Biochimica et Biophysica Acta, 769 (1984) 404–410
Elsevier

BBA 71977

ENDOGLYCOSIDASE F CLEAVES THE OLIGOSACCHARIDES FROM THE GLUCOSE TRANSPORTER OF THE HUMAN ERYTHROCYTE

GUSTAV E. LIENHARD, JOSEPH H. CRABB and KATHLEEN J. RANSOME

Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03756 (U.S.A.)

(Received July 26th, 1983)

Key words: Endoglycosidase F; Glucose transporter; (Human erythrocyte)

The glucose transporter from human erythrocytes is a heterogeneously glycosylated protein that runs as a very broad band of average apparent M_r 55000 upon sodium dodecyl sulfate polyacrylamide gel electrophoresis. When the purified preparation of transporter, solubilized in Triton X-100, was treated with endoglycosidase F, much of it ran as a sharp band of M_r 46000 upon electrophoresis. Moreover, endoglycosidase F released 80% of the radioactivity in a preparation of the transporter labeled in its oligosaccharides with galactose oxidase and tritiated borohydride, and almost none of the remaining radioactivity was located in the M_r 46000 band. These results suggest that endoglycosidase F can release virtually all of the carbohydrate linked to the transporter polypeptide. A quantitative analysis of the gels was complicated by partial aggregation of polypeptides that occurs due to prolonged incubation in Triton X-100, but at least 65% of the protein in the preparation of purified transporter is the 46 kDa polypeptide. The extracellular domain of the transporter is very resistant to proteolysis; no cleavage occurred upon treatment of intact erythrocytes with seven different proteases at high concentration.

Introduction

The glucose transporter from human erythrocytes is a glycoprotein that is about 15% carbohydrate by weight [1]. Previously we have shown that there is heterogeneity in the structure of the oligosaccharide chain or chains linked to the transporter polypeptide [2]. There is evidence that these oligosaccharides are branched chains of alternating units of *N*-acetylglucosamine and galactose that are linked to asparagine residues via the typical mannose, *N*-acetylglucosamine core structure [1–6]. Some of the chains probably bear the ABH determinants at the nonreducing end [1–7].

Studies on the structure of the transporter polypeptide would be facilitated by a method for complete removal of the oligosaccharides. Although we have found that the enzyme endo-β-galactosidase cleaves the chains, it is not very satisfactory in this regard, since a substantial portion of many chains remain attached to the protein [2]. Recently, Elder and Alexander described an endoglycosidase (endoglycosidase F) that appears to catalyze the hydrolysis of the glycosidic bond of the *N*-acetylglucosamine($\beta 1 \rightarrow 4$)*N*-acetylglucosamine linked to asparagine in glycoproteins with both high mannose and complex carbohydrates [8]. In this paper we report that this enzyme cleaves the oligosaccharides from the glucose transporter. In addition, we present results indicating that the extracellular domain of the glucose transporter is very resistant to proteolysis.

Materials and Methods

Materials

Two preparations of the glucose transporter were used. The unlabeled transporter was purified

from human erythrocytes according to the procedure that we have recently described [9]. The radiolabeled transporter was a portion of the preparation that we previously characterized [2]; it had been isolated, by a slightly different procedure, from erythrocytes that had been labeled on their surface carbohydrates by treatment with galactose oxidase followed by tritiated borohydride. Endoglycosidase F was a gift from Drs. Elder and Alexander; it was the concentrate from the ACA 54 chromatography step in 50 mM EDTA/50% glycerol described by them [8]. The sources of other reagents were: bovine pancreatic trypsin, pronase, proteinase K, and thermolysin, Boehringer Mannheim; α-chymotrypsin (type CDI) and elastase (type ESFF), Worthingtion Biochemical Corp.; and V8 protease, Miles.

Endoglycosidase F treatment and gel electrophoresis

Except where noted, the preparation of purified glucose transporter (10 μg protein) was incubated for 18 h at room temperature with 5 μl of the enzyme stock (about 0.8 μg protein) in a total volume of 50 μl containing 0.5% Triton X-100/0.05% SDS/75 mM mercaptoethanol/100 mM sodium phosphate/50 mM EDTA, pH 6.1. The reaction mixtures were prepared for electrophoresis by the addition of an equal volume of 10% (w/v) alkyl sulfates/20% (w/v) glycerol/75 mM dithiothreitol/2 mM EDTA/0.004% bromophenol blue/100 mM Tris-HCl, pH 6.8. Electrophoresis was performed according to the procedure of Laemmli [10], with 3 and 10% acrylamide in the stacker and separating gels, respectively. For both sample preparation and electrophoresis a mixture of 12, 14, and 16 carbon alkyl sulfates ('lauryl sulfate' from Pierce Chemical) was substituted for pure sodium dodecyl sulfate, in order to decrease aggregation of the transporter [9].

Gels were stained with Coomassie blue as described in Ref. 11, with the exception that alkyl sulfates were first removed from the gels by a 6 h incubation with isopropanol/acetic acid/water (25 : 10 : 65, by volume). Tube gels were scanned at 550 nm, and the relative areas of peaks were obtained by cutting and weighing copies of the scans. In each instance where quantitation of the various peaks was desired, at least two gels were run, one with twice the volume of sample as the other. It was found that the areas of the various peaks were proportional to the amount of protein loaded; and the values given in Results, which are the averages of at least duplicate determinations, do not differ from the individual values by more than ±5%. The gels that contained radiolabeled transporter were sliced into 2 mm segments; the segments were digested with 600 μl 30% (w/v) hydrogen peroxide in 20 ml glass vials at 75°C for 12 h; and the samples were counted with 10 ml Aquasol II (New England Nuclear).

Protease treatment of intact erythrocytes

Erythrocytes from human blood freshly drawn into citrate were washed three times with 150 mM NaCl/20 mM Hepes, pH 7.8, by centrifugation at 2500 × *g* for 10 min. Washed cells at 20% hematocrit (500 μl total volume) were incubated with 1 mg/ml of each protease for 2 h at 23°C. Calcium chloride at 2 mM was included with the thermolysin. At the end of the incubation the cells were pelleted; the supernatant was aspirated; and the cells were resuspended in 500 μl of the buffer to which was introduced 4 mM EDTA and 1 mM diisopropyl fluorophosphate. After 15 min, the cells were again pelleted and resuspended in 1 ml of the buffer. A small portion of the resuspended cells (80 μl) was added directly to 1 ml of 2% alkyl sulfates/750 mM mercaptoethanol/10% glycerol/1 mM EDTA/0.002% bromophenol blue/50 mM Tris-HCl, pH 6.8, already at 95°C, and the mixture was held at this temperature for 1.5 min. Portions of these samples (7-μl amounts) were used for slab gel electrophoresis and subsequent detection of the transporter by the Western blot procedure. The remaining cells were pelleted and then lysed at 2°C by the addition of 2 ml of 5 mM sodium phosphate, pH 8. The erythrocyte membranes were washed three times with 5 mM sodium phosphate, pH 8, by centrifugation at 27000 × *g* for 15 min and then prepared for electrophoresis by solubilization at 100°C for 2 min in 400 μl of the denaturing buffer given above. Portions of these samples (15 μl) were run on slab gels and stained with Coomassie blue or subjected to the Western blot procedure.

The methods for slab gel electrophoresis and for identification of the transporter by the means of rabbit anti-transporter antiserum in the West-

ern blot procedure were those that we have previously described [12]. The antiserum used was also the one employed previously [12]. We have shown that antibodies in this serum combine with the transporter, by demonstrating that the IgG fraction will immunoprecipitate the cytochalasin B binding sites on the transporter [9]; the immunoprecipitation procedure was similar to the one that we used to characterize the immune serum from another rabbit [13].

Results and Discussion

Endoglycosidase F treatment of the glucose transporter

When the purified glucose transporter, solubilized in 0.5% Triton X-100/0.05% SDS, was treated with endoglycosidase F, its behavior upon gel electrophoresis was dramatically altered. The treated protein migrated as a sharp band with a mobility corresponding to M_r 46000 in contrast to the untreated transporter, which runs as a very broad band in the M_r range 76000 to 49000 (Fig. 1).

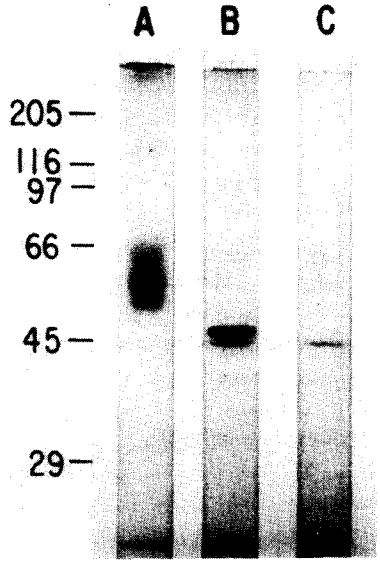

Fig. 1. SDS gel electrophoresis of the purified glucose transporter alone (A, 2.5 μg), of the transporter (2.5 μg) treated with endoglycosidase F (B), and of the endoglycosidase alone (C). See Methods for details. In each case, the protein was incubated for 18 h in Triton X-100/SDS/mercaptoethanol. The scale, which is given in kilodaltons, shows the mobilities of the following standard polypeptides (from the top): rabbit muscle myosin, *E. coli* β-galactosidase, rabbit muscle phosphorylase B, bovine serum albumin, egg albumin, and bovine erythrocyte carbonic anhydrase (kit SDS 6H, from Sigma Chemical).

In order to be certain that this change in the electrophoretic behavior of the transporter is due to the release of the oligosaccharides linked to the transporter, we treated transporter radiolabeled in its oligosaccharides with the glycosidase. The radiolabeled transporter had been prepared by labeling the surface oligosaccharides of intact erythrocytes with galactose oxidase/NaB^3H_4 and then purifying the transporter therefrom [2]. The results in Fig. 2 show that there is virtually no label in the 46 kDa band. Moreover, most of the labeled oligosaccharide in the preparation was released by endoglycosidase F. In two experiments of this type, the percentages of label that remained in the region of the gel between the origin and M_r 40000 after enzyme treatment were 23 and 19% of the total, respectively.

For many purposes, it would be useful to be able to release the oligosaccharides from the transporter without solubilizing the transporter with detergent. Our preparation of purified transporter

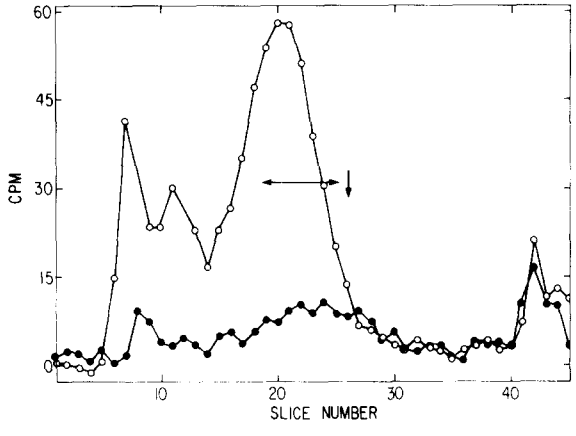

Fig. 2. The effect of endoglycosidase F upon the oligosaccharides of the glucose transporter. Transporter labeled in its carbohydrate (50 μg/ml; 20500 cpm ^3H/ml) was incubated in the absence (○) and presence (●) of the glycosidase, as described in the Methods; and 2.5 μg amounts were applied to each gel. Slices 1–5 comprise the stacking gel. The horizontal and vertical arrows mark the main peaks of Coomassie blue stain for the glycosylated and deglycosylated transporter, respectively. The small peak of label at slice 42 is probably due to radiolabeled lipids that fractionate with the transporter. The radiolabel in slices 6–14 of the untreated transporter is due to aggregates that form under the incubation condition (see Fig. 3).

consists of the transporter reconstituted into a phospholipid bilayer [9]. It was treated with the glycosidase exactly as described in Methods, with the exception that Triton X-100/SDS was omitted from the mixture. In this case, the Coomassie blue profile of the treated transporter upon SDS electrophoresis was virtually identical with that of an untreated control (data not shown), and thus unfortunately no significant cleavage occurred. This result probably cannot be explained by the assumption that the oligosaccharide chains are located within sealed vesicles, since 84% of the sialic acid in our preparation that is released by neuraminidase can be released without solubilization with Triton X-100 (Appleman, J.A. and Lienhard, G.E., unpublished results).

The percentage of glucose transporter polypeptide in the purified preparation

Because the glucose transporter runs as such a broad band upon electrophoresis, it has been difficult to establish the purity of our preparation. The findings that the purified transporter binds 0.70 mol of cytochalasin B per 46 kDa, that N-terminal analysis gave only methionine, albeit in a yield of only 0.4 mol per mol 46000 kDa protein, and that C-terminal analysis with carboxypeptidase A released 0.63 mol valine and 0.09 mol alanine per mol protein [9] indicate that the preparation consists of at least 70% of one polypeptide chain. In an effort to obtain more information concerning purity, gels of the transporter were analyzed quantitatively through spectrophotometric scanning of the Coomassie blue stain and measurement of the areas beneath peaks.

Scans of gels of the glycosidase-treated transporter, of the glycosidase alone, and of the untreated transporter alone are presented in Fig. 3. In addition to the sharp main band at M_r 46000 (band d, Fig. 3A), the scan of the treated transporter shows a number of minor bands due to the polypeptides in the endoglycosidase F preparation (compare Fig. 3A and Fig. 3B) and other bands (designated a, b, c, e) that must arise from the transporter preparation itself. The scan of the untreated transporter (Fig. 3C) shows the main peak centered at M_r 55000 (designated d), as well as other bands (a, b, c) of lower mobility that appear to correspond to bands a–c seen with the glycosidase-treated transporter. Bands a–c probably arise from aggregation of the transporter polypeptide under the solubilizing conditions required for the glycosidase treatment (see below). In the case of each of these, the mobility seen with the glycosidase-treated sample is greater than that seen with the untreated one. This finding and the disap-

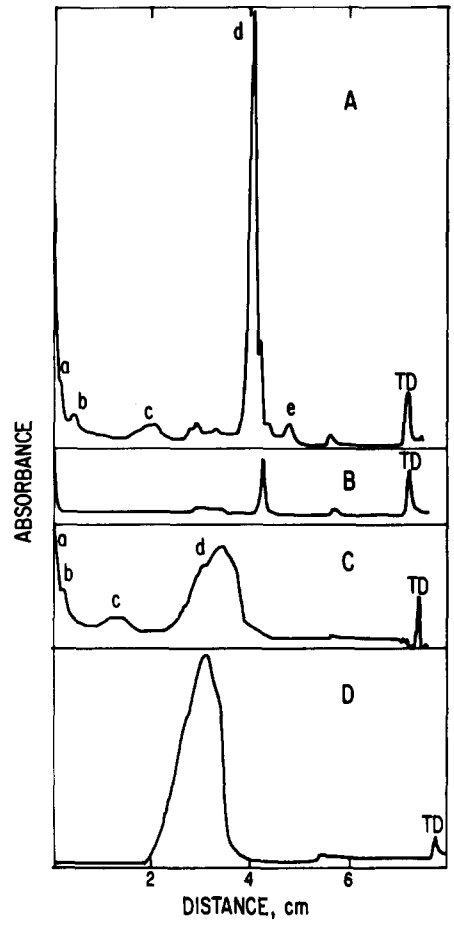

Fig. 3. Spectrophotometric scans of SDS gels. (A) Glucose transporter (5 μg) and endoglycosidase F (0.4 μg) after 18 h incubation in the Triton X-100 mixture (see Methods). (B) Endoglycosidase F (0.4 μg) after 18 h incubation in the Triton X-100 mixture. (C) Glucose transporter (5 μg) after 18 h incubation without glycosidase in the Triton X-100 mixture. (D) Glucose transporter (2.5 μg) after 18 h incubation in 100 mM sodium phosphate/50 mM EDTA, pH 6.1, without Triton X-100/SDS/mercaptoethanol. The gels in A–C were scanned with the fullscale absorbance setting at 2.0 on the recorder; the height of peak d in A is 1.76 absorbance units. The gel in D was scanned with the full scale setting at 0.8 units, and the peak height is 0.34. TD marks the position of the tracking dye.

pearance of radiolabel from this region of the gel (Fig. 2) indicate that some oligosaccharide has been cleaved from the aggregates.

The results shown in Fig. 3, as well as those in Fig. 1 and 2, are those for the limit reaction catalyzed by the glycosidase. When the treatment was carried out under identical conditions, except for 2 h rather than 18 h, the SDS gel of this incubation mixture showed peaks of glycosylated and deglycosylated transporter that were about equal in area (data not given, but these peaks are the same as peak d in Figs. 3C and A, respectively); thus in 2 h about 50% conversion occurred. When the treatment was for 12 h, rather than 18 h, about 90% conversion occurred.

The main polypeptide seen after glycosidase treatment (peak d, Fig. 3A) constitutes 67% of the total area of the scan, after subtraction of the contribution from the glycosidase itself (area of Fig. 3B). Most of the remaining stain occurs in the region of the putative aggregates (a–c, Fig. 3A), which constitute 29% of the total area. The last 4% of the area occurs in peak e. In the case of the untreated transporter, 63% of the area is located in the main peak (d, Fig. 3C) and 37% in the aggregates (a–c, Fig. 3C). The glycosidase treatment did not result in any loss of protein, since the total area of the scan of the treated sample, after subtraction of the contribution of the enzyme (Fig. 3, A − B) is the same as the total area of the scan of the untreated protein (Fig. 3C). Thus, on the basis of this analysis, it is certain that at least 67% of the protein in the purified preparation is the transporter polypeptide. The true value may be higher if, as appears to be the case (see below), the aggregates contain the transporter polypeptide.

Aggregation of polypeptides in the glucose transporter preparation

Fig. 3D shows the scan of a gel of the transporter preparation that has been incubated under the conditions for endoglycosidase F treatment, with the exception that 0.5% Triton X-100/0.5% SDS/75 mM mercaptoethanol was omitted from the incubation mixture. 94% of the stain is located in the major broad band, and it is evident by comparison with Fig. 3C that incubation in the Triton X-100 mixture leads to the formation of a series of aggregates. The band designated c has a mobility corresponding to M_r 120000 and is thus probably a dimer. We have attempted, without success, to find conditions under which the transporter preparation would be solubilized and so be a substrate for the glycosidase, but would not show aggregates upon electrophoresis. It seems worthwhile to state briefly the results and conclusions without providing experimental details.

Aggregation is not an artifact of the method of sample preparation, since when Triton X-100/mercaptoethanol was added to the transporter just after the 10% alkyl sulfate mixture (see Methods), no aggregation was seen. The aggregation requires more than several minutes, since when the 0.5% Triton X-100/0.05% SDS/75 mM mercaptoethanol was added just before the 10% alkyl sulfate mixture, no aggregation occurred. It seems unlikely that the aggregation is due to the formation of disulfide bonds because (i) the SH concentration in the incubation mixture, measured colorimetrically [14], did not change during the 18 h period; (ii) preparation of samples for electrophoresis with 100 mM dithiothreitol, rather than the normal 37 mM, with or without heating at 100°C for 2 min, did not significantly decrease the amount of the aggregates; (iii) the extent of aggregation was the same when the 18 h incubation mixture contained transporter at 100 and 200 μg/ml. It also seems unlikely that aggregation is due to the formation of reactive radicals or aldehydes arising from the oxidation of unsaturated lipids in the preparation, since neither the omission of the reductant mercaptoethanol from the incubation mixture nor the inclusion of 100 mM semicarbazide, an aldehyde trapping reagent [15], substantially altered the degree of aggregation.

A number of other detergents were examined in an attempt to find one in which aggregation did not occur. However, roughly the same extent of aggregation as seen with Triton X-100 was observed when the transporter, at 100–200 μg/ml, was incubated for 18 h in 100 mM sodium phosphate/50 mM EDTA/50–75 mM mercaptoethanol, pH 6.1, with the following: 1% octyl β-D-glucopyranoside; 0.5% octaethyleneglycol *n*-dodecyl ether; 0.5% *p*-(1,1,3,3-tetramethylbutyl)phenoxynonaoxyethylene glycol (purified from Triton X-100); 1% *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate; 1% *N*-tetradecyl-*N*,*N*-di-

methyl-3-ammonio-1-propanesulfonate; 1% *N*-hexadecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate; and 1.25% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate.

Protease sensitivity of the extracellular domain of the glucose transporter

If the oligosaccharides were linked to the polypeptide chain near its amino or carboxy terminus, protease treatment of the extracellular domain of the glucose transporter, where the carbohydrates are located [2], might release them but leave a major portion of the polypeptide intact. Moreover, as an initial step in the elucidation the transmembrane folding pattern of the glucose gransporter polypeptide, it would be useful to be able to introduce specific cleavages at sites in the extracellular domain. The effect of protease treatment of intact erythrocytes upon the transporter polypeptide cannot be determined simply by SDS electrophoresis of the membrane proteins followed by Coomassie blue staining because the transporter constitutes only 3% of the total membrane protein and runs as such a broad band [9]. However, very small amounts of transporter on SDS gels can be detected through the use of antiserum against the transporter [12] in the Western blot procedure [16]. In this procedure the polypeptides are electrophoretically transferred from the gel to nitrocellulose paper, and the blot is treated sequentially with rabbit antiserum against the transporter and ^{125}I-labeled goat anti-rabbit IgG.

The results of treating intact erythrocytes with chymotrypsin, trypsin, elastase, V8 protease, thermolysin, pronase, and proteinase K are shown in Fig. 4. In agreement with the literature [17] chymotrypsin, thermolysin, and pronase cleaved band 3 of the erythrocyte membrane to yield a 60 kDa fragment, whereas trypsin did not. Proteinase K also split band 3 (upper panel, Fig. 4). On the other hand the glucose transporter was resistant to all of the proteases (lower panel, Fig. 4). In each case, the intensity of the transporter band upon the autoradiogram is about the same as the control; and it was found by cutting and counting the radioactive transporter band and an adjacent blank area that the lavel in the lane from each protease-treated sample was 70% or more of the corresponding control value. Moreover, no new polypeptides of lower molecular weight, which would be expected if cleavage had occurred, are evident in the autoradiograms.

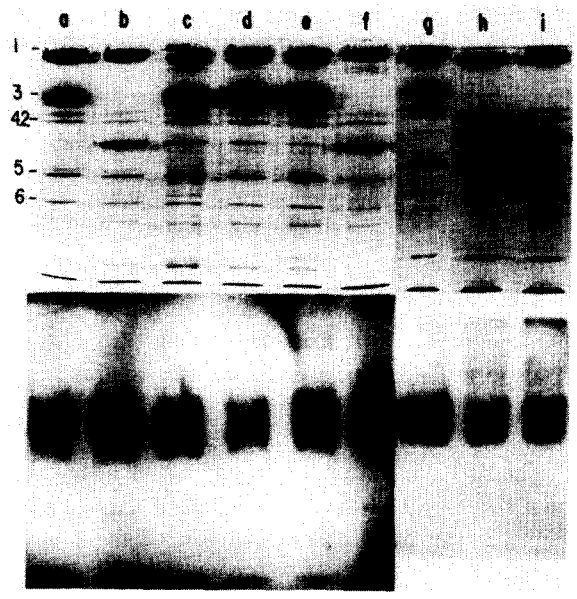

Fig. 4. The effect of external proteases upon the glucose transporter. Upper panel. Coomassie blue stained SDS gels of membranes isolated from human erythrocytes that had been incubated with: (a) no addition, (b) chymotrypsin, (c) trypsin, (d) elastase, (e) V8 protease, (f) thermolysin, (g) no addition, (h) pronase and (i) proteinase K. Lanes a–f and g–i are separate gels. The band numbers on the left are those according to Steck [21]. Lower panel. Autoradiograms of the nitrocellulose blots of SDS gels of the total protein from erythrocytes incubated as described directly above. The blots were treated with rabbit antiserum against the glucose transporter and ^{125}I-goat anti-rabbit IgG. Lanes a–f and g–i are separate blots. The major labeled band has the mobility expected for the glucose transporter. See Methods for the details.

The blots shown in the lower panel of Fig. 4 are those of whole cells denatured at 100°C. These were used in order to avoid proteolysis, known to occur at the cytoplasmic domain of the transporter [18], by residual protease during isolation of the erythrocyte membranes. In fact, the membranes isolated after the protease treatments were also carried through the Western blot procedure; and these also showed no cleavage of the transporter, with the exception of the appearance of a very weak band at about M_r 30000 in the case of trypsin (data not presented). It is worth noting that these experiments were performed with a high

concentration of each protease (1 mg/ml) that was only slightly less than the concentration of the erythrocyte membrane protein (20% v/v cells is equivalent to about 1.6 mg/ml membrane protein [19]). Thus, the extracellular domain is very resistant to proteolysis.

Conclusions

Our results show that endoglycosidase F cleaves the complex oligosaccharides from the glucose transporter polypeptide. Since the anion transporter of the human erythrocyte, band 3, probably bears the same type of oligosaccharides [5,6,20], the endoglycosidase may prove able to release the oligosaccharides from this protein also. The finding that the extracellular domain of the glucose transporter is resistant to proteolytic cleavage is disappointing, since it appears that large peptides cannot be obtained in this way. However, the experimental approach of combining protease treatment with the Western blot procedure may be useful in the investigation of other membrane proteins that are present in small amounts.

Acknowledgement

We are deeply indebted to Drs. John H. Elder and Stephen Alexander of the Research Institute of Scripps Clinic for the gift of purified endoglycosidase F. This research has been supported by a grant (GM 22996) from the National Institutes of Health.

References

1 Sogin, D.C. and Hinkle, P.C. (1978) J. Supramol. Struct. 8, 447–453
2 Gorga, F.R., Baldwin, S.A. and Lienhard, G.E. (1979) Biochem. Biophys. Res. Commun. 91, 955–961
3 Jarnefelt, J., Rush, J., Li, Y.-T. and Laine, R.A. (1978) J. Biol. Chem. 253, 8006–8009
4 Krusius, T., Finne, J. and Rauvala, H. (1978) Eur. J. Biochem. 92, 289–300
5 Fukuda, M.N., Fukuda, M. and Hakomori, S. (1979) J. Biol. Chem. 254, 5458–5465
6 Mueller, T.J., Li, Y.-T. and Morrison, M. (1979) J. Biol. Chem. 254, 8103–8106
7 Finne, J. (1980) Eur. J. Biochem. 104, 181–189
8 Elder, J.H. and Alexander, S. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 4540–4544
9 Baldwin, S.A., Baldwin, J.M. and Lienhard, G.E. (1982) Biochemistry 21, 3836–3842
10 Laemmli, U.K. (1970) Nature 227, 680–685
11 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
12 Lienhard, G.E., Kim, H.H., Ransome, K.J. and Gorga, J.C. (1982) Biochem. Biophys. Res. Commun. 105, 1150–1156
13 Baldwin, S.A. and Lienhard, G.E. (1980) Biochem. Biophys. Res. Commun. 94, 1401–1408
14 Riddles, P.W., Blakeley, R.L. and Zerner, B. (1979) Anal. Biochem. 94, 75–81
15 Jencks, W.P. (1959) J. Am. Chem. Soc. 81, 475–481
16 Burnette, W.N. (1981) Anal. Biochem. 112, 195–203
17 Cabantchick, Z.I., Knauf, P.A. and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239–302
18 Baldwin, S.A., Lienhard, G.E. and Baldwin, S.A. (1980) Biochim. Biophys. Acta 599, 699–714
19 Zoccoli, M.A. and Lienhard, G.E. (1977) J. Biol. Chem. 252, 3131–3135
20 Tsuji, T., Irimura, T. and Osawa, T. (1980) Biochem. J. 187, 677–686
21 Steck, T. (1974) J. Cell Biol. 62, 1–19