SELECTIVE EXTRACTION OF AN INTRINSIC FAT-CELL PLASMA-MEMBRANE GLYCOPROTEIN BY TRITON X-100

Correlation with [^{3}H]cytochalasin B binding activity

Carla J. GREENBAUM, Michael F. SHANAHAN, Dennis J. PILLION and Michael P. CZECH
Section of Physiological Chemistry, Division of Biology and Medicine, Brown University, Providence, RI 02912, USA

Received 1 September 1977

1. Introduction

Cytochalasin B is an extraordinarily potent inhibitor of glucose-transport systems which operate by facilitated diffusion in a variety of mammalian cell types studied [1–10]. In red cells, most of the high-affinity binding of cytochalasin B can be inhibited by addition of unlabelled glucose, indicating competition for similar binding sites [11]. In fat cells [10] and fibroblasts [12] kinetics characteristic of competitive interaction between cytochalasin B and glucose are not observed, although binding of [^{3}H]cytochalasin B to high affinity sites does parallel transport inhibition [10]. These findings have suggested that high affinity cytochalasin B-binding sites may be related to one or more protein components of the plasma-membrane hexose-transport system in a given cell type.

We have recently found [13] that all of the fat-cell plasma-membrane proteins, except the 94 000 and 78 000 dalton glycoproteins and a minor 56 000 dalton fraction, are eluted from the membrane upon treatment with dimethylmaleic anhydride (DMMA) as described for erythrocyte membranes [14,15]. This extracted membrane preparation was shown to retain stereospecific hexose-transport activity which was sensitive to inhibition by cytochalasin B. Similarly, [^{3}H]cytochalasin B binding to this membrane exhibited a dissociation constant identical to that observed with intact fat-cell plasma membranes. The present report describes our development of methodology designed to further differentially elute protein components from the DMMA-extracted adipocyte membrane and to correlate such effects with [^{3}H]-cytochalasin B-binding capacity.

2. Materials and methods

2.1. *Isolation of fat cells and membranes*

White fat cells were obtained [13,16] by enzymatic digestion of the parameterial adipose tissue of 200–700 g female rats (Charles River CD strain and NJ Camm Research strain) fed laboratory chow ad libitum, as previously described. Tissue, 8–10 g/10 ml Krebs-Ringer phoshate buffer containing 3% bovine serum albumin (Armour Lot #N10101), and 1 mg/ml crude collagenase (Clostridium histolyicum, Worthington), was incubated for 60 min at 37°C. The Krebs-Ringer phosphate buffer (pH 7.4) contained 128 mM NaCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, 5.2 mM KCl and 10 mM Na_2HPO_4. The crude preparations of plasma membranes were prepared by homogenization of cells in ice-cold buffer A in a loose-fitting glass homogenizing tube with five up-and-down strokes using a Teflon pestle [10]. The homogenate was centrifuged at 8500 × *g* for 10 min and the supernatant, as well as a small amount of fluffy white material collected from the surface of the brown (mitochondrial) pellet, was then centrifuged at 40 000 × *g* for 40 min and the resulting pellet resuspended in ice-cold 1 mM EDTA, 5 mM Tris (pH 7.5).

2.2. *Glucose transport and [^{3}H]cytochalasin B binding in membrane vesicles*

Assay of D-[^{3}H]glucose uptake by membranes was routinely performed on 50 μl of membrane suspensions in Krebs-Ringer phosphate buffer in glass test tubes (12 × 100 mm) as described in detail previously [10]. Assay of [^{3}H]cytochalasin B binding to membranes was performed essentially as described [10]. The membranes were incubated at 4°C instead of room temperature in 50 μl indicated buffer and 0.5 μl or 1 μl of [^{3}H]cytochalasin B in ethanol was added. After 30 min 2 ml ice-cold buffer were added and the mixture quickly decanted onto HA Millipore filters and the filters washed with 4 ml of ice-cold buffer.

2.3. *Sodium dodecyl sulfate–polyacrylamide gel electrophoresis*

Gel electrophoresis of plasma membranes was performed using polyacrylamide gels prepared as described [18] except that 7.5% acrylamide and half the amount of cross-linker were used. Protein was estimated by the method [17] using bovine serum albumin as a standard.

2.4. *Membrane extractions*

DMMA-extracted membranes were prepared as described [13]. For Triton extractions, frozen DMMA-extracted membranes were thawed and centrifuged at 40 000 × *g* for 40 min. The pellet was resuspended in 500 μl 36 mM sodium phosphate buffer, pH 7.4. 0.5% Triton in this buffer was added in 5-fold excess to the preparation, and incubated at 4°C for 45 min. The material was centrifuged as above and the resulting pellet washed with Krebs-Ringer phosphate buffer, and centrifuged again to remove the Triton X-100. This pelleted material was resuspended in Krebs-Ringer phosphate buffer while the supernatant was diluted 1:1 with the same buffer and passed through a column (5 × 1 cm) of bio-beads (Bio Rad) 10–15 times. The solution was then concentrated on an Amicon PM 30 filter to vol. 1 ml. For recombination of Triton X-100-solubilized proteins and residual pelleted material, the 1 ml concentrated solution of solubilized protein was added to one-half the volume of the resuspended, pelleted. extracted, membrane material and the combination was subjected to two 5 s homogenization–sonication sessions with a Brinkman Polytron. The membranes were diluted with Krebs-Ringer phosphate buffer, centrifuged for 40 min at 40 000 × *g*, and finally resuspended in the same buffer.

3. Results

Figure 1 presents dodecyl sulfate gels stained with Coomassie Blue or Schiff reagent, containing the various membrane preparations used in the present studies. As previously reported in detail [13], incubation of fat-cell plasma membranes with DMMA followed by centrifugation resulted in a membrane pellet containing only two major glycoprotein bands which migrate at the 94 000 and 78 000 dalton regions of dodecyl sulfate gels (A). Often we have also observed a minor band of 56 000 daltons which always constitutes 5% or less of the DMMA-extracted membrane protein; however in the gels depicted in fig.1 this fraction is barely detectable. Incubation of the DMMA-extracted membrane preparation with 0.5% Triton X-100 at 4°C for 45 min prior to centrifugation resulted in a markedly decreased content of the 94 000 dalton glycoprotein relative to the 78 000 dalton fraction staining intensity with respect to both Coomassie Blue or Schiff reagent (B gels). It was crucial that the incubation be performed at low temperature since treatment at room temperature resulted in non-selective extraction of both glycoprotein species from the membrane (not illustrated). No consistent increase in protein material which failed to penetrate the gel was observed in these experiments and a parallel increase in the 94 000 dalton glycoprotein fraction could be recovered in the supernatant fraction subsequent to extraction of membranes with Triton X-100 and centrifugation.

When the supernatant fraction was diluted, passed through a column of SM-2 bio beads to remove Triton X-100, concentrated using an Amicon PM 30 filter, and recombined with the Triton X-100-extracted pellet, dodecyl sulfate gels indicated reincorporation of 94 000 dalton material into the membrane fraction (C gels). Increased staining intensity at 94 000 daltons compared to the membranes extracted with Triton X-100 was observed using both Coomassie Blue or Schiff reagent. The degree to which reincorporation was obtained varied somewhat in various experiments

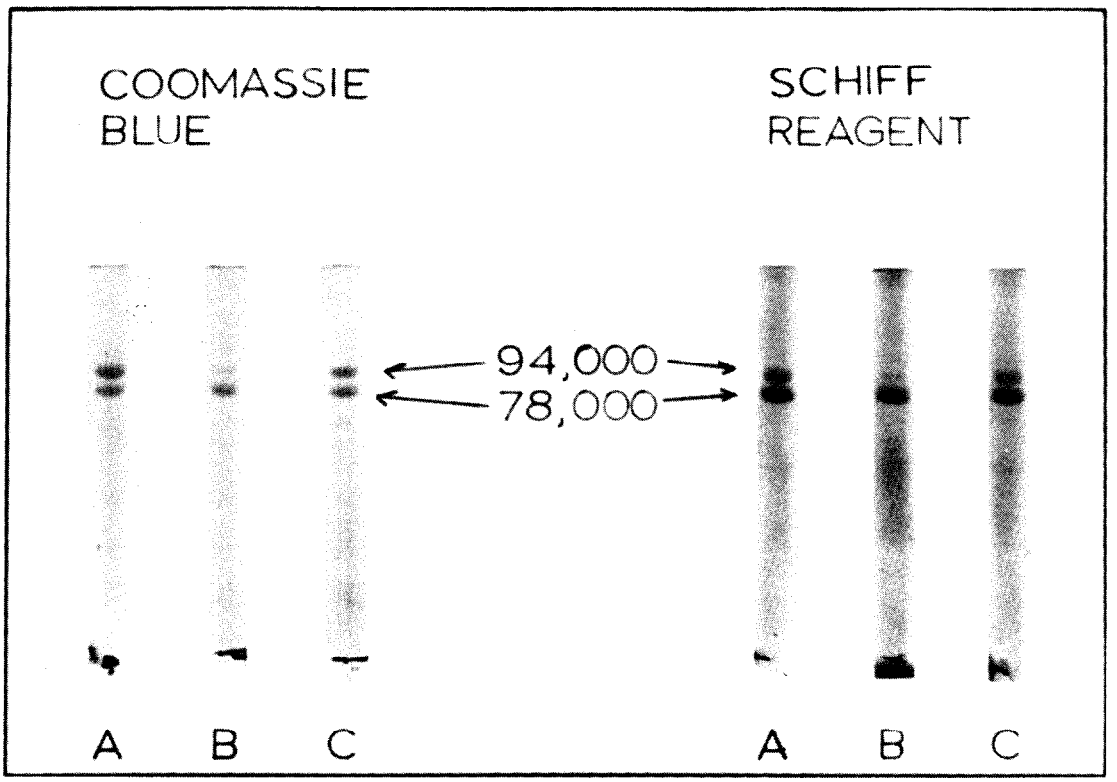

Fig.1. Selective extraction of the 94 000 dalton glycoprotein fraction from the DMMA-extracted fat-cell plasma membrane and its reincorporation into the membrane. (A) DMMA-treated fat-cell plasma membrane. (B) Membrane pellet A after extraction with Triton X-100. (C) Membrane pellet B after recombination with Triton X-100 solubilized membrane fraction, centrifugation and washing.

and in the gels depicted in fig.1. Reversal to the control condition was extensive but not entirely complete. Table 1 provides a quantitative estimate of the average relative staining intensities of the two glycoprotein fractions in the various membrane preparations for 3 experiments. While control DMMA-extracted membranes exhibited about twice as much 94 000 dalton glycoprotein material compared to that at 78 000 daltons, extraction with Triton X-100 yielded a membrane preparation containing two-thirds of the protein in the lower molecular weight fraction. The Triton X-100 extracted membrane pellet which was sonicated in the presence of the supernatant fraction reverted to nearly the same relative protein concentrations observed in the original DMMA-extracted membrane.

Since the Triton X-100 extraction procedure described above was found to dramatically enrich the DMMA-extracted membrane preparation in the 78 000 dalton glycoprotein fraction, it was interesting to determine the effect of this procedure on the specific activity of hexose transport system activity in this membrane. In the present studies it was found that the Triton X-100-extracted membrane preparation was devoid of hexose-transport activity as was the recombined preparation. In order to determine whether specific [^{3}H]cytochalasin B binding activity was altered by the membrane extraction and reincorporation procedures described in this report, a rapid filtration method was used to assay high affinity [^{3}H]cytochalasin B binding to each of the extracted membrane preparations. Binding to control DMMA-extracted membranes on a per mg protein basis is taken as 100% and binding to the other

Table 1
Correlation of [^{3}H]cytochalasin B binding with the content of the 94 000 dalton glycoprotein fraction in various extracted fat-cell plasma-membrane preparations

Membrane preparation	[^{3}H]cytochalasin B bound	Relative coomassie intensity (% contribution to total)	
	% control	94 000 dalton band	78 000 dalton band
Control DMMA-extracted membrane	100	66	34
Triton X-100-extracted membrane pellet	43 ± 11	32	68
Triton X-100-extracted membrane recombined with solubilized protein	81 ± 18	62	38

Membranes (20–50 μg protein) were incubated in 50 μl Krebs-Ringer phosphate buffer at 4°C for 30 min in the presence of 0.26 μM [^{3}H]cytochalasin B and binding assayed by rapid filtration. The values presented are the means ± SE of three separate experiments with different membrane preparations and are normalized as percents of the values obtained for DMMA-extracted membranes. All values were calculated on a per mg membrane protein basis. Membranes were electrophoresed on dodecyl sulfate gels, stained with Coomassie Blue and densitometric patterns determined. The relative staining intensity for each glycoprotein band was then determined based on its percent contribution to the combined staining intensity of both bands

preparations is given as the percent of this control value to normalize the data from the individual experiments. Binding per mg membrane protein was reduced by an average of almost 60% in the Triton X-100-extracted membranes, while reincorporation of the 94 000 dalton glycoprotein restored [^{3}H]cytochalasin B binding to within 20% of control values.

4. Discussion

Previous studies with erythrocyte membranes demonstrated selective extraction of extrinsic proteins by agents such as NaI, dimethylmaleic anhydride, or alkali treatment [14], while intrinsic proteins were selectively eluted from the membrane with Triton X-100 [20]. The present studies with fat-cell plasma membranes are striking in that the two major glycoprotein fractions which are retained in the membrane subsequent to treatment with DMMA exhibited differential sensitivity to elution by Triton X-100. In red cells the DMMA-resistant glycoprotein components are uniformly extracted from the membrane by the detergent [20]. The utility of these sequential extraction procedures with fat-cell membranes is the reduction of the number of protein components from ten or more major peptide bands in the native plasma membrane [21] to one major glycoprotein fraction of 78 000 daltons in the doubly-extracted membrane preparation (gel B in fig.1). This simple procedure should be of great value in attempts to both purify fat-cell plasma-membrane glycoprotein fractions to homogeneity and the characterize these components structurally and enzymatically.

While the loss of transport activity paralleled the extraction of the 94 000 dalton fraction by Triton X-100, the possibility that the detergent irreversibly denatured and inhibited the transport system cannot be ruled out. The failure to find transport activity in preparations made by recombining Triton X-100-extracted membranes with the supernatant from such extractions makes precise interpretation impossible. It seems likely that in spite of the apparant association of the solubilized 94 000 dalton glycoprotein fraction with the extracted membrane under our recombination conditions, proper reconstitution of this fraction to its native orientation within

the membrane structure is not achieved. We are thus currently pursuing solubilization and reconstitution experiments similar to those recently reported for the red-cell D-glucose transport system [22,23].

In contrast to the results obtained with transport activity, [^{3}H]cytochalasin B binding to the various membrane preparations did parallel, to a remarkable degree, the presence of the 94 000 dalton glycoprotein fraction. The binding values reported in table 1 are standardized on a per mg membrane protein basis and provide strong support for the notion that [^{3}H]cytochalasin B-binding sites are selectively eluted from the membrane by the Triton X-100 extraction procedure. We have previously characterized the binding of [^{3}H]cytochalasin B to intact [10] and DMMA-extracted [13] fat-cell plasma membranes by Scatchard analysis and, assuming the presence of both high- and low-affinity binding sites, have shown that most of the binding observed under the conditions of the present experiments involves high affinity sites. It is also noteworthy that these high-affinity binding sites involve only about 5% of the protein molecules in the DMMA-extracted membrane, assuming mol. wt 100 000, which eliminates the possibility that the 94 000 dalton glycoprotein fraction is a homogeneous, cytochalasin B-binding species. Nonetheless, the data are consistent with the hypothesis that this fraction contains such a binding protein and that this component may be intimately related to the hexose-transport activity of the fat-cell plasma membrane.

Acknowledgements

This investigation was supported by a grant (AM-17893) from the National Institutes of Health. M.P.C. is a recipient of a Research Career Development Award (AM-00249) of the USPHS. The authors wish to thank Gerlinde Celona for excellent secretarial assistance.

References

[1] Mizel, S. B. and Wilson, L. (1972) J. Biol. Chem. 247, 4102–4105.
[2] Estensen, R. D. and Plagemann, P. G. W. (1972) Proc. Natl. Acad. Sci. USA 69, 1430–1434.
[3] Kletzien, R. F., Perdue, J. F. and Springer, A. (1972) J. Biol. Chem. 247, 2964–2966.
[4] Cohn, R. H., Banerjee, S. D., Shelton, E. R. and Bernfield, M. R. (1972) Proc. Natl. Acad. Sci. USA 69, 2865–2869.
[5] Zigmond, S. H. and Hirsch, J. G. (1972) Science 176, 1432–1434.
[6] Kletzien, R. F. and Perdue, J. F. (1973) J. Biol. Chem. 248, 711–719.
[7] Czech, M. P., Lynn, D. G. and Lynn, W. S. (1973) J. Biol. Chem. 248, 3636–3641.
[8] Bloch, R. (1973) Biochemistry 12, 4799–4801.
[9] Taverna, R. D. and Langdon, R. G. (1973) Biochim. Biophys. Acta 323, 207–219.
[10] Czech, M. P. (1976) J. Biol. Chem. 251, 2905–2910.
[11] Lin, S. and Spudich, J. A. (1974) J. Biol. Chem. 249, 5778–5783.
[12] Atlas, S. J. and Lin, S. (1976) J. Cell. Physiol. 751–756.
[13] Shanahan M. F. and Czech, M. P. (1977) J. Biol. Chem. in press.
[14] Steck, T. L. and Yu, J. (1973) J. Supramol. Struct. 1, 220–232.
[15] Kahlenberg, A. (1976) J. Biol. Chem. 251, 1582–1590.
[16] Rodbell, M. (1964) J. Biol. Chem. 239, 375–380.
[17] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[18] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[19] Zacharius, R. M., Zell, T. E., Morrison, J. H. and Woodlock, J. J. (1969) Anal. Biochem. 30, 148–152.
[20] Yu, J., Fischman, D. A. and Steck, T. L. (1973) J. Supramol. Struct. 1, 233–247.
[21] Czech, M. P. and Lynn, W. S. (1973) J. Biol. Chem. 240, 3892–3898.
[22] Kasahara, M. and Hinkle, P. C. (1976) Proc. Natl. Acad. Sci. USA 73, 396–400.
[23] Zala, C. A. and Kahlenberg, A. (1976) Biochem. Biophys. Res. Commun. 72, 866–874.