Biochem. J. (1990) 272, 99–105 (Printed in Great Britain)

Identification and characterization of glucose transport proteins in plasma membrane- and Golgi vesicle-enriched fractions prepared from lactating rat mammary gland

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Plasma membrane- and Golgi vesicle-enriched membrane fractions were prepared from day-10 lactating rat mammary glands. Each fraction was found to contain a single set of D-glucose-inhibitable cytochalasin B-binding sites: plasma membranes and Golgi vesicles bound 20 ± 2 and 53 ± 4 pmol of cytochalasin/mg of membrane protein (means $\pm$ S.E.M.), with dissociation constants of 259 ± 47 and 520 ± 47 nM respectively. Anti-peptide antibodies against the *C*-terminal region (residues 477–492) of the rat brain/human erythrocyte glucose transporter labelled a sharp band of apparent M_r 50000 on Western blots of both fractions. Treatment with endoglycosidase F before blotting decreased the apparent M_r of this band to 38000, indicating that it corresponded to a glycoprotein. Confirmation that this immunologically cross-reactive band was a glucose transporter was provided by the demonstration that it could be photoaffinity-labelled, in a D-glucose-sensitive fashion, with cytochalasin B. Quantitative Western blotting studies yielded values of 28 ± 5 and 23 ± 3 pmol of immunologically cross-reactive glucose transporters/mg of membrane protein in the plasma membrane and Golgi vesicle fractions respectively. From comparison with the concentration of cytochalasin B-binding sites, it is concluded that a protein homologous to the rat brain glucose transporter constitutes the major glucose transport species in the plasma membranes of mammary gland epithelial cells. Glucose transporters are also found in the Golgi membranes of these cells, at least half of them being similar, if not identical, to the transporters of the plasma membrane. However, their function in this location remains unclear.

INTRODUCTION

The rate of glucose supply to the mammary gland is the main factor determining the rate of milk production in both ruminants and monogastrics [1,2]. Threadgold & Kuhn [3] have proposed that uptake of glucose across the epithelial cell plasma membrane is the rate-limiting step in the mammary utilization of carbohydrate in the rat, and a facilitated diffusion system for such uptake has been described in both rat and mouse [4,5]. However, the mechanism by which the glucose then crosses the membranes of Golgi vesicles to reach the site of lactose synthesis is less clear. Some studies have yielded evidence that passive sugar uptake into these vesicles occurs via proteinaceous pores that are non-stereospecific, in contrast with the great discrimination between D- and L-glucose shown by the plasma membrane transport system [6,7].

Most other mammalian cell types so far investigated have also been found to take up glucose by the passive process of facilitated diffusion [8], although some epithelial cells in the kidney and small intestine also exhibit active, sodium-linked, glucose uptake [9]. As is the case for the mammary gland epithelial cells [4,5], the passive process is potently inhibited by the mould metabolite cytochalasin B [10], and the binding of this ligand has been used to quantify transporter numbers in many different cell types. Binding is competitively inhibited by the substrate D-glucose but not by L-glucose, and is normally reversible [11]. However, upon irradiation with u.v. light a proportion of the inhibitor molecules become covalently linked to their binding sites [12,13]. This phenomenon has been exploited to identify the transporters in a number of tissues.

The kinetics of passive glucose transport, its regulation by hormones and other factors, and the potency of cytochalasin B as an inhibitor differ in different tissues [9,14]. However, the recent cloning of the passive glucose transporters from a number of mammalian tissues has revealed that despite their different properties they are members of a family of related proteins. The best-characterized of these proteins is the human erythrocyte glucose transporter (GLUT1 in the terminology of Fukumoto *et al.* [15]), which is also abundant in brain, placenta and a number of other tissues [16,17]. The three other members of the mammalian glucose transporter family so far identified comprise a protein (GLUT2) found primarily in liver, small intestine and the β-cells of the pancreatic islets of Langerhans [18,19], a transporter (GLUT3) found in brain, placenta and other tissues [15], and an insulin-regulated transporter (GLUT4) found in adipose tissue, heart and skeletal muscle [20,21]. These four proteins each comprise between 492 and 524 amino acids and range in M_r from 54000 to 57500. However, their apparent M_r on SDS/polyacrylamide gels differs from tissue to tissue because of variable extents of glycosylation. For example, the heterogeneously glycosylated human erythrocyte glucose transporter (GLUT1) normally migrates as a very broad band of apparent M_r 45000–70000, but migrates as a sharp band of apparent M_r 46000 following enzymic deglycosylation [22].

In the present study we have utilized both cytochalasin B and antibodies specific for the erythrocyte/brain type of glucose

Abbreviations used: TBS, Tris-buffered saline (20 mM-Tris/HCl/500 mM-NaCl, pH 7.5); TTBS, Tris-buffered saline containing 0.05% (v/v) Tween 20; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane.

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transporter (GLUT1) to characterize and quantify the glucose transporters of lactating rat mammary gland epithelial cells. By examining the subcellular distribution of the transporters we have sought to confirm the presence of a facilitated diffusion system for glucose entry across the plasma membrane, and have investigated the possibility that a similar system is involved in the uptake of glucose into the Golgi apparatus.

Parts of this work were presented at the 629th and 633rd Meetings of the Biochemical Society, held in December 1988 and December 1989.

EXPERIMENTAL

Animals

Nulliparous female Wistar rats (A. Tuck and Son, Rayleigh, Essex) were mated and at parturition (day 0 of lactation) the litters were adjusted to 8 pups. Animals were fed on Labsure irradiated CRM diet (Labsure, Poole, Dorset) and water *ad libitum* in rooms with a daily photoperiod of 12 h.

Materials

[4-^{3}H]Cytochalasin B (33 Ci/g) and ^{125}I-F(ab')$_2$-labelled donkey anti-(rabbit IgG) were purchased from Amersham International (Amersham, Bucks., U.K.). Endoglycosidase F from *Flavobacterium meningosepticum* was obtained from Boehringer (Lewes, Sussex, U.K.). Out-dated human blood was provided by the blood bank of the Royal Free Hospital and by the Scottish Blood Transfusion Service (Law Hospital, Carluke, Lanarkshire, U.K.).

Membrane preparations

Inguinal and abdominal mammary tissue was removed from rats between days 10 and 14 of lactation and was used to produce either plasma membrane- or Golgi vesicle-enriched membrane fractions according to the methods of Clegg [23] and West [24] respectively. Human erythrocyte membranes were prepared by hypo-osmotic lysis according to the method of Dodge *et al.* [25]. The low-density microsomal fraction from basal rat adipocytes was prepared by the method of Simpson *et al.* [26], and plasma membranes from rat liver were prepared by the method of Neville [27]. After removal of samples for immediate marker enzyme assay, all membrane preparations were rapidly frozen in liquid N_2 and stored at -70 °C.

Marker enzyme assays

5'-Nucleotidase and galactosyltransferase activities were assayed as markers for plasma membranes and Golgi vesicle membranes respectively, as described elsewhere [28,29]. Protein concentrations were determined by the method of Bradford [30] or that of Lowry *et al.* [31] using BSA as a standard (these methods yielded identical results).

Cytochalasin B binding assay

A centrifugal method for determining the binding of [4-^{3}H]cytochalasin B to membranes was developed, based upon the method of Lavis *et al.* [32]. Incubations were performed in 0.5 ml polypropylene microcentrifuge tubes. The reaction mixture had a final volume of 500 μl and contained the following: 0.02 μCi of [4-^{3}H]cytochalasin B (equivalent to 2.6 nM), unlabelled cytochalasin B serially diluted to give a range of concentrations from 62 to 4000 nM, 500 mM-D-glucose or -L-glucose, 10 μM-cytochalasin E, 10 mM-$CaCl_2$, 20 mM-Tris/HCl, pH 8.0, and either 50–60 μg or 750–850 μg of protein for human erythrocyte membranes or rat mammary membrane preparations respectively. After incubation for 25 min at 25 °C the mixtures were centrifuged for 2 min at 11000 $g_{av.}$ in an Eppendorf microfuge. A sample (250 μl) of the supernatant was taken for determination of free cytochalasin B concentration by liquid scintillation counting. The remaining supernatant was then aspirated and discarded before resuspension of the pellet in 50 μl of 1% (w/v) SDS and its transfer to a scintillation vial. The tube was subsequently washed twice with 50 μl volumes of 1% (w/v) SDS, which were transferred to the same scintillation vial for determination of bound cytochalasin B concentration by liquid scintillation counting. Assays were performed in duplicate and specific binding of cytochalasin B was calculated from the observed differences between the counts in the presence of D-glucose and L-glucose. The data were analysed by the method of Scatchard [33] and the best fits to straight lines were determined by linear-regression analysis.

Photoaffinity labelling and immunoprecipitation

Photoaffinity labelling of membranes with cytochalasin B was performed by the method of Kasanicki *et al.* [34]. For immunoprecipitation experiments, the photolabelled membranes (1.35 mg) were solubilized at 4 °C in 5.4 ml of 50 mM-sodium phosphate (pH 7.4)/100 mM-NaCl/1 mM-EDTA (buffer B) containing 0.25% (w/v) SDS, 2.5% (v/v) Triton X-100, 1 mM-phenylmethanesulphonyl fluoride, 1.5 μM-pepstatin A and 0.1 mM-E-64. The mixtures were centrifuged at 100000 $g_{av.}$ for 1 h to remove traces of insoluble material and then were treated with 50 μg of control rabbit IgG or 50 μg of affinity-purified antibody raised against the *C*-terminal region (residues 477–492) of the rat brain/human erythrocyte glucose transporter [35], bound to 12.5 μl of Protein A–Sepharose CL-4B. After overnight incubation on a rotary mixer the Sepharose samples were washed three times by centrifugation with buffer B containing 1% (v/v) Triton X-100 and 0.1% (w/v) SDS, and once with buffer B containing 0.1% (v/v) Triton X-100 and 0.1% (w/v) SDS. Bound proteins were then eluted from the Sepharose in 100 μl of gel sample buffer [50 mM-Tris/HCl (pH 6.8)/1 mM-EDTA/2% (w/v) SDS/10 mM-dithiothreitol/6 M-urea/10% (v/v) glycerol/pyronin Y (12 mg/ml). Samples (90 μl) were then electrophoresed on an SDS/12% (w/v) polyacrylamide gel and the distribution of radioactivity on the gel was determined as previously described [34].

Western blotting

For identification and quantification of glucose transporters in mammary gland membrane fractions, membrane samples (10 μg) were electrophoresed on SDS/12% (w/v) polyacrylamide gels by the procedure of Laemmli [36]. For quantitative Western blotting, samples of human erythrocyte membranes containing known amounts (5–25 ng) of glucose transporter were run on the same gel to act as standards. These human erythrocyte membranes had been treated with alkali to remove peripheral membrane proteins [37], and their transporter content had been determined by Scatchard analysis of D-glucose-inhibitable cytochalasin B binding.

Polypeptides were electrophoretically transferred from the gels to nitrocellulose, and then the blots were blocked by incubation for 2 h in 20 mM-Tris/HCl/500 mM-NaCl, pH 7.5 (TBS), containing 5% (w/v) low-fat milk powder. Blots were incubated overnight with control rabbit IgG or affinity-purified antibodies against residues 477–492 of the rat brain/human erythrocyte glucose transporter. For some experiments, affinity-purified antibodies raised against synthetic peptides corresponding to residues 507–522 of the rat liver glucose transporter (GLUT2) or against residues 494–509 of the rat adipocyte glucose transporter (GLUT4) were employed. The antibodies were diluted to a concentration of 2 μg/ml in TBS containing 1% low-fat milk powder (antibody buffer). After extensive washing of the blots in

TBS containing 0.05% (v/v) Tween 20 (TTBS), ^{125}I-F(ab')$_2$-labelled donkey anti-(rabbit IgG) (2.5 μCi in 10 ml) was applied in antibody buffer containing 0.05% (v/v) Tween 20 and incubated for 2 h. Finally, after extensive washing in TTBS, the blots were dried and autoradiographed for 16–72 h at −70 °C. For quantification of the immunoblots, autoradiographs were aligned over the nitrocellulose and radioactive bands were cut out and counted in a γ-radiation counter. An adjacent non-radioactive piece of nitrocellulose of equal area to the radioactive bands was also cut from the blot in order to determine background values. The amount of ^{125}I specifically bound to the human erythrocyte glucose transporter band was linearly proportional to the amount of transporter standard applied to the gel over the range used (5–25 ng). Linear-regression analysis was therefore used to create a calibration curve from the human erythrocyte standard data, and hence to estimate the concentration of glucose transporter in mammary membrane samples.

Enzymic deglycosylation

Before digestion with endoglycosidase, human erythrocyte membranes and rat mammary membranes (100 μg of protein) were dissolved in 200 μl of 100 mM-sodium phosphate (pH 6.1)/50mM-EDTA/75mM-2-mercaptoethanol/0.1mM-phenylmethanesulphonyl fluoride/0.05% (w/v) SDS/1% (v/v) Triton X-100. Samples were then incubated with or without 0.2 units of endoglycosidase F at 22 °C for 18 h. Digestion was terminated by denaturing the proteins with SDS in preparation for polyacrylamide-gel electrophoresis and Western blotting as outlined above.

RESULTS

Characterization of the isolated membrane fractions

Golgi vesicle fractions prepared as described in the Experimental section were found to be enriched 19-fold with galactosyltransferase activity when compared with the initial homogenate. The specific activity of the plasma membrane marker 5'-nucleotidase was only 12% of that found for the 'purified' plasma membrane fraction (see below), indicating that a maximum of 12% of the membranes in the Golgi fraction represented contaminating plasma membranes. The plasma membrane fraction prepared as described in the Experimental section was found to be enriched 55-fold in 5'-nucleotidase activity, somewhat greater than the enrichment of approx. 12-fold reported by Clegg [23]. However, this fraction was also heavily contaminated with the Golgi marker galactosyltransferase, which was enriched 37-fold relative to the original homogenate.

D-Glucose-inhibitable cytochalasin B binding

In order to estimate the concentration of glucose transport proteins in mammary gland membrane fractions, cytochalasin B binding was measured over a range of cytochalasin concentrations in the presence of D- or L-glucose. The efficacy of the binding assay was first tested using human erythrocyte membranes, yielding a value for the concentration of D-glucose-sensitive cytochalasin B binding sites [660±31 pmol/mg of protein (mean±S.E.M., $n = 5$)] comparable with values previously reported in the literature [32,37]. The measured K_d for the binding (282±24 nM) was somewhat higher than values of 100–200 nM previously reported [11,37,38], possibly because of the composition of the buffer used in the present assay. Application of this assay to both of the mammary gland membrane fractions revealed that D-glucose-displaceable binding of cytochalasin B to each was saturable, and Scatchard plot analysis indicated the presence of a single class of binding sites in both (Fig. 1). The number and affinity of the binding sites in each fraction are compared with those of human erythrocyte membranes in Table 1. The Golgi vesicle-enriched fraction had nearly three times as many sites per mg of protein as the plasma membrane fraction, but bound cytochalasin B with only half the affinity of the plasma membrane sites. The affinity of cytochalasin B binding to the latter was comparable with the affinity of binding to human erythrocyte membranes.

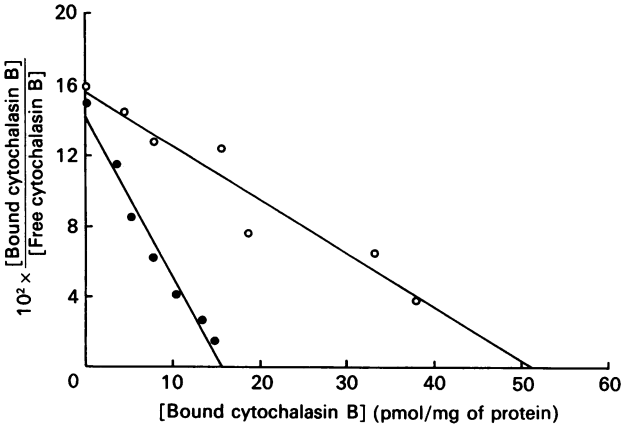

Fig. 1. Representative Scatchard-plot analysis of D-glucose-inhibitable cytochalasin B binding to plasma membrane- and Golgi vesicle-enriched fractions from lactating rat mammary gland

D-Glucose-inhibitable cytochalasin B binding to plasma membranes (●) and to Golgi vesicles (○) was measured as described in the Experimental section. Each point represents the mean of duplicate assays. The straight lines are the best fits derived by linear regression analysis of the data.

Immunologically cross-reactive transporters in the mammary gland

The cytochalasin B binding experiments described above indicated the presence of putative glucose transport proteins in both the plasma membrane-enriched and Golgi vesicle-enriched

Table 1. Quantification of glucose transport proteins in lactating rat mammary gland membrane fractions by assay of D-glucose-inhibitable cytochalasin B binding and by Western blotting

The concentration of binding sites ($B_{max.}$) and their affinity for cytochalasin B (K_d) were determined as described in the Experimental section. Values are means±S.E.M. for (n) determinations. *Significantly different from the $B_{max.}$ for rat mammary plasma membranes at the $P < 0.001$ level; †significantly different from the K_d values for human erythrocyte membranes and rat mammary plasma membranes at the $P < 0.01$ level, as determined using Student's unpaired t test. The concentration of immunologically cross-reactive transporter was determined by Western blotting as described in the Experimental section, using human erythrocyte membranes as a standard.

	Human erythrocyte membranes	Rat plasma membranes	Rat Golgi membranes
Cytochalasin B:			
$B_{max.}$ (pmol/mg of protein)	660±31 (5)	20±2 (5)	53±4* (3)
K_d (nM)	282±24 (5)	259±47 (8)	520±43† (3)
Western blotting:			
Transporter concn. (pmol/mg of protein)	–	28±5 (9)	23±3 (7)

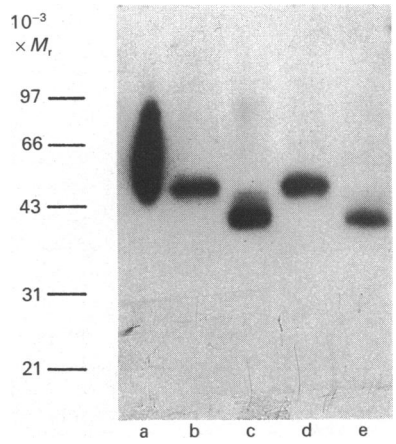

Fig. 2. Recognition of mammary gland membrane proteins, before and after deglycosylation, by anti-(glucose transporter) antibodies on Western blots

Lactating rat mammary gland membrane fractions were treated with (c, e) or without (b, d) endoglycosidase F as described in the Experimental section. Samples containing 30 μg of plasma membranes (b, c) or 40 μg of Golgi vesicle membranes (d, e) or 5 μg of untreated human erythrocyte membranes (a) were then electrophoresed on an SDS/12% (w/v) polyacrylamide gel, transferred to nitrocellulose and stained with antibodies against the rat brain glucose transporter, as described in the Experimental section. The positions of M_r markers are indicated.

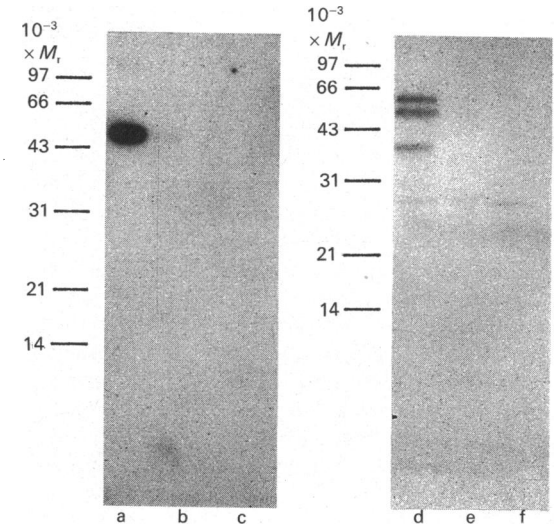

Fig. 3. Western blotting of mammary gland proteins using antibodies against rat adipocyte and liver glucose transporter isoforms

Samples (100 μg) of crude homogenate of lactating rat mammary gland (b, e) and of a crude membrane fraction (P3 in the terminology of Clegg [23]) prepared from the homogenate (c, f), as well as samples (10 μg) of basal rat adipocyte low-density microsomes (a) and of rat liver plasma membranes (d) were subjected to Western blotting as described in the legend to Fig. 2. Samples a, b and c were stained with antibody against the rat adipocyte glucose transporter (GLUT4), and samples d, e and f were stained with antibodies against the rat liver glucose transporter (GLUT2).

fractions from lactating rat mammary glands. In order to confirm this identification and provide an independent estimate of the concentration of transport proteins, Western blotting was performed using antibodies raised against a synthetic peptide corresponding to residues 477–492 of the human erythrocyte glucose transporter [35]. An identical sequence is found at the *C*-terminus of the rat brain transporter [16,17]. These antibodies labelled a single sharp band of apparent M_r 50000 on Western blots of both mammary gland fractions (Fig. 2). No labelling was seen with control rabbit IgG (results not shown). When quantitative Western blotting was performed as described in the Experimental section, using human erythrocyte membranes as standards of known transporter content, the results shown in Table 1 were obtained. In the plasma membrane fraction there was a close correspondence between the concentration of transporters measured by assay of cytochalasin B binding and by Western blotting. However, quantitative Western blotting yielded a value for the transporter content of the Golgi vesicle-enriched fraction that was only half that estimated from cytochalasin B binding measurements. A possible explanation for such a finding might be that more than one transporter isoform is present in mammary gland epithelial cells. Therefore Western blotting experiments were also carried out using anti-peptide antibodies raised against the *C*-terminal regions of the rat adipocyte (GLUT4) and the rat liver (GLUT2) transporter isoforms (Fig. 3). Both of the antibodies strongly stained bands corresponding to the transporters in samples of adipocyte and liver membranes respectively. However, neither recognized any proteins in large (100 μg) samples of either a tissue homogenate or a crude membrane fraction (P3 in the terminology of Clegg [23]) prepared from mammary gland. Both mammary gland samples stained extremely strongly with the antibodies against the *C*-terminus of GLUT1 (results not shown).

The sharpness of the bands seen with mammary membrane fractions stained with anti-GLUT1 antibodies was in contrast with the pattern of labelling of human erythrocyte membranes on blots, where the characteristically broad band of the glucose transporter of M_r 45000–70000 was seen (Fig. 2). This broadness is known to stem from heterogeneous glycosylation of the transporter, which migrates as a sharp band of apparent M_r 46000 after treatment with endoglycosidase F [22]. In the present study, treatment of both the Golgi vesicle-enriched and the plasma membrane-enriched fractions of the mammary gland with endoglycosidase F before Western blotting was found to increase the mobility of the labelled band, so that it migrated with an apparent M_r of 38000 (Fig. 2).

Photoaffinity labelling of mammary gland glucose transporters

The close correspondence between the concentration of glucose transporters measured by cytochalasin B binding assays and by Western blotting with anti-GLUT1 antibodies, at least for the plasma membrane-enriched fraction from mammary gland, suggested that both techniques were identifying the same, genuine, glucose transport protein. In order to confirm that the protein identified as a transporter on Western blots did indeed correspond to at least a proportion of the cytochalasin B binding proteins of this tissue, photoaffinity labelling experiments with [^{3}H]cytochalasin B were performed. The inefficiency of the labelling process [12,13], coupled with the low concentration of transporters in the mammary gland, precluded the direct analysis of membranes by SDS/polyacrylamide-gel electrophoresis after labelling: the level of non-specific labelling of membrane proteins in general was comparable with the specific labelling of glucose transporters. Therefore membranes were solubilized in detergent after photolabelling and subjected to immunoadsorption with control IgG or antibodies against residues 477–492 of the human erythrocyte glucose transporter before analysis of the immunoadsorbed proteins by gel electrophoresis. The results of these experiments are shown in Fig. 4. Immunoadsorbates of photolabelled human erythrocyte membranes, which were used

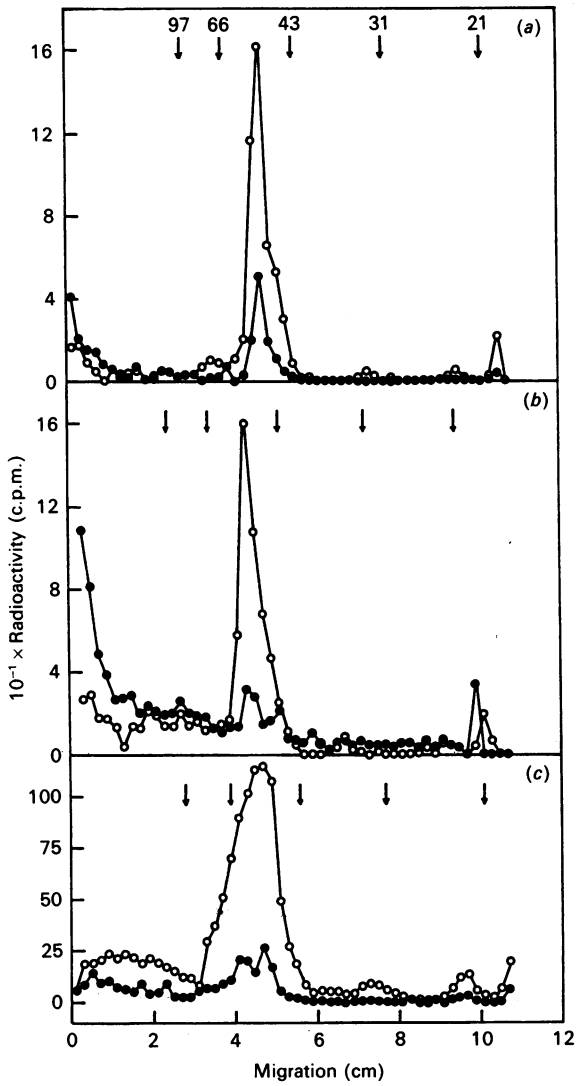

Fig. 4. Electrophoretic profiles of photoaffinity-labelled glucose transporters immunoprecipitated from (*a*) rat mammary gland plasma membranes, (*b*) rat mammary gland Golgi membranes and (*c*) human erythrocyte membranes

Membranes were labelled with [^{3}H]cytochalasin B in the presence of 500 mM-L-glucose (○) or 500 mM-D-glucose (●), solubilized and incubated with Sepharose-bound anti-(glucose transporter) antibodies, and the adsorbed proteins were electrophoresed on an SDS/12% (w/v) polyacrylamide gel. The radioactivity of 2 mm gel slices was determined by liquid scintillation counting. Arrows indicate the positions of M_r markers.

as a control, yielded the expected broad band of labelling of apparent M_r 45000–70000 on SDS/polyacrylamide gels. The incorporation of label into this peak was very substantially decreased by the presence of 500 mM-D-glucose during the photolabelling procedure. Immunoadsorbates of both Golgi vesicle-enriched and plasma membrane-enriched mammary fractions yielded a single, much sharper, peak of labelling of apparent M_r 50000 on SDS/polyacrylamide gels. The mobility of this peak corresponded exactly to that of the band labelled by antibodies on Western blots of the mammary gland fractions. The incorporation of label into this peak was also very substantially decreased by the presence of 500 mM-D-glucose during the photolabelling procedure. No labelled material was seen with either of the mammary membrane fractions or human erythrocyte membranes when immunoadsorption was performed with control rabbit IgG (results not shown).

DISCUSSION

In the present study a number of techniques were used to identify and quantify the glucose transporters present in the plasma membranes and Golgi vesicle membranes of lactating rat mammary gland epithelial cells. Important considerations for the interpretation of our findings are the nature and purity of the two subcellular fractions isolated from the mammary epithelial cells. The Golgi vesicle-enriched fraction contained only about a 12% contamination with plasma membrane, as indicated by the activity of the marker enzyme 5′-nucleotidase. However, the plasma membrane-enriched fraction was substantially enriched in galactosyltransferase activity. The enrichment of this fraction in the Golgi marker was expected from the previous studies of Clegg [23], who showed that the distribution of 5′-nucleotidase and galactosyltransferase on continuous sucrose density gradients of mammary microsomal fractions substantially overlapped. It is possible that the plasma membranes themselves contain galactosyltransferase activity, as has been demonstrated for the plasma membranes of some other cells [39,40]. Certainly, if the cytochalasin B binding sites detected in the Golgi vesicle-enriched fraction are actually located on Golgi membranes (see below), then a true contamination of the plasma membrane fraction with Golgi membranes would have resulted in a much greater concentration of cytochalasin B binding sites within the plasma membrane fraction than was actually observed.

The above considerations suggest that the two subcellular fractions obtained did correspond primarily to plasma membranes and Golgi vesicle membranes from the mammary gland epithelial cells. Western blotting using antibodies against the *C*-terminus of the human erythrocyte/rat brain glucose transporter revealed the presence of a putative glucose transporter which migrated as a sharp band of apparent M_r 50000 in both fractions. In its sharpness and mobility this band exactly resembled that exhibited by the rat brain glucose transporter on Western blots [41]. The increase in mobility resulting from treatment with endoglycosidase F indicated that the band corresponded to a glycosylated protein, and the apparent M_r of the deglycosylated form, 38000, was identical with that reported for the deglycosylated human erythrocyte protein [42]. (The erythrocyte protein migrates with an apparent M_r of either 38000 or 46000, depending upon the precise conditions used to make up the sample for gel electrophoresis [22,42].)

The conclusion that the band identified on Western blots corresponded to a glucose transporter was confirmed by the ability of the antibodies to immunoadsorb a radiolabelled protein of M_r 50000 from detergent extracts of membranes photoaffinity-labelled with [4-^{3}H]cytochalasin B, and by the inhibition of labelling brought about by the presence of D-glucose. Studies of non-covalent cytochalasin B binding revealed that the plasma membrane fraction contained a single class of D-glucose-inhibitable sites which bound cytochalasin B with an affinity that was essentially identical with that of the human erythrocyte glucose transporter. Furthermore, there was a close correspondence between the number of glucose transporters measured by cytochalasin B binding assays and by quantitative Western blotting. It is therefore very likely that the rat brain/human erythrocyte-type glucose transporter (GLUT1), or a closely related species, represents the major glucose transporter isoform in rat mammary gland epithelial cell plasma membranes. However, cytochalasin B binding to the Golgi vesicle-enriched fraction indicated a single class of D-glucose-inhibitable sites, with an affinity for cytochalasin B which was significantly lower than the

affinity of the plasma membrane sites. This finding might reflect the different membrane environments of the sites, different extents of covalent modification of the proteins at the two sites, or the presence of a different/additional transporter in the Golgi vesicle fraction. Comparison of the results of non-covalent binding studies with those of quantitative Western blotting indicated that only about half of the sites could be accounted for by the presence of glucose transporters which cross-reacted with the antibodies against the GLUT1 transporter isoform. One possible explanation for this discrepancy would be the presence of a second, non-cross-reactive, transporter species in the Golgi vesicle fraction. However, the Western blotting experiments illustrated in Fig. 3 indicate that if such a species exists, it does not correspond to either the liver glucose transporter isoform (GLUT2) or the adipocyte transporter isoform (GLUT4).

The number of cytochalasin B binding sites per mg of protein within the Golgi vesicle fraction was found to be more than twice that in the plasma membrane fraction. The presence of such a large number of transporters in an intracellular location resembles the situation in insulin-regulatable tissues such as adipocytes and muscle. In these tissues insulin appears to stimulate glucose transport primarily by bringing about a translocation of transporters from an intracellular store to the plasma membrane [9,14]. Insulin is known to stimulate the production of fatty acids from glucose in the mammary gland [43], but the extent to which this hormone regulates glucose transport is less clear. Glucose uptake by acini *in vitro* is apparently little affected either by insulin and other hormones or by previous starvation of the animals from which the acini were prepared [3]. Similarly, insulin has been reported not to stimulate glucose uptake acutely in mammary epithelial cells isolated from lactating mice [5]. However, studies of the metabolic clearance rate of glucose in lactating rats suggest that glucose uptake by the mammary gland *in vivo* is insulin-responsive [44]. Furthermore, the uptake *in vivo* of 3-*O*-methylglucose by the gland, which is greatly decreased in starved animals, is restored towards normal not only by refeeding but also by insulin [3]. The rapidity of this restoration, and its insensitivity to cycloheximide [3], suggest that it does not involve synthesis of new transporter molecules.

Tentative evidence for the presence of a translocatable pool of intracellular transporters, resembling that in fat and muscle, has been provided by a study of the decrease in $V_{max.}$ for hexose uptake by mouse mammary epithelial cells caused by fasting [45]. In this study, starvation was found to decrease the number of plasma membrane transporters, measured by a cytochalasin B binding assay, possibly as a result of their removal into an intracellular location. Our present study has provided the first direct evidence that such a pool of intracellular glucose transporters exists in the rat mammary gland epithelial cell. Further experiments are required to explore the possibility that some or all of the glucose transporters that we have identified in the Golgi vesicle-enriched fraction are translocated between the cell surface and the interior in response to starvation and/or the presence of insulin.

An alternative/additional role for the intracellular transporters may be in supplying glucose for lactose synthesis within the lumen of the Golgi apparatus. However, we cannot yet be certain that the vesicles containing glucose transporters in the Golgi membrane-enriched fraction actually correspond to those involved in lactose synthesis. Indeed, other studies have reported that the Golgi membrane discriminates only poorly between different sugars of $M_r < 300$, suggesting the presence of pores, rather than specific transporters for glucose [6,7]. The possibility that the transporters that we have identified in the Golgi vesicle-enriched fraction do play a role in lactose synthesis requires further investigations.

We thank the Medical Research Council and the Department of Agriculture and Fisheries for Scotland for project grant support, and the Science and Engineering Research Council for studentships to A. D. and S. M. We are grateful to Dr. Roger Clegg and Dr. David West for useful discussions and help in preparing rat mammary membranes, to Dr. Colin Wilde and Dr. Paul Watt for help in marker enzyme assays, and to Lee Fryer, Liz Woolston and Dr. Yolanta Kruszynska for help in preparation of antibodies against GLUT2 and GLUT4, and for the gift of adipocyte membranes.

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Received 30 March 1990/11 July 1990; accepted 20 July 1990