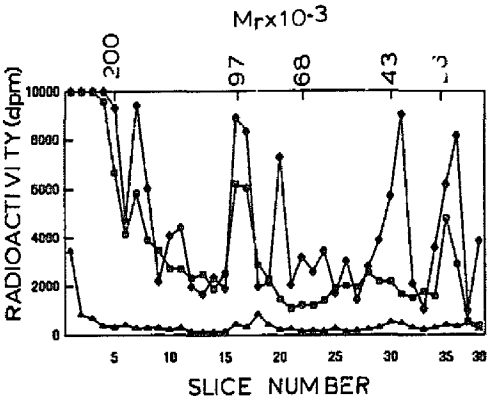

Fig. 7. Retention of ^{125}I-labelled band 3 by MAb-430 column. Analysis by gel slicing and counting. The lanes from the duplicate gel matching that shown in Fig. 6 were sliced into 2 mm segments and analyzed for radioactivity in a scintillation counter. ◆, octylglucoside extract; □, MAb-430 column retentate; ▲, nonimmune column retentate.

found in other than its normal position on the gels.

Discussion

Although our previous work [1–4] and that of others [30–32] has strongly supported the hypothesis that band 3 contains the major, if not only, glucose transporter present in the human erythrocyte membrane, this has remained controversial because evidence has been advanced from several laboratories which has been interpreted as indicating that band 4.5 is the transporter or contains it. The evidence for this is of several kinds, but the primary evidence consists of reports that phospholipid vesicles constituted with purified band 4.5 in their membranes exhibited glucose transport activity [15–18,21]. Apparently, however, no attempts were made to demonstrate what fraction of the total reconstitutable transport activity of unfractionated erythrocyte membrane extracts was recovered in band 4.5. When we investigated this question we found [4] that a very small fraction of the total activity was recovered in band 4.5, but a major fraction, sufficient to account for approx. 95% of the total, was recovered in purified band 3.

It has been reported that polyclonal and monoclonal [21] antibodies raised against purified band 4.5 removed band 4.5 and the reconstitutable glucose transport activity from nondenaturing detergent solutions of purified band 4.5. This circular argument is flawed by the failure of the investigators to determine what fraction of the total reconstitutable glucose transport activity present in unfractionated membrane extracts could be removed by their antibodies. In the present work we have investigated the specificities for proteins and abilities of polyclonal and monoclonal antibodies to band 3 to remove band 3 and reconstitutable glucose transport activity from unfractionated octylglucoside extracts of erythrocyte membranes. We found that these antibodies specifically removed band 3 (Figs. 4–7) and removed approx. 95% of the reconstitutable glucose transport activity from these unfractionated extracts (Fig. 3). On Western blots of unfractionated detergent extracts of erythrocyte membranes (Figs. 1 and 2) we found that a polyclonal antibody to band 4.5, which had been used to probe a cDNA hepatoma line for clones which were presumed to code for the glucose transporter [22], reacted with six discrete protein bands in the 4.5 region; one of these had a M_r or approx. 55000. This does not agree with published data [19–20] which indicated that this antibody reacts with a broad, featureless band. The reason for this discrepancy is not clear, but it does call into question the identity of the protein for which the cDNA may code. Moreover, we have found (Figs. 1 and 2) that both the polyclonal and monoclonal antibodies to band 3 react with a band 4.5 component of M_r approx. 55000. We and others have reported [3,4,30–33] that a M_r 55000 fragment of band 3, which bears the attachment site of the covalent affinity label maltosyl isothiocyanate [3,33], is generated by endogenous proteolysis or by digestion of intact erythrocytes with chymotrypsin. The M_r 55000 chymotryptic fragment has been shown to possess glucose transport activity when reconstituted into phospholipid vesicles [34]. These data strongly suggest that band 4.5 is a mixture of polypeptides, one of which is a fragment of band 3; the presence of this fragment in band 4.5 may confer upon it the small amount of glucose transport activity which has been observed.

It is well accepted that band 3 is the anion transporter of the erythrocyte membrane [35], and evidence exists to support the hypothesis that it is also responsible for water translocation through the erythrocyte membrane [36]. In a previous report we speculated that band 3 might contain a family of transport proteins of closely similar structure [4]. However, the fact that a monoclonal antibody removes essentially all of band 3 strongly suggests, as do sequencing data [37], that band 3 is a single protein. If it is a single protein, rather than similar proteins sharing a common epitope recognized by a monoclonal antibody, it follows that it is a polyfunctional protein which catalyzes the transport of glucose, anions, and water. This possibility is supported by recent publications showing interactions between glucose transport and the transport of anions [38] and osmotic water flow [39] through the erythrocyte and corneal epithelial membranes which are difficult to explain by alternative hypotheses. In these reports it has been shown that cytochalasin B, as well as other inhibitors of glucose transport, are also inhibitory to anion and water translocation. However, we have found [4] that cytochalasin B, although an effective inhibitor of glucose transport into vesicles reconstituted with unfractionated erythrocyte membrane extract, is not an effective inhibitor of glucose transport into vesicles reconstituted with purified band 3. A possible explanation which might reconcile these observations is that the cytochalasin B binding protein may be a transport-modulating protein which is associated with band 3 in the membrane, as are several other proteins [40,41], and which, if if has bound to it a ligand inhibitory to glucose transport, may alter the ability of the transporter to undergo the conformational change which has been proposed to be necessary for its function [42].

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