Vol. 109, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1982 Pages 408-413

IDENTIFICATION OF THE GLUCOSE TRANSPORT PROTEIN OF THE MICROVILLOUS MEMBRANE OF HUMAN PLACENTA BY PHOTOAFFINITY LABELLING

Larry W. Johnson and Carl H. Smith

Departments of Pediatrics and Pathology
Washington University School of Medicine
and St. Louis Children's Hospital
St. Louis, Missouri 63110

Received October 12, 1982

The initial step in transfer of glucose from mother to fetus is facilitated diffusion transport across the microvillous membrane of the placental syncytium (1). We have used ^{3}H-cytochalasin B as a photoaffinity label to identify the transport protein involved. Two binding proteins were present, one of which is apparently the glucose transport protein and one of which is actin. The two were identified by competition labeling with D-glucose, and cytochalasin E. They were separated by selective extraction with dimethyl maleic anhydride. The glucose transport protein is apparently a single molecular species of 52,000 molecular weight.

Transport of nutrients from mother to human fetus occurs almost exclusively via the placental trophoblast. To help elucidate the mechanism of transport of essential glucose across the placenta we have prepared plasma membrane vesicles from the first-encountered membrane of this tissue, the microvillous membrane of the syncytium. We have demonstrated that this membrane possess a facilitated diffusion system for glucose whose properties can account for the supply of glucose to the fetus (1).

Recently a new method has emerged for identification of proteins involved in glucose transport. It uses a well characterized competitor of glucose transport, cytochalasin B, as a photoaffinity radiolabel for red blood cells (2,3). In the belief that this new method might identify the transport protein of the microvillous membrane of placenta, we have applied it to membrane vesicles derived from fresh human placenta. We here report successful labeling and separation of two protein bands on polyacrylamide gel electrophoresis. Cytochalasin B binding to only one of these is glucose sensitive, and hence we believe it to be from the glucose transport site. The problem of

0006-291X/82/220408-06$01.00/0

labeling of actin, abundant in this membrane, gave the work additional interest. This problem was overcome by dimethylmaleic anhydride extraction and cytochalasin E competition labeling.

MATERIALS AND METHODS

Membrane Preparation

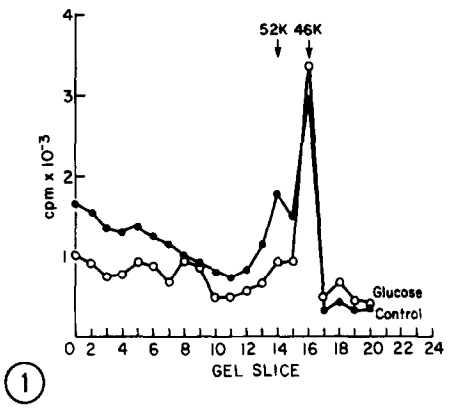
Human placentas were obtained immediately after delivery and microvillous membrane was prepared, as previously described, by gentle shearing and differential centrifugation. The final pellet consisting of closed vesicles of microvillous membrane has been characterized and found to contain right-side-out microvilli which have sealed to form vesicles (4).

Photoaffinity Labeling

Microvillous membrane vesicles were suspended in 5 mM NaH_2PO_4 1 mM EDTA, 250 mM sucrose pH 7.4 at approximately 1 mg of protein/ml. Aliquots containing 200 µg of protein were removed and equilibrated with 500 mM, D or L glucose or with 500 mM sorbitol. In order to introduce [^{3}H]-cytochalasin B in the absence of organic solvent it was dried as a thin film to which vesicle suspensions were then added. After vigorous vortex mixing the suspensions were incubated 30 minutes at 4°C. Photoaffinity crosslinking was initiated by exposing the [^{3}H]-ligand-bound vesicles to the intense light of a high-pressure mercury arc lamp of 450 watts (Canrad-Hanovia, Inc., Newark, N.J.) for 20 minutes at 15 cm distance. Cooling of both lamp and sample was accomplished by blowing a stream of 4°C air through the apparatus. The samples were diluted immediately with 1 ml of 10 mM Tris HCl, 1 mM EDTA, 320 mM sucrose pH 7.4 and centrifuged 40 minutes 15,000 x g to isolate the membranes. Electrophoresis was performed on 8% polyacrylamide slab gels containing 0.1% sodium dodecyl sulfate (5) and gels were stained with Coomassie blue. The slab gels were dried onto filter paper *in vacuo* and sliced into 5 mm segments for scintillation counting in H_2O/Protosol/Omnifluor 1:10:50 (New England Nuclear Corp., Boston, MA).

RESULTS

Membrane vesicles were equilibrated with 2 x 10^{-6}M [^{3}H]-cytochalasin B. When membrane vesicles with bound [^{3}H]-cytochalasin B were then irradiated with a mercury arc lamp for up to 20 minutes increasing amounts of [^{3}H]-label became covalently attached. Polyacrylamide gel electrophoresis demonstrated that the attachment was primarily in two peptide bands, with apparent molecular weights of 46,000 and 52,000 (Figure 1). Moreover, if D-glucose was present as a ligand competitor during photolysis, cytochalasin B binding to the 52,000 molecular weight band was markedly decreased while that to the 46,000 molecular weight band was unaffected. Sorbitol or L-glucose (data not shown), did not compete with [^{3}H]-cytochalasin B for the attachment site during photolabeling. The minor labeling in the entire upper portion of the



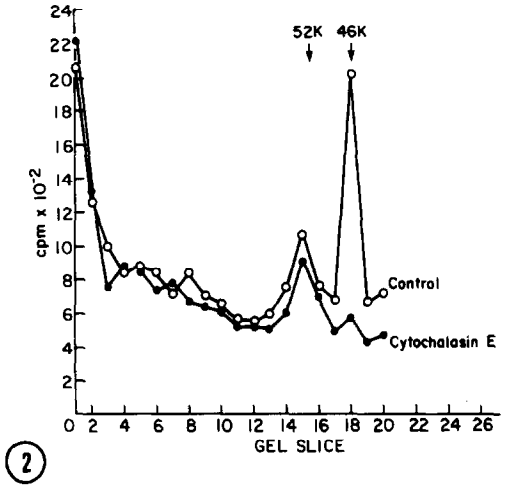


Figure 1. Photo-labeled protein distribution after polyacrylamide gel electrophoresis. [^{3}H]-cytochalasin B was bound to membrane in the presence of sorbitol (control) or D-glucose and then irradiated to activate covalent linkage. Glucose prevents labeling of the 52,000 molecular weight protein but not of the 46,000 molecular weight protein.

Figure 2. Effects of cytochalasin E on photo-labeling membrane proteins with [^{3}H]-cytochalasin B. Competition virtually eliminated labeling of the 46,000 molecular weight protein, but had little effect on the 52,000 molecular weight protein.

gels may be due to aggregation of transport protein in sodium dodecyl sulfate as was seen with erythrocyte transporter (6).

Placental microvilli give a dense Coomassie blue-stained band believed to be actin (7). Its mobility coincides with the 46,000 molecular weight affinity labeled band. We therefore attempted to decrease cytochalasin B affinity labeling of the 46,000 molecular weight band by adding an excess (10^{-4} M) of cytochalasin E before photolysis. This did eliminate the large peak of radioactivity covalently attached to that material (Figure 2). The 52,000 molecular weight band, however, was unaffected by cytochalasin E. D-glucose still reduced the labeling of the 52,000 molecular weight peptide in a manner unaffected by the presence of cytochalasin E (data not shown).

To further elucidate the nature of the two cytochalasin B binding bands, we attempted to extract them selectively with dimethyl maleic anhydride. Membrane vesicles were first photo-labeled in the presence or absence of D-glucose and then treated with solid dimethyl maleic anhydride (2 mg/200 µg of protein) as previously described (7). The extracted proteins showed only

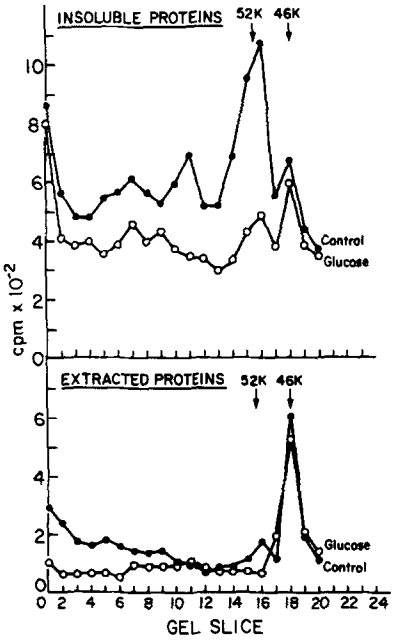


Figure 3. Dimethyl maleic anhydride extraction of photo-labeled protein. Following photo-labeling in the presence or absence of D-glucose, membranes were frozen, thawed, and extracted with dimethyl maleic anhydride. The glucose-sensitive protein at 52,000 molecular weight was not extracted, whereas the 46,000 molecular weight protein was largely dissociated from membrane by this treatment. Minor peaks of higher molecular weight were labelled inconsistently.

one dominant band of radioactivity at 46,000 molecular weight (Figure 3). This band was unaffected by the presence of glucose during photolabeling. The residual non-extracted proteins showed a dominant band of radioactivity at 52,000 molecular weight with only a small band remaining at 46,000 molecular weight. D-glucose competition during photolabeling greatly decreased the labeling of the major non-extracted 52,000 molecular weight band.

DISCUSSION

The reversible binding of cytochalasin B to glucose transport protein in microvillous membrane has previously been inferred from two lines of evidence: 1. [^{3}H]-cytochalasin B bound to the membranes can be displaced by D-glucose (8, and unpublished observation of the authors). 2. Cytochalasin B at low concentrations inhibits glucose transport across the membranes (1,9). By photoaffinity labelling with [^{3}H]-cytochalasin B we have now identified what is apparently the glucose transport protein in this membrane.

We have previously shown that the microvillous membrane contains longitudinal microfillaments and yields a dominant Coomassie blue-stained band which has the same molecular weight as actin. This protein as well as the filaments can be extracted from the membrane with dimethyl maleic anhydride (7). The observations that two distinct bands of apparent molecular weights 52,000 and 46,000 were photolabeled, and that one of them coincided with the stained actin band, suggested that both actin and the glucose transport protein had been labelled. This was substantiated by three pieces of evidence: 1) D-glucose, stereospecifically, competed for the binding of [^{3}H]-cytochalasin B to the 52,000 molecular weight protein but not the 46,000 molecular weight protein. 2) Cytochalasin E, known to bind to actin (10), did not affect ^{3}H-cytochalasin B binding to the 52,000 molecular weight band but competed strongly for the 46,000 molecular weight site. 3) Dimethyl maleic anhydride, which extracts actin as well as extrinsic membrane proteins (7,11), selectively removed the 46,000 molecular weight labeled protein. We, therefore, conclude that the 52,000 molecular weight band which is apparently intrinsic is derived from the glucose transport proteins of the membrane.

The placental microvillous transport protein as demonstrated here is similar in apparent molecular weight to those of the erythrocyte, chicken fibroblast and the adipocyte (12). Unlike these, it is apparently a single molecular species. Purification of the placental protein and study of its regulation (1) should be greatly facilitated by the studies reported in this paper. In addition recognition that there are two major proteins which bind cytochalasin B should help in the interpretation of cytochalasin B binding data (8) and glucose transport studies where cytochalasin B is used.

Note: After this work was completed an abstract appeared describing labeling of placental glucose transport protein (13). Those studies although somewhat similar were apparently not as extensive and failed to resolve the two major cytochalasin B-binding proteins of the membrane.

ACKNOWLEDGEMENT

This work was supported by USPHS grant HD-07562.

REFERENCES

1. Johnson, L.W., and Smith, C.H. (1980) Am. J. Physiol. 238 (Cell Physiol. 7), C160-C168.
2. Carter-Su, C., Pessin, J.E., Mora, R., Gitomer, W., and Czech, M.P. (1982) J. Biol. Chem. 257, 5419-5425.
3. Shanahan, M.F. (1982) J. Biol. Chem. 257, 7290-7293.
4. Smith, C.H., Nelson, D.M., King, B.F., Donohue, T.M., Ruzycki, S.M., and Kelley, L.K. (1977) Am. J. Obstet. Gynecol. 128, 190-196.
5. Laemmli, U.K. (1970) Nature 227, 680-685.
6. Baldwin, S.A., Baldwin, J.M., and Lienhard, G.E. (1982) Biochemistry 21, 3836-3842.
7. Kelley, L.K., King, B.F., Johnson, L.W., and Smith, C.H. (1979) Exp. Cell Res. 123, 167-176.
8. Bissonnette, J.M., Ingerman, R.L., and Koch, P.A. (1982) Fed. Proc. 41, 1414.
9. Bissonnette, J.M., Black, J.A., Wickham, W.K., and Acott, K.M. (1981) J. Membrane Biol. 58, 75-80.
10. Lin, D.C., Tobin, K.D., Grumet, M., and Lin, S. (1980) J. Cell Biol. 84, 455-460.
11. Steck, T.L., and Yu, J. (1973) J. Supramol. Struct. 1, 220-232.
12. Shanahan, M.F., Olson, S.A., Weber, M.J., Lienhard, G.E. and Gorga, J.C. (1982) Biochem. Biophys. Res. Comm. 107, 38-43.
13. Ingermann, R.L., and Bissonnette, J.M. (1982) The Physiologist 25, 295.