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Rat skeletal muscle, liver and brain have different fetal and adult forms of the glucose transporter

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Rabbit antibodies made against the human erythrocyte glucose transporter were used to determine whether or not embryonic glucose transporters of rat skeletal muscle, liver and brain are identical to the transporters of adult animals. The results indicate that in both skeletal muscle and liver, the transporter switches from a highly antibody-reactive embryonic form to a low antibody-reactive adult form within 2 days of birth. This suggests that there are two different forms of glucose transporter in embryonic and adult skeletal muscle and liver. In contrast, these antibodies have equal reactivity toward the glucose transporters of embryonic and adult brain. In embryonic brain, two forms of the transporter coexist, with different molecular weights (M_r = 45000 and 40000), while in the adult brain the M_r = 40000 form is predominant. The dissociation constant for glucose for the embryonic liver transporter was measured by displacement of bound cytochalasin B. The results indicate that the embryonic liver transporter has a low affinity for glucose and for cytochalasin B, similar to the adult liver transporter, even though the antibody reactivity toward these two transporters is different.

Introduction

The glucose transporter is an intrinsic membrane glycoprotein which catalyzes the translocation of glucose across the plasma membrane of eukaryotic cells in an energy-independent manner (for a review, see Ref. 1).

Considerable evidence supports the view that, in rat, the glucose transporter is tissue-specific. In the first place, both the glucose affinity and the mRNA of the liver transporter are different from those of transporters of other tissues [2–4]. Secondly, the extent of glycosylation of transporters can be extensive (erythrocytes, kidney cells [5–8]) or less pronounced (brain cells, adipocytes, skeletal muscle [8–11]). In the third place, the glucose transporters of skeletal muscle cells and adipocytes differ from those of brain cells in their reactivity with a specific antibody [8]. These results suggest that there are at least three different types of glucose transporter.

Another, and so far unexplored, potential cause of heterogeneity of glucose transporters is the existence of distinct fetal and adult proteins, as is found in hemoglobin [12], myosin heavy chains [13,14], tropomyosin [15], troponin T [16] and the acetylcholine receptor [17]. This has raised the question of whether the expression of glucose transporters in a given cell or tissue might be developmentally regulated. One reason to examine this possibility is that in rat diaphragm the ability

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of insulin to stimulate glucose transport is relatively low at birth and increases to a maximum at 20 days of age [18].

This phenomenon is not dependent on the number of insulin receptors and their protein kinase activity [18,19] or the number of intracellular glucose transporters [18]. It was of interest therefore, to determine whether insulin-sensitive glucose transport in muscle might depend on the replacement of fetal transporters by adult ones. Here, we show that the glucose transporter of embryonic rat skeletal muscle is different from that of adult rats. Nevertheless, the switch from fetal to the adult form is completed by the 3rd day of life, indicating that the insensitivity to insulin of the glucose transporters at birth is not due to the presence of an embryonic form of the glucose transporter. In addition, we show that the embryonic liver also contains a transporter different from that in adult rat liver.

Materials and Methods

Materials. [^{3}H]Cytochalasin B, ^{125}I-labeled protein A, and endoglycosidase F (Endo F) were purchased from New England Nuclear (Boston, MA). Triton X-100, Nonidet P40, leupeptin, antipain, benzamidine, Trasylol, chymostatin and cytochalasin B and E were obtained from Sigma (St. Louis, MO). All other chemicals were from Sigma.

Preparation of microsomes from erythrocytes, skeletal muscle, brain and liver. Erythrocyte ghosts were prepared as described in Ref. 8. Peripheral membrane proteins were stripped by washing the ghosts with 2 mM EDTA (pH 12). This method removes approx. 40–50% of the total membrane protein. The liver, brain, diaphragm or thigh muscle were dissected from embryonic (18–22 days of gestation), newborn or adult rats. The fetal and adult muscle and adult brain microsomes were prepared as described previously [8]. The embryonic liver and brain microsomes were prepared as described by Brennan [20]. The concentration of glucose transporters in these preparations was assayed by D-glucose inhibitable cytochalasin B binding as described [8,21]. The deviation of these measurements are usually 5–30% of the total displaceable sites; i.e., $(\Delta L + \Delta D)/(L - D) \leq 30\%$, where L and D are the averages of triplicate measurements of cytochalasin B bound in the presence of L- and D-glucose, respectively; ΔL and ΔD are the standard deviations of these measurements.

Affinity purification of anti-glucose transporter antibodies and immunoblotting. Antibodies were prepared against human erythrocyte glucose transporters and were affinity purified using glucose transporter-Sepharose CL-4B resin as described [8]. Alternatively, protein-depleted human erythrocyte membranes were used as an immobilized matrix for antibody purification essentially as described by Schroer et al. [22]. Affinity purified antibodies obtained by both methods gave identical results. Immunoblotting was performed by the method of Towbin et al. [23].

Displacement of cytochalasin B bound to microsomes by D-glucose. Erythrocyte membranes, liver, brain and muscle microsomes were resuspended in 2–5 vol. of 1% NaCl and cytochalasin E (final concentration of 10 μM). Equal aliquots were withdrawn and mixed with [^{3}H]cytochalasin B and glucose in a total volume of 120 μl. The concentration of D-glucose was varied from 0 to 800 mM, while the total D-glucose plus L-glucose was held at 800 mM. The mixture was incubated at room temperature for 15 min, and centrifuged at $100000 \times g$ for 30 min. The supernatant was aspirated and discarded. The pellet was dissolved in 1% SDS and 75 mM NH_4HCO_3 before subjecting to liquid scintillation counting with 10 vol. of Aquasol (New England Nuclear). The [^{3}H]cytochalasin B counts in the pellet with 800 mM of D-glucose were regarded as nondisplaceable binding and subtracted. The binding at other concentrations of D-glucose was calculated accordingly.

Treatment with endoglycosidase F. Aliquots (100 μg/ml) of membranes were incubated in 100 mM Na_2HPO_4 (pH 6.1) with 50 mM EDTA, 1% Nonidet P40, 1% 2-mercaptoethanol, 0.1% SDS, and 1.2 U Endo F. In addition, 2 ml of a mixture of leupeptin (1 mg/ml), antipain (2 mg/ml), benzamidine (10 mg/ml), Trasylol (10^4 kallikrein inhibitor units/ml), chymostatin (1 mg/ml), and pepstatin (1 mg/ml) were added to each tube. Samples were incubated at 37°C for 16 h. The reaction was stopped by boiling in electrophoresis sample buffer.

Data analysis. Cytochalasin B has been shown to be a competitive inhibitor of D-glucose binding to the glucose transporter [24,25]. After mixing these two ligands with the transporter, the equilibration can be described as follows:

$$T + G \rightleftharpoons T_g$$

$$T + C \rightleftharpoons T_c$$

where T is the transporter, G and C are D-glucose and cytochalasin B; T_g and T_c are transporters associated with glucose and cytochalasin B, respectively. The dissociation constants are defined by the following two equations:

$$k_g = [T]\cdot[G]/[T_g] \quad (1)$$

$$k_c = [T]\cdot[C]/[T_c] \quad (2)$$

$[T_o]$ is the total concentration of glucose transporter:

$$[T_o] = [T] + [T_g] + [T_c] \quad (3)$$

Substituting Eqns. 1 and 2 into Eqn. 3, one finds that

$$[T_c](1 + k_c/[C](1 + [G]/k_g)) = [T_o] \quad (4)$$

According to this equation, there should be a linear relationship between $1/[T_c]$, the reciprocal of the amount of the cytochalasin B-transporter complex, and [G], the glucose concentration at any value of [C], the concentration of cytochalasin B. The intercept of this line on the ordinate is $(1 + k_c/[C])/[T_o]$; and the intercept on the abscissa is $-k_g(1 + [C]/k_c)$. If we define $K_{app} = k_g(1 + [C]/k_c)$, K_{app} is the concentration of D-glucose which displaces 50% of the cytochalasin B bound to the transporter at a concentration [C].

Using this method, one can determine the affinity constants and the total number of glucose transporters by performing displacement experiments with two different concentrations of cytochalasin B.

Results

Certain antibodies made against the human erythrocyte glucose transporter can distinguish transporters from several rat tissues, including skeletal muscle [8]. Using these antibodies, we undertook to determine whether in the fetal rat the transporter in skeletal muscle is similar to or different from that in the adult rat. Therefore, we prepared skeletal muscle microsomes from rats at different stages of development and measured the number of transporters in these preparations. Subsequently, aliquots containing similar amounts of glucose transporters were used for immunoblotting. The results of these experiments are shown in Fig. 1; panel A shows the protein stain (Amido black) of microsomal proteins separated by SDS gel electrophoresis, and panel B is the autoradiogram of the immunoblot. Here the binding of ^{125}I-labeled protein A reflects the relative reactivity of the antibodies to the transporter. While the patterns in panel A are all similar to one another, the immunoblots show that the amount of antibody bound by a given amount of transporter decreases: lanes a and b (1 day before birth and at birth, respectively) as compared to lanes c, d, and

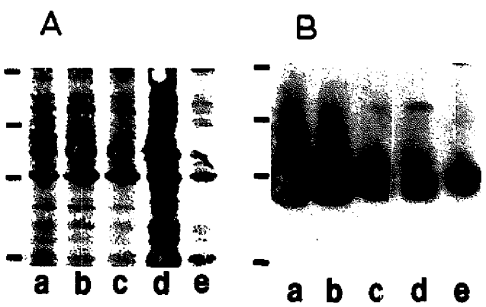

Fig. 1. Switching of glucose transporter form during development. Novel antibodies were used as a probe to determine when the muscle transporter switches from the embryonic to the adult form. The Amido black stain (A) and the corresponding autoradiogram of the Western blot (B) are shown here. Microsomes are from 21-day embryonic rats (a), 0.5-day postnatal (b), 3-day postnatal (c), 8-day postnatal (d), and 22-day postnatal (e). The amount of microsomes applied to the SDS gel was adjusted to similar amounts of D-glucose-inhibitable cytochalasin B binding (40–60 fmol). Affinity-purified antibodies were incubated with the proteins transferred to nitrocellulose. The ratio of ^{125}I-protein A bound to cytochalasin-B binding sites are: 4.5 (a), 3.6 (b), 1.0 (c), 0.87 (d), and 0.95 (e). By 3 days after birth, the transporters have completely switched to the adult form. Only a portion of the gel is shown here; the molecular weight standards are phosphorylase *b* (94000), bovine serum albumin (68000), ovalbumin (43000), and carbonic anhydrase (30000).

e (3-day-old, 8-day-old and 22-day-old rats). The difference in the ratio of ^{125}I-labeled protein A bound per cytochalasin B binding site (4.5 in lane a as compared to 0.95 in lane e, see legend to Fig. 1) suggests that the fetal transporter is different from the adult transporter. It appears that the switching of the transporter is complete before postnatal day 3 (Fig. 1B, lane c). In addition, similar results were obtained with a muscle plasma membrane-enriched fraction (data not shown). Assuming that all the transporters are the embryonic form at 21 days of gestation (Fig. 1B, lane a), and the adult form at 22 days after birth (Fig. 1B, lane e), we can estimate that 25% of the transporters are the adult form by 12 h after birth (Fig. 1, legend). One of us reported previously [18] that insulin stimulation of glucose uptake in rat diaphragm is low at birth and increases linearly during the first 15–17 days after birth. This phenomenon is not related to changes in the number of insulin receptors [18], receptor kinase activity [19], or intracellular glucose transporter pool size [18]. As shown in Fig. 1, this lower level of insulin-stimulated glucose uptake in young rats also is not due to the presence of a fetal form of the transporter, because insulin-stimulated glucose uptake is still low at a time when the adult form of the transporter is predominant (3 days after birth). These observations are consistent with the hypothesis made previously that either the signaling mechanism or the translocation machinery is not well developed at birth.

To determine whether switching of glucose transporter forms during development occurs in other cell types, glucose transporter containing membranes were isolated from liver and brain. In liver, the situation is similar to that in muscle, with the exception that the antibodies used do not recognize the transporters of adult liver. In Fig. 2, panel A shows the amido black stain, panel B is the autoradiogram of the immunoblot. It is evident that these antibodies react readily with transporters from fetal liver (Fig. 2B, lanes a and b) and poorly with transporters from livers of 2-day-old rats (Fig. 2B, lane c). Under these experimental conditions, the antibodies showed no reactivity towards adult liver glucose transporters (Fig. 2B, lane d). Since these microsomal preparations do not contain significant amounts of anion transporter (the major membrane protein of erythrocytes), the much higher reactivity of the antibodies towards fetal liver transporters is not due to contamination with membranes from erythroid cells (data not shown). From these results, it appears that the polypeptide itself undergoes a change from fetal to adult forms in liver during development. The functional significance of this phenomenon was investigated by determining the affinity constants of D-glucose and cytochalasin B for the transporter.

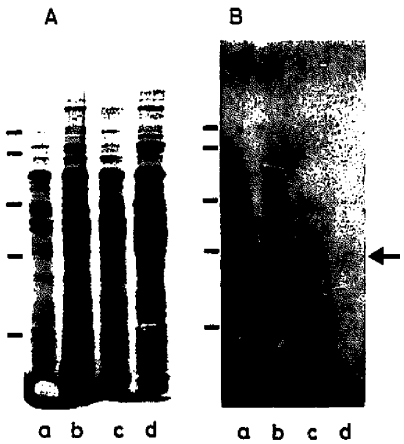

Fig. 2. Expression of liver glucose transporters is developmentally regulated. Microsomes were isolated from embryonic, newborn and adult rat liver. The amount of transporter was determined by D-glucose inhibitable cytochalasin B binding. The concentration of cytochalasin B ranged from 30–100 nM. Liver microsomes containing equal binding activity (900 fmol of transporter) from 18-day embryonic (a), 22-day embryonic (b), 2-day-old (c) and adult rats (d) were immunoblotted with affinity purified antibodies. The Amido black stain (A) and the corresponding autoradiograms (B) are shown here. Although the protein staining pattern is similar at the different stages of development, the antibody staining pattern is strikingly different.

Displacement of cytochalasin B binding by D-glucose was used to determine the dissociation constants, k_g and k_c, of the glucose transporter for glucose and cytochalasin B, respectively (see Materials and Methods for details). The feasibility of this approach was demonstrated by using the human erythrocyte transporter as a model system (Fig. 3A). It appears that the k_{app} values corresponding to 5 and 50 nM cytochalasin B are 19 and 28 mM, respectively. From these results, it

was calculated that $k_g = 18$ mM for glucose and $k_c = 90$ nM for cytochalasin B. Both of these values are in agreement with the values reported in the literature. For instance, k_m of glucose efflux in intact human erythrocyte is 15–25 mM [26,27] and in ATP-depleted ghost is 10 mM [27]; k_d of cytochalasin B is 50–100 nM [6,8].

The same approach was applied to embryonic liver transporters (Fig. 3B). The results indicate that the k_{app} are 91 mM and 115 mM for 30 nM and 240 nM cytochalasin B respectively. The k_g and k_c were estimated to be 90 mM and 800 nM, respectively. These values are similar to those obtained for adult liver transporters (data not shown), which are in good agreement with results reported by other investigators [2,3]. Thus, we conclude that the affinity of cytochalasin B and glucose of the embryonic liver transporter is similar to that of adult liver transporters. Fig. 3C also shows the results of displacement experiments with brain and muscle transporters, suggesting that transporters in these tissues have ligand-binding properties similar to those of erythrocytes.

In contrast to the situation in muscle and liver, in brain the ability of these antibodies to recognize transporters in fetal and adult cells was similar. Interestingly, however, two forms of the glucose transporter exist in fetal brain. Fig. 4A compares 18-day fetal brain with adult brain, panel B compares 18-fetal, 22-day fetal and 2-day post-

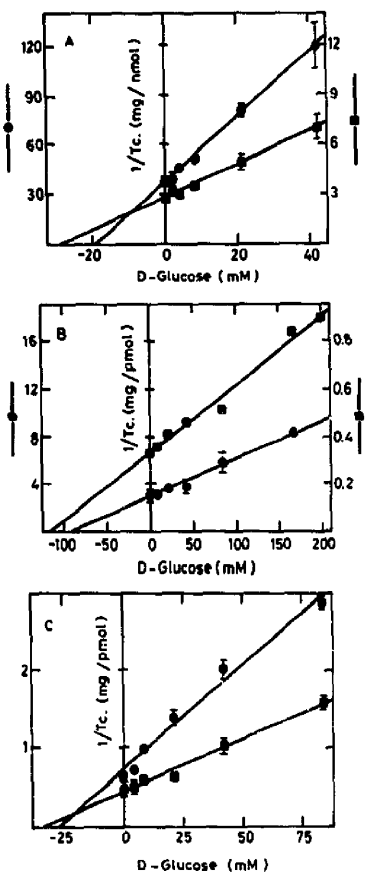

Fig. 3. Determination of ligand dissociation constants for the glucose transporter. The k_c of cytochalasin B and the k_g of D-glucose for the transporter were determined by displacement of cytochalasin B bound to the transporter with different concentrations of D-glucose as described in Materials and Methods. The concentration of cytochalasin B remains essentially constant for each experiment. Under these conditions, theoretically, k_g and k_c can be calculated by measuring k_{app} at two different concentrations of cytochalasin B. Experiments were performed on human erythrocyte transporters (A), 22-day embryonic liver transporters (B), and adult brain and muscle transporters (C). The amount of protein used for each single determination is: 15 μg for protein depleted human erythrocytes; 150 μg for 22 day embryonic liver microsomes; 50 μg for brain microsomes, and 75 μg for skeletal muscle microsomes. Each point represents the average of triplicate determinations and the bar is the standard deviation; note that, frequently, the deviation is smaller than the dimension of the points in the graph. The non-displaceable binding and trapping of cytochalasin B in the pellets comprised 5%, 70–80%, 60%, and 70% of the total for human erythrocyte membranes, and embryonic liver, brain, and muscle microsomes, respectively. The concentrations of cytochalasin B for human erythrocyte transporters (A) and 50 nM (squares) and 5 nM (circles); the corresponding K_{app} values are 28 mM and 19 mM, respectively. The calculated k_g and k_c are 18 mM and 90 nM. The total number of binding sites is 450 pmol/mg (see Fig. 2, for a comparison). The concentrations of cytochalasin B used for the embryonic liver microsomes (B) are 240 nM (squares) and 30 nM (circles). The k_{app} values are 115 mM and 91 mM, respectively. The calculated k_g and k_c are 88 mM and 770 nM. The total number of glucose transporter in these microsomes is 10–12 pmol/mg. In C, the concentration of cytochalasin B was 45 nM for both brain (circles) and muscle (squares) microsomes. The k_{app} values are 27 mM and 33 mM, respectively. Assuming $k_c = 100$ nM [8,31], the calculated k_g values for brain and muscle are 19 mM and 23 mM, respectively, and the total binding sites for brain and muscle are 4.5 pmol/mg and 7.2 pmol/mg, respectively.

natal brain. Two distinctive forms of glucose transporter exist in embryonic brain (Fig. 4A, lane a; Fig. 2B, lanes a and b). The lower-molecular-weight form has a mobility identical to that of adult brain ($M_r = 40000$); the other form has a lower mobility ($M_r = 45000$). Furthermore, the M_r 45000 form gradually decreases in amount during embryonic development, comprising 70% and 40% of the total transporter population at 18 and 22 days of gestation, respectively. The higher-molecular-weight form disappears by 2 days after birth (Fig. 4B, lane c).

A simple explanation for the presence of two glucose transporters in fetal brain, as compared to one transporter in adult brain, might be that in fetal brain the glucose transporter is glycosylated at more sites than it is in adult brain. To investigate this possibility, the microsomes of adult and fetal brains were treated with endoglycosidase F. The results with the adult brain transporters shown in Fig. 5, lanes a and b, indicate that a fraction of these transporters can be deglycosylated, producing a species with $M_r = 37000$. The situation with the fetal brain transporters shown in Fig. 5, lanes c and d, is complicated after the treatment with endoglycosidase F; there is a decrease in the $M_r = 45000$ species and the appearance of a band at $M_r = 37000$. It is not clear, however, whether the M_r 37000 band derives from the M_r 45000 species or from the M_r 40000 species.

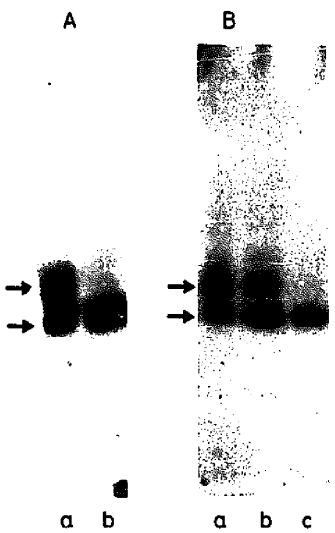

Fig. 4 Immunoblotting of glucose transporters of rat brain during development. Two autoradiograms of immunoblots with affinity purified antibodies are shown here. (A) Microsomes from 18-day embryonic brain (a) and adult brain (b) containing equal amounts of glucose transporters. The embryonic brain contains two different forms of glucose transporters ($M_r = 45000$ and 40000) indicated by the arrow, while the adult brain has primarily the lower-molecular-weight form. Binding of ^{125}I-protein A was quantitated by densitometric scanning of the autoradiogram. While protein A binding in the embryonic and the adult forms is essentially equal, the ratio of binding to the M_r 45000 form to M_r 40000 is 3:2, respectively. (B) Brain microsomes from 18-day (a) and 22-day embryonic (b) rats, and 2-day postnatal rats (c). It appears that the ratio of M_r 45000 form to M_r 40000 decreases from 2:1 in (a) to 2:3 in (b) and the $M_r = 45000$ becomes undetectable in 2-day-old animals (c). The amount of protein A bound per unit transporter remains constant during development.

Fig. 5. Removal of asparagine-linked oligosaccharide chains. Adult (a and b) and embryonic (c and d) rat brain microsomes were treated with (+) or without (−) Endo F as described in Materials and Methods. The treated samples were subjected to gel electrophoresis, transferred to nitrocellulose, and immunoblotted with affinity-purified glucose transporter antibodies. Molecular weight markers are β-galactosidase (130000), phosphorylase *b* (94000), bovine serum albumin (68000), ovalbumin (43000), and carbonic anhydrase (30000).

Discussion

Antibodies were used as a probe to investigate whether the expression of glucose transporters in three different rat tissues – skeletal muscle, brain and liver – is developmentally regulated. Several conclusions can be drawn from this study. First, in skeletal muscle and liver, the antibodies react more strongly with embryonic transporters than with adult ones. These results indicate that there are two forms of the transporters: embryonic and adult. The mechanism for the generation of this diversity in transporters, whether due to multiple genes or alternative splicing of mRNA, has yet to be established.

It is clear that liver expresses almost exclusively the adult form of the transporter within a few days after birth, as judged by the reactivity of the transporter antibody. Also on the basis of antibody reactivity, it is reasonable to conclude that in muscle there is a similar switching of transporter form during postnatal life. The possibility cannot be ruled out, however, solely on the basis of antibody recognition, that both embryonic and adult forms are expressed in adult muscle. Recently it was reported that two distinctive types of glucose transporter coexist in adult rat adipocytes [28–30]. Furthermore, the insulin-responsive form is present in both the intracellular vesicles and the plasma membrane, while the nonresponsive form is located exclusively in intracellular vesicles [29]. We do not know whether or not this is also the case for skeletal muscle.

The second conclusion is that there are two types of glucose transporter with different molecular weights, M_r 40000 and 45000, in embryonic brain tissue. Interestingly, the M_r 45000 form disappears completely by 2 days after birth. In contrast to the transporters of skeletal muscle and liver, the antibodies showed similar reactivity towards the embryonic and adult brain transporters. Dick et al. [31] have described the properties of the brain microvessel glucose transporter which might contribute to the staining observed in this study (Fig. 4). We think this is unlikely for two reasons: first, the brain microsomes used here do not contain the microvessel form of the insulin receptor, another integral membrane protein [20]; and second, the microvessel transporter has a higher apparent molecular weight and stains more diffusely compared to the embryonic brain transporter. As indicated earlier, the relationship of the M_r 45000 and the M_r 40000 forms of the brain glucose transporters to each other, and to the M_r 37000 form obtained by treatment with endoglycosidase F is not clear. We can not distinguish between the possibilities that there are two different polypeptides or one polypeptide with different extents of glycosylation.

The third question addressed in this study is whether or not the affinity of glucose and cytochalasin B for the embryonic liver transporter is similar to that of the adult transporter. Since the function of the transporters is to regulate the flux of glucose, it is important to determine the affinity constants of these two ligands. We first showed that both the k_g and k_c of the human erythrocyte transporter obtained by displacement of cytochalasin B by glucose are in good agreement with the values previously reported using other techniques. Measurements of the k_c and k_g of cytochalasin B and glucose, respectively, of embryonic liver transporters reveals that, as with the adult liver transporters, the dissociation constants for cytochalasin B and glucose ($k_c = 800$ nM and $k_g = 90$ mM) are much higher than those of the erythrocyte glucose transporter. Therefore, we conclude that the affinities of the embryonic liver transporter for its ligands are similar, if not identical, to those of the adult liver transporter, although the antibody recognition sites are clearly different. It should be noted that the assumption that the cytochalasin B binding assay reflects the actual number of glucose transporters is based on the data from the human erythrocyte transporter. Therefore, the validity of this method for quantitating transporters in other tissue has yet to be established. Recently, however, Joost et al. [30] have reported that in adipocyte membrane vesicles, there is a good correlation between cytochalasin B binding and actual transporter activity. This suggests that cytochalasin B binding reflects at least the relative, if not the absolute, number of glucose transporters.

In summary, we have demonstrated that, in skeletal muscle and liver, the expression of glucose transporters is developmentally regulated. Thus the glucose transporter joins the growing list of

cellular proteins which exist in developmentally specific forms (e.g., hemoglobin, myosin, tropomyosin, tropin T, and the acetylcholine receptor). As is the case for these other proteins, the functional significance of this developmental regulation of the glucose transporter has yet to be elucidated.

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