Cytochalasin B as a probe for the two hexose-transport systems in rat L6 myoblasts

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We have recently demonstrated that two hexose-transport systems are present in undifferentiated rat L6 myoblasts: D-glucose and 2-deoxy-D-glucose are preferentially transported by the high-affinity system, whereas 3-O-methyl-D-glucose is transported primarily by the low-affinity system. Mutant D23 is found to be defective only in the high-affinity hexose-transport system. The low-affinity transport system is much more sensitive to inhibition by cytochalasin B (CB). The present study examines the identity, properties and regulation of the CB-binding sites by measuring CB binding to both whole cells and plasma membrane. Scatchard analysis of the binding data revealed the presence of two CB-binding sites, namely CB_H and CB_L. These two sites differ not only in their affinity for CB, but their levels can also be differentially altered by various biochemical, physiological and genetic manipulations. CB_L resembles the high-affinity hexose-transport system in that it is absent in mutant D23 and is present in larger quantities in glucose-starved cells. Moreover, CB binding to this site is inhibited by D-glucose and 2-deoxy-D-glucose, the preferred substrates of the high-affinity hexose-transport system. On the other hand, CB_H is found to be unaltered in mutant D23, which also retains the normal low-affinity hexose-transport system. CB_H also resembles the low-affinity transport system in that it is not elevated in glucose-starved cells. Furthermore, binding of CB to this site can be inhibited by 3-O-methyl-D-glucose, the preferred substrate of the low-affinity transport system. It should be noted that 2-deoxy-D-glucose does not have much effect on CB_H, and vice versa. Studies with purified membrane preparations indicate that both CB-binding sites are present in similar ratios in the plasma membrane and the low-density microsomal fraction. Plasma-membrane studies also reveal that D-glucose 6-phosphate, but not 2-deoxy-D-glucose 6-phosphate, is very effective in activating CB binding. Data presented suggest that CB binding may be regulated by sugar analogues in an allosteric manner.

INTRODUCTION

Hexose transport has been characterized in a variety of eukaryotic cells (Czech, 1976a,b; Olefsky, 1978; Baldwin & Lienhard, 1981; Griffin *et al.*, 1982; Pessin *et al.*, 1982). These studies revealed the presence of more than one hexose transport system in each type of cell. For example, two hexose-transport systems were detected in chick-embryo fibroblasts (Christopher *et al.*, 1976; Pessin *et al.*, 1984), rat adipocytes (Olefsky, 1978; Foley *et al.*, 1980), and mouse 3T3 cells (Colby & Romano, 1975). A number of affinity ligands have been used to identify the hexose transporters. These range from monoclonal antibodies to a variety of sugar analogues (Taverna & Langdon, 1973; Lin & Spudich, 1974; Fannin *et al.*, 1981; Allard & Lienhard, 1985; Kay, 1985; Weber & Eichholz, 1985). Of these, cytochalasin B (CB) is the best characterized ligand. It is a very specific and potent hexose-transport inhibitor (Czech, 1976a,b; Pessin *et al.*, 1982). Although it binds to three different sites in human red blood cells and in rat adipose cells (Lin & Spudich, 1974; Czech, 1976a,b; Jung & Rampal, 1977; Wardzala *et al.*, 1978; Carter-Su & Okamoto, 1985), only one site is sensitive to the hexose-transport

substrates; the other two sites can be blocked by cytochalasin E (CE). The hexose-sensitive CB-binding site is therefore thought to be associated with the hexose transporter. CB has also been used as a photoaffinity label for the glucose transporter in human red blood cells, chick-embryo fibroblasts, rat adipocytes, human placenta and rat skeletal muscle (Johnson & Smith, 1982; Klip *et al.*, 1983; Carter-Su & Okamoto, 1985). In chick-embryo fibroblasts, two hexose-sensitive membrane components have been identified by photolabelling studies with CB (Pessin *et al.*, 1982). Most of these studies were carried out with either crude or semi-purified membrane preparations (Jung & Rampal, 1977). In adipocytes, at least 80 % of the CB-binding sites were located in the low-density microsomal fraction (LDMF), the rest being in the plasma membrane and the high-density microsomal fraction (HDMF) (Karnielli *et al.*, 1981; Simpson & Cushman, 1986).

A combination of biochemical and genetic approaches has recently been used to examine hexose transport in undifferentiated rat myoblasts (Klip & Walker, 1983; D'Amore & Lo, 1986a,b; D'Amore *et al.*, 1986a,b). These studies reveal the presence of a high- and a low-affinity hexose-transport systems in these cells (D'Amore

Abbreviations used: CB and CE, cytochalasins B and E; dGlc, 2-deoxy-D-glucose; MeGlc, 3-O-methyl-D-glucose; PBS, phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8.1 mM-Na₂HPO₄/1.5 mM-KH₂PO₄, pH 7.4); LDMF, low-density microsomal fraction; HDMF, high-density microsomal fraction; PMSF, Phenylmethanesulphonyl fluoride; CB_L, low-affinity cytochalasin B-binding site; CB_H, high-affinity cytochalasin B-binding site; cPBS, complete phosphate-buffered saline containing 0.9 mM-CaCl₂ and 0.5 mM-MgCl₂, pH 7.4; Na⁺,K⁺-ATPase, ouabain-insensitive Na⁺,K⁺-ATPase.

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& Lo, 1986a,b; D'Amore *et al.*, 1986a). D-Glucose and 2-deoxy-D-glucose (dGlc) are transported preferentially by the high-affinity system, whereas 3-O-methyl-D-glucose (MeGlc) is transported primarily by the low-affinity system. In common with that in chick-embryo fibroblasts, the high-affinity system is much more sensitive to thiol reagents (Christopher *et al.*, 1976; D'Amore & Lo, 1986b). Plasma-membrane-vesicle studies show that D-glucose and dGlc are transported against a concentration gradient, whereas MeGlc only equilibrates across the membrane (Cheung & Lo, 1984; Mesmer *et al.*, 1986). These two systems also respond differently to glucose starvation (D'Amore & Lo, 1986a; D'Amore *et al.*, 1986b). Different genetic elements are responsible for the two transport systems, as mutants defective only in the high- but not the low-affinity hexose-transport system have been isolated (D'Amore *et al.*, 1986a). Finally, the low-affinity system is much more sensitive to CB than is the high-affinity system (D'Amore & Lo, 1986c). The K_i value for CB inhibition of the high-affinity transport system is $0.16 \mu\text{M}$, whereas that for the low-affinity transport system is $0.016 \mu\text{M}$. This observation suggests that specific CB-binding site(s) may be involved in the regulation of hexose transport. The present paper reports our attempts at establishing the relationship between the CB-binding sites and the hexose-transport systems.

Part of this work was presented at a symposium at the Second Rossiter Research Conference (D'Amore & Lo, 1986c).

METHODS AND MATERIALS

Cell lines and culture media

Yaffe's L6 rat skeletal myoblast (Yaffe, 1968) was maintained in Alpha medium (Flow Laboratories) supplemented with 10% (v/v) horse serum (Flow Laboratories) and gentamycin ($50 \mu\text{g/ml}$; Gibco) as previously described (Lo & Duronio, 1984a,b). Transfers were routinely made every 3 days (before fusion), 0.1% trypsin being used to detach cells from the plates. The glucose starvation medium (or referred to as the 'fructose medium') comprised essentially the same ingredients, except that glucose was replaced with 0.1% fructose and that the horse serum was previously dialysed extensively against complete phosphate-buffered saline (cPBS).

Cytochalasin B binding to whole cells

The oil-phase-separation method was used to measure CB binding to intact whole cells. This is actually a modification of the method used for studying hexose uptake in cell suspensions (Klip *et al.*, 1982). Cells were grown on tissue-culture dishes ($20 \text{ mm} \times 140 \text{ mm}$) in the glucose or fructose medium and harvested before reaching confluence. After dissociation from each plate by incubating with 5 ml of citrate/saline ($134 \text{ mM-KCl}/15 \text{ mM-trisodium citrate}$, pH 7.8) at 37°C for 15–20 min, cells were pelleted by centrifugation at $1000 g$ for 5 min. The cell pellet was suspended in cPBS at a density of 2×10^7 cells/ml and kept at 4°C to minimize cell damage. Just before the binding assay, cells were incubated at 37°C for 5 min; $100 \mu\text{l}$ of this suspension was then incubated with an equal volume of cPBS containing [^3H]CB (0.01 – $2.5 \mu\text{M}$), and $10 \mu\text{M-CE}$ for 6 min at 37°C . Sugar analogues for inhibitors were included in this buffer whenever necessary. The sugar concentration in the binding buffer was adjusted to 0.6 M with sorbitol. The

reaction mixture was then transferred to an Eppendorf micro test tube containing 1 ml of dibutyl phthalate and centrifuged immediately at 4°C for 2 min. The cell pellet was separated from the unbound [^3H]CB by the oil phase. After carefully aspirating off the liquid, the cell pellet was solubilized in $100 \mu\text{l}$ of 0.5 M-NaOH for 4 h or longer and counted for radioactivity in 10 ml of scintillation fluid supplemented with 0.7% acetic acid. Background radioactivity for each assay was determined by adding unlabelled CB ($10 \mu\text{M}$) or phloretin (0.5 mM). In order to determine the response of the CB-binding sites to D-glucose, the latter was not included in the background-radioactivity determinations. All CB binding experiments were carried out in duplicate and each was repeated two or three times. Unless stated otherwise, values are results of representative experiments, background radioactivity having been subtracted from the crude data. The above procedures provide quite reproducible data.

Measurement of intracellular free sugar and sugar phosphate

Intracellular free sugar and sugar phosphate were determined as previously described (D'Amore & Lo, 1986a), with some modifications. Cells were incubated with 400 mM of the radioactive sugar analogues (dGlc, D-glucose and MeGlc) in the presence of non-radioactive CB ($0.5 \mu\text{M}$) under CB binding conditions. After 6 min of incubation, cells were separated from the reaction mixture by the oil-phase-separation procedure. The cell pellet was immediately washed with $200 \mu\text{l}$ of ice-cold cPBS. The internalized substrate was then released by treatment with 1 ml of ice-cold 10% (w/v) trichloroacetic acid. After centrifugation, the trichloroacetic acid-soluble materials were extracted three times with 1 vol. of ethyl ether to remove the trichloroacetic acid. After removal of ethyl ether by heating at 60°C for 10 min, $200 \mu\text{l}$ of this extract was then loaded on to a column ($1.7 \text{ cm} \times 14 \text{ cm}$) of Dowex 1-X8 (200–400 mesh, Cl^- form) resin (Bio-Rad) (D'Amore & Lo, 1986a). It should be noted that the column was not saturated under these conditions (results not shown). The unphosphorylated sugar was first eluted with 40 ml of water, and the sugar phosphate was then eluted with 40 ml of 0.1 M-HCl plus 0.1 M-NaCl ; 2 ml fractions were collected. The amounts of free sugar and sugar phosphate were then determined.

Estimation of intracellular space

The intracellular space under CB binding conditions was determined as described previously (D'Amore & Lo, 1986a) with some modifications. Cells were incubated with 400 mM radioactive MeGlc under conditions similar to those used in CB binding experiments. The amounts of MeGlc internalized at different times were determined. At equilibrium, the amount present inside the cells was $51.6 \text{ nmol}/10^5$ cells. This indicated an intracellular volume of 1.29 pl/cell . We have previously obtained a value of 1.86 pl/cell for cells in suspension (Lo, 1979). The observed decrease in cell volume is probably due to the very high sugar concentration used under CB binding conditions.

Preparation of plasma-membrane fractions

Plasma-membrane fractions were prepared from undifferentiated L6 cells as described previously (Cheung & Lo, 1984), with some modifications. Briefly, rat myoblasts

were grown in 245 mm × 245 mm × 20 mm tissue-culture plates (Nunc, Kamstrup, Denmark) at 37 °C with air/CO₂ (19:1). Cells were washed twice with PBS containing PMSF (0.5 mM), EDTA (1 mM), leupeptin (2 µg/ml) and pepstatin A (1 µg/ml), harvested by scraping them off the plates, and centrifuged at 700 *g* for 10 min at 25 °C. All remaining steps were carried out at 4 °C. Cells were homogenized (15–20 strokes) with a tight-fitting (pestle A) Dounce homogenizer in ice-cold buffer A [0.25 M-sucrose/10 mM-Hepes (pH 7.0)/1 mM-EDTA/0.5 mM-PMSF/leupeptin (2 µg/ml)/pepstatin A (1 µg/ml)]. The extent of cell breakage was monitored with a phase-contrast microscope. Unbroken cells were removed by centrifugation at 700 *g* for 5 min. After centrifugation at 33000 *g* for 15 min, the supernatant was kept for microsome preparation, whereas the pellet was resuspended in ice-cold buffer A and layered over a discontinuous sucrose density gradient, containing 3 ml portions of 35, 29 and 24 % (w/w) sucrose in buffer B [Hepes (10 mM)/PMSF (0.5 mM)/EDTA (1 mM)/leupeptin (2 µg/ml)/pepstatin A (1 µg/ml), pH 7.0]. The gradient was centrifuged at 33000 rev./min for 2 h in a Beckman SW40 rotor. The interfaces between buffer A and 24 % sucrose (fraction A), and that between 24 and 29 % sucrose (fraction B) were collected and diluted with buffer B and centrifuged at 33000 *g* for 20 min. The pellets were resuspended in the same buffer and stored at –80 °C. Both fractions A and B exhibit a 5–7-fold enrichment of the ouabain-sensitive Na⁺,K⁺-ATPase (plasma-membrane marker enzyme) specific activity over that of the initial cell-free extract; these two fractions have negligible activities of hexokinase, lactate dehydrogenase, succinate-dependent cytochrome *c* reductase and NADPH-dependent cytochrome *c* reductase (Cheung & Lo, 1984).

Preparation of microsomal fractions

Microsomal membranes were prepared as described by (Carter-Su & Okamoto, 1985), with some modifications. Cell-free homogenates were prepared as described above. The HDMF was pelleted by centrifuging the 33000 *g* supernatant at 48000 *g* for 20 min. The pellet was then resuspended in 10 mM-Hepes buffer, pH 7.0. The specific activity of Na⁺,K⁺-ATPase in this fraction was 30–40 % of the plasma membrane. The LDMF was harvested by further centrifuging the 48000 *g* supernatant at 250000 *g* for 1 h. The Na⁺,K⁺-ATPase activity in LDMF was about 2–5 % of the plasma-membrane activity.

CB binding to membrane preparations

Equilibrium CB binding was initiated by the addition of membrane preparation (suspended in 10 mM-phosphate buffer, pH 7.4, and 1 mM-EDTA) to the binding-assay buffer. The latter contains 10 mM-phosphate buffer (pH 7.4), CE and [³H]CB. The total volume of the reaction mixture was 160 µl. The final membrane protein concentration was between 0.5 and 1.25 mg/ml, and the final concentrations of CE and CB were 10 µM and 0.011–3.0 µM respectively. The reaction mixture was incubated at 4 °C for 90 min in polycarbonate microcentrifuge tubes (7 mm × 20 mm; Beckman). This was then centrifuged at 436000 *g* for 40 min at 4 °C in a Beckman TL-100 ultracentrifuge. Centrifugation at such high centrifugal force was found to be essential to obtain a tightly packed pellet. The amount of

unbound (i.e. free) CB was determined by sampling 60 µl of the resulting supernatant. In order to remove the residual CB, the tubes were washed four times with 180 µl of sucrose/phosphate buffer [0.25 M-sucrose/10 mM-sodium phosphate (pH 7.4)/1 mM-EDTA]. The pellet was dissolved overnight at 80 µl of 0.5 M-NaOH, and counted for radioactivity in scintillation fluid containing 0.7 % acetic acid. The background radioactivity was determined by the inclusion of 30 µM unlabelled CB and 100 µM-phloretin. Similar background radioactivity was obtained when the unlabelled CB concentrations varied from 10 to 100 µM (results not shown). Since the effect of D-glucose on the two CB-binding sites was to be examined, D-glucose was not used to determine the background radioactivity. All the results presented represent specific binding, background radioactivity having been subtracted from the crude data. All CB binding studies were carried out in duplicate. Unless otherwise stated, the results presented illustrate results of representative experiments, which were repeated at least twice and usually three or more times. Results were found to be very consistent in all cases.

Protein determination

Protein determination was by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Materials

2-Deoxy-D-[2,6-³H]glucose (38.7 Ci/mmol), 3-O-[1-³H]methyl-D-glucose (2.1 Ci/mmol) and [4-³H(n)]CB (15.5 Ci/mmol) were purchased from Amersham Canada Ltd. D-[³H]Glucose was from New England Nuclear. Scinti Verse E was from Fisher Scientific Co. Cytochalasins, CBs, glucose analogues and other chemicals were from Sigma Chemical Co. All chemicals were obtained from commercial sources and were of the highest available purity.

RESULTS

CB binding to glucose-grown rat L6 myoblasts

We have recently demonstrated that the two hexose-transport systems in rat myoblast L6 are inhibited to different extents by CB (D'Amore & Lo, 1986c). This suggests that the two hexose transporters may have their own specific CB-binding sites. This possibility was tested directly by binding studies using radioactive CB. Although CE can bind to proteins similar to those to which CB binds, it differs from the latter in its inability to recognize the glucose transporter (Carter-Su & Okamoto, 1985). Thus CB binding to the glucose transporters was determined by the inclusion of 10 µM-CE in the binding assay.

Two different CB binding assays have been used by various workers (Klip & Walker, 1983). These included removal of unbound CB from the reaction mixture by filtration or by centrifugation. Although not indicated here, the oil-phase-separation method was found to yield higher specific binding than the filtration method. Table 1 shows the effect of various sugar analogues and hexose-transport inhibitors on CB binding to L6 cells. Whereas sorbitol (at different concentrations) and L-glucose have no effect on CB binding, both MeGlc and dGlc inhibit CB binding to a significant extent. Phloretin, a very potent hexose-transporter inhibitor, is just as effective as 10 µM-CB in blocking specific CB binding. It should be

Table 1. Effects of various sugar analogues and a transport inhibitor on CB binding to whole cells and plasma membrane

Experiments were carried out as described in the text. Values are means $\pm$ S.E.M. for four different experiments. The concentration of CB used in this study was $0.5 \mu\text{M}$. The control value of whole-cell studies was $0.85 \pm 0.009 \text{ pmol}/10^6 \text{ cells}$, and the specific radioactivity of the radioactive CB was $2.40 \times 10^3 \text{ d.p.m.}/\text{pmol}$. In the case of the plasma-membrane studies, the control value was $16.0 \pm 0.42 \text{ pmol}/\text{mg}$ of protein, and the specific radioactivity of the radioactive CB was $1 \times 10^4 \text{ d.p.m.}/\text{pmol}$. Abbreviation: N.D., not determined.

Addition	CB bound (%)	
	Whole cells	Plasma membrane
Control	100 ± 1.1	100 ± 2.6
Sorbitol		
0.2 M	97 ± 2.9	N.D.
0.4 M	N.D.	99 ± 3.0
0.6 M	99 ± 0.4	N.D.
L-Glucose (0.4 M)	95 ± 1.9	98 ± 3.0
MeGlc (0.4 M)	62 ± 1.5	49 ± 3.1
dGlc (0.4 M)	59 ± 4.7	43 ± 2.0
Phloretin		
0.1 mM	N.D.	23 ± 1.9
0.5 mM	0 ± 1.2	N.D.

mentioned that significant inhibition of the binding of $0.5 \mu\text{M}$ -CB to the cells could be observed only when the concentrations of dGlc or MeGlc were above 200 mM.

Fig. 1(a) is a Scatchard plot of CB binding to glucose-grown rat L6 myoblasts. Since the background radioactivity at each CB concentration was determined and subtracted from the raw data, the results presented indicate specific binding. It may be apparent that the slope of the binding curve at low CB concentrations (SL-1) is significantly higher than that at high CB concentrations (SL-2). This indicates the presence of a high- and a low-affinity CB binding site in rat myoblasts. These binding sites will be referred to as 'CB_H' and 'CB_L' respectively. Since different parts of the binding curve represented various extents of contribution by the two CB-binding sites, their binding affinities were therefore determined by the vectorial analysis of a curved Scatchard plot as described by Munck (1976). This approach gave K_d values of 0.11 and $1.07 \mu\text{M}$, and B_{max} (maximal specific binding) values of 0.4 and $1.07 \text{ pmol}/10^6 \text{ cells}$ for CB_H and CB_L respectively. The data were also analysed by a SCAFIT program written for microcomputers to analyse multi-component Scatchard plots (Biomedical Computing Technology Information Center, Vanderbilt Medical Center, Nashville, TN, U.S.A.). This analysis yielded K_d values of 0.11 ± 0.02 and $1.01 \pm 0.03 \mu\text{M}$, and B_{max} values of 0.41 ± 0.07 and $1.06 \pm 0.09 \text{ pmol}/10^6 \text{ cells}$, for CB_H and CB_L, respectively. Thus the two sites differ significantly in their CB-binding affinity.

If two CB-binding sites are indeed present, one may also be able to distinguish them by differential inhibition (Clark & Peck, 1979). To this end, the effects of specific hexose-transport substrates were tested. Fig. 1(a) shows that whereas SL-1 is not significantly affected, SL-2 is

considerably decreased by 400 mM-dGlc. Under the above CB binding conditions, the intracellular concentrations of dGlc and dGlc 6-phosphate were found to be 322 mM and 45 mM respectively. Thus the present study indicates that dGlc is effective in abolishing binding to CB_L; however, it does not have much effect on CB_H. Fig. 1(a) also shows that MeGlc exerts quite a different effect on CB binding. SL-1, but not SL-2, is lowered considerably by 400 mM-MeGlc. Thus CB_H seems to be much more sensitive to MeGlc than CB_L.

CB binding to glucose-starved rat L6 myoblasts

We have recently demonstrated that only the high-affinity hexose-transport system was stimulated during glucose starvation (D'Amore & Lo, 1986a; D'Amore *et al.*, 1986b). If the CB-binding sites are associated with the respective transporters, the corresponding changes may be observed. Fig. 1(b) shows that the CB binding curve for glucose-starved cells has a much higher B_{max} value. This binding curve is similar to SL-2 of glucose-grown cells, not only in its slope, but also in its sensitivity to dGlc. In fact, CB_H becomes apparent only when CB_L is inhibited by dGlc (Fig. 1b). When $0.8 \mu\text{M}$ -CB was used, a 50% decrease in the CB binding could be brought about by 420 mM-dGlc. On the other hand, 400 mM-MeGlc lowered the amount of CB bound without altering the binding affinity (Fig. 1b). The above study shows that CB_L is present at a much elevated level in glucose-starved cells. After correcting for the inhibitory effect of 400 mM-dGlc, the level of CB_H is similar to that in glucose-grown cells.

CB binding to a hexose-transport mutant

The above studies on the preferential inhibition of CB binding by the two specific hexose-transport substrates, and on the glucose-starved cells, suggest that the CB binding sites may be associated with the two hexose transporters. A more definitive test of this finding comes from studies with a hexose-transport mutant. Mutant D23 is defective in the high-affinity hexose-transport system (D'Amore & Lo, 1986c; D'Amore *et al.*, 1986a). The low-affinity hexose-transport system is not altered in this mutant. Fig. 2(a) shows that the slope of the CB binding curve for glucose-grown D23 is quite similar to SL-1 of glucose-grown L6 cells; CB_L can hardly be detected in this mutant. The CB-binding site in this mutant is similar to CB_H in glucose-grown L6 cells in its binding capacity and sensitivity to MeGlc (Fig. 2a). At a CB concentration of $0.4 \mu\text{M}$, 50% inhibition of CB binding occurs in the presence of 420 mM-MeGlc. Both glucose starvation (Fig. 2b) and dGlc (Fig. 2a) are also found to have not much effect on the CB-binding site in this mutant. Thus CB_L is either absent or present at a very low level in mutant D23.

CB binding to purified plasma membrane from rat L6 myoblasts

In order to define the location of the CB-binding sites and to carry out binding studies in the absence of various metabolic events, CB binding studies were carried out with purified membrane preparations. The properties of the plasma membrane prepared by our established procedures have been described elsewhere (Cheung & Lo, 1984). They are essentially devoid of marker enzymes for other cellular organelles. Fig. 3 shows the binding of CB to purified plasma membrane (fractions A and B).

Fig. 1. CB binding to rat myoblast L6 cells

(a) and (b) are Scatchard plots for CB binding in the presence and absence respectively of various sugar analogues. The concentration range of CB used was from 10 nM to 2.5 μM. CB binding was determined by the oil-phase-separation method as described in the text. Results represent the amount of CB specifically bound to the cells, the amount of non-specific background radioactivity at each CB concentration having been subtracted. Values were the means for duplicate estimates from one typical experiment. The average S.E.M. was about 5%. (a) Indicates the CB binding curve for glucose-grown L6 cells: ●, control experiment where 400 mM-sorbitol was present; △ and ○, CB binding in the presence of 400 mM-dGlc and 400 mM-MeGlc respectively. (b) Indicates the binding curve for glucose-starved cells: ●, control experiment, where 400 mM-sorbitol was present; □ and △, CB binding in the presence of 400 mM-dGlc and 400 mM-MeGlc respectively.

Fig. 2. CB binding to hexose-transport mutant D23

(a) and (b) are Scatchard plots illustrating the CB binding properties of the hexose-transport mutant D23. Experiments were carried out as described in Fig. 1. (a) Indicates the properties of the glucose-grown D23: ●, control experiment, where 400 mM-sorbitol was present; ▽ and □, binding in the presence of 400 mM-dGlc and 400 mM-MeGlc respectively. The broken line indicates the binding curve of glucose-grown L6 cells, as illustrated in Fig. 1. (b) Indicates the CB binding curve of glucose-starved cells: ○ and ●, CB binding curves for mutant D23 and its parental strain, L6 respectively. Values are means of duplicate determinations from one typical experiment.

Similar to whole cells, two CB binding sites (CB_H and CB_L) can be detected on this purified plasma membrane. Vectorial analysis (Munck, 1976; Clark & Peck, 1979) gave K_d values of 0.10 and 1.44 μM, and B_{max} values of 12.1 and 43 pmol/mg of protein for CB_H and CB_L, respectively. Similar values were also obtained by the SCAFIT program, which yielded K_d values of 0.09 ± 0.01 and 1.63 ± 0.29 μM, and B_{max} values of 11.4 ± 1.4 and 44.6 ± 5.4 pmol/mg of protein respectively. This represents a 16–23-fold increase in the number of CB-binding sites per mg of protein when compared with whole cells (Fig. 1). Since this is a fairly pure plasma-membrane preparation, it may be surmised that both

CB_L and CB_H are present on the plasma membrane. The ratio of the B_{max} values of CB_L and CB_H is about 3.91.

Fig. 3 also shows the curve of CB binding to plasma membrane prepared from mutant D23. Analysis of this binding curve by both vectorial analysis and by the SCAFIT program yielded similar data. The K_d values were calculated to be 0.13 ± 0.01 and 4.18 ± 0.29 μM, and the B_{max} values were 11.4 ± 0.70 and 18.1 ± 0.91 pmol/mg of protein for CB_H and CB_L respectively. The ratio of the B_{max} values of CB_L and CB_H is around 1.59. Thus the K_d and B_{max} values of CB_L in mutant D23 differ significantly from those of L6-myoblast plasma membrane (Fig. 3), whereas those for CB_H remain unaltered.

CB binding to microsomal fractions

CB binding was assessed by the same procedure as that used for the plasma membrane. As for plasma membrane, a biphasic curve was also observed with HDMF (Fig. 4). Analysis by both vectorial analysis and by the SCAFIT program yielded similar results: the HDMF exhibited K_d values of 0.09 ± 0.03 and $1.49 \pm 0.10 \mu\text{M}$, and B_{max} values of 6.1 ± 0.07 and $22.6 \pm 1.90 \text{ pmol/mg}$ of protein, for CB_H and CB_L respectively. The ratio of the B_{max} values for CB_L and CB_H was about 3.7. Both CB_H and CB_L are also found in LDMF, albeit at a much reduced level (Fig. 4). Analysis of the data revealed the CB binding sites of LDMF exhibited K_d values of 0.11 ± 0.01 and $1.6 \pm 0.07 \mu\text{M}$, and B_{max} values of 1.75 ± 0.02 and $6.75 \pm 0.61 \text{ pmol/mg}$ of protein for CB_H and CB_L respectively. The ratio of the B_{max} values of CB_L and CB_H was 3.86.

Effect of glucose analogues on CB binding to purified plasma membrane

Similar to the situation in whole cells, CB binding to purified plasma membrane is inhibited only by very high concentrations of MeGlc and dGlc. Whereas 400 mM-sorbitol and 400 mM-L-glucose have no effect on CB binding, 400 mM-dGlc or 400 mM-MeGlc can bring about a 50–60% inhibition of CB binding (Table 1); furthermore, 77% inhibition can be brought about by 0.1 mM-phloretin.

Differences between whole cells and purified plasma membranes became apparent when the effect of D-

Fig. 3. CB binding to purified plasma membrane from glucose-grown cells

Preparation of plasma membrane and CB binding assays were carried out as described in the text. CB binding was determined at concentrations ranging from $0.011 \mu\text{M}$ to $3.0 \mu\text{M}$. Data represent binding to specific sites. The amount of non-specific binding (background radioactivity) was determined for each concentration and subtracted from the raw data. ● and ○, Binding to plasma membrane prepared from glucose-grown L6 and D23 cells. Values presented are the means of duplicate estimates from one typical experiment. The average S.E.M. was about 3%.

glucose on CB binding was examined. Fig. 5(a) shows that 400 mM-D-glucose has no effect on CB binding to glucose-grown L6 cells. This is surprising in view of the response of these cells to the various sugar analogues (Table 1). The major difference between D-glucose and the sugar analogues tested is that the former is converted into different metabolites upon entrance into the cells. In order to examine the effect of D-glucose in the absence of its metabolites, binding studies were carried out with purified plasma membrane, which has been demonstrated to be devoid of hexokinase (Cheung & Lo, 1984). Fig. 5(b) shows that, in the presence of 400 mM-D-glucose, the K_d values of CB_H and CB_L were $0.10 \pm 0.01 \mu\text{M}$ and $5.00 \pm 0.34 \mu\text{M}$, and the corresponding B_{max} values were 4.2 ± 0.30 and $54 \pm 5.70 \text{ pmol/mg}$ of protein, respectively. The ratio of the B_{max} values of CB_L and CB_H is 12.9. Thus whereas D-glucose does not have much effect on the binding affinity of CB_H , it diminishes the latter's binding capacity by 63%; however, the reverse is true for CB_L .

Effects of sugar phosphate on CB binding

Data presented above suggest that the lack of effect of D-glucose on CB binding to whole cells may be due to the activation of CB binding by the glucose metabolites. When cells were incubated with 400 mM-D-glucose under CB binding conditions, the intracellular concentrations of glucose and glucose phosphate were found to be about 200 mM and 30 mM respectively. The effect of glucose 6-phosphate was therefore tested. Table 2 shows that 40, 100 and 150 mM-glucose 6-phosphate can bring about 41, 77 and 103% activation of CB binding to the plasma membrane respectively. When plasma-membrane CB binding studies were carried out in the presence of 400 mM-D-glucose and 40 mM-glucose 6-phosphate, only 14% inhibition was observed under these conditions (Table 2). This is similar to the effect of D-glucose on whole cells. It is therefore conceivable that the lack of significant inhibition by D-glucose on CB binding to whole cells may be due to the activation of CB binding by D-glucose 6-phosphate. Table 2 also shows that glucose 6-phosphate has no effect on CB binding to

Fig. 4. CB binding to HDMF and LDMF from glucose-grown L6 myoblasts

Preparation of HDMF and LDMF and CB binding were carried out as described in the text and in Fig. 3. ○ and ▲, CB binding to HDMF and LDMF respectively.

Fig. 5. Effect of D-glucose on CB binding to whole cells and purified plasma membrane from glucose-grown L6 myoblasts

CB binding to whole cells and plasma membrane was carried out as described in Figs. 1 and 3 respectively. (a) Indicates CB binding to glucose-grown L6 cells: ● and △, CB binding in the presence of 400 mM-sorbitol and 400 mM-D-glucose respectively. (b) Indicates CB binding to plasma membrane from glucose-grown L6 myoblasts: ● and ○, CB binding in the presence of 400 mM-sorbitol and 400 mM-D-glucose respectively.

Table 2. Effects of sugars and sugar phosphates on CB binding to whole cells and plasma membrane

Experiments were carried out as described in Table 1. Values are means $\pm$ S.E.M. for four different experiments. The concentration of CB used in this study was $0.5 \mu\text{M}$. The control value of whole-cell studies was $0.85 \pm 0.009 \text{ pmol}/10^6 \text{ cells}$, and the specific radioactivity of the radioactive CB was $2.40 \times 10^3 \text{ d.p.m.}/\text{pmol}$. In the case of the plasma-membrane studies, the control value was $16.0 \pm 0.42 \text{ pmol}/\text{mg}$ of protein, and the specific radioactivity of the radioactive CB was $1 \times 10^4 \text{ d.p.m.}/\text{pmol}$. Abbreviations: N.D., not determined; 6-P, 6-phosphate.

Addition	CB bound (%)	
	Whole cells	Plasma membrane
Control	100 ± 1.1	100 ± 2.6
D-Glucose (0.4 M)	89 ± 2.0	43 ± 2.0
Glucose 6-P		
40 mM	101 ± 2.5	141 ± 3.2
100 mM	108 ± 3.5	177 ± 1.0
150 mM	99 ± 1.5	203 ± 3.0
D-Glucose (0.4 M) + glucose		
6-P (40 mM)	N.D.	86 ± 2.1
dGlc (0.4 M)	59 ± 4.7	43 ± 2.0
dGlc 6-P		
40 mM	96 ± 0.5	104 ± 2.7
100 mM	101 ± 4.5	113 ± 1.0
150 mM	105 ± 2.5	120 ± 6.1
dGlc (0.4 M) + dGlc 6-P (45 mM)	N.D.	53 ± 2.1

whole cells, this indicates that the glucose 6-phosphate binding site is not accessible to the external environment.

Table 2 shows that dGlc differs from D-glucose in its inhibition of CB binding to whole cells. This suggests that dGlc or its metabolite may be acting on the CB-binding sites in a different manner. Table 2 shows that

Fig. 6. Effect of trypsin on CB binding to sealed right-side-out and inside-out-plasma membrane preparations

Plasma-membrane fractions A and B were prepared as described in the text. Membranes were treated with trypsin (0–500 $\mu\text{g}/\text{ml}$) in 10 mM-phosphate buffer, pH 7.4, containing 1 mM-EDTA at 37 °C for 1 h. CB binding was initiated by adding buffer containing 10 μM -CE, 0.5 μM -[^3H]CB (final concns.). The reaction mixture was incubated at 4 °C for 90 min, the remaining steps having been described in Fig. 3. ○, Corrected specific CB binding to right-side-out sealed vesicles; ▲, corrected specific CB binding to inside-out vesicles or plasma-membrane fragments. Results represent specific binding. Values are the means ($\pm$ S.E.M.) for three separate experiments. The specific radioactivity of CB used was $10^4 \text{ d.p.m.}/\text{pmol}$.

dGlc 6-phosphate is not as effective as D-glucose 6-phosphate in activating CB binding to the plasma membrane; 40, 100 and 150 mM-dGlc 6-phosphate could bring about only 4, 13 and 20% activation of specific CB binding respectively. Whereas 400 mM-dGlc inhibits CB binding to the plasma membrane by 57%, the inclusion of

45 mM-dGlc 6-phosphate did not cause significant changes in CB binding (Table 2). The extent of inhibition was quite similar to that of whole cells.

Effect of trypsin treatment on the CB-binding sites

Although not indicated here, treatment of cells with trypsin (1 mg/ml) for 30 min at 37 °C was found to have no effect on the amount of CB bound and on the response of the CB binding sites to various sugar analogues. This suggests that the CB-binding sites are either not accessible to the external environment or that they are resistant to cell-surface trypsin treatment. These possibilities were further explored with sealed and open plasma-membrane preparations. We have previously demonstrated that sealed right-side-out (fraction A) and open vesicles (fraction B) can be separated by sucrose-gradient centrifugation (Cheung & Lo, 1984). After correction for the presence of the contaminating species, Fig. 6 shows that CB binding to the sealed right-side-out vesicles is relatively resistant to trypsin treatment; however, the reverse is true with the inside-out or leaky membrane preparations. Incubation of membrane with trypsin (50 µg/ml) for 1 h resulted in a 50 % decrease in CB binding to inside-out or leaky vesicles, whereas only a 3 % decrease was observed with the sealed right-side-out vesicles.

DISCUSSION

The mechanism and regulation of hexose transport have been studied quite extensively in human red blood cells, adipocytes and chick-embryo fibroblasts (Czech, 1976a,b; Olefsky, 1978; Baldwin & Lienhard, 1981; Griffin *et al.*, 1982; Pessin *et al.*, 1982). CB has been used as affinity ligand to identify and to quantify the glucose transporter (Cushman & Wardzala, 1980; Foley *et al.*, 1980; Mullins & Langdon, 1980; Simpson & Cushman, 1986). Upon photolysis, CB is covalently linked to a diffuse 45–70 kDa band; such labelling is partially blocked by 500 mM-D-glucose. Since the human red-blood-cells glucose transporter has a molecular mass of about 45 kDa, it is thought that the CB-labelled protein may actually be the glucose transporter (Pessin *et al.*, 1982). The observation that a very high concentration of D-glucose is required to produce close to 50 % inhibition of CB binding raises the question as to whether CB is actually binding to the glucose binding site of the transporter. The uncertainty of this CB-binding site does throw some doubts on the identity of the CB-binding component.

Two hexose-transport systems have recently been demonstrated in undifferentiated rat myoblasts (D'Amore & Lo, 1986a,b,c; D'Amore *et al.*, 1986a,b). These systems differ not only in their transport affinity and substrate specificity, but also in their expression under various growth conditions. Mutants defective in only one hexose-transport system have been isolated (D'Amore *et al.*, 1986a). It is therefore conceivable that these mutants can be used as unique tools in determining the identity of the CB-binding components.

In the present investigation, CB binding studies were carried out with whole cells and purified membrane preparations. Whole-cell studies have several distinct advantages. (i) Whole-cell studies require only a relatively small number of cells, whereas a much larger number of

cells are required for plasma-membrane preparation (Cheung & Lo, 1984). (ii) Whole-cell studies allow a direct comparison of the transport and the CB-binding properties. (iii) Unless purified plasma membrane is used, crude membrane preparations do not have distinct advantage over whole cells, as they still contain a substantial amount of microsomal and mitochondrial contamination. (iv) A comparison of the CB-binding properties of whole cells with those of purified plasma-membrane preparations may provide additional information on the effect of various metabolites. On the other hand, studies with purified membrane preparations allow one to define the location, and possibly the orientation, of the CB-binding sites, and to measure CB binding in the absence of various metabolites. The present results indicate that both whole-cell and plasma-membrane studies yield essentially similar information on the intrinsic properties of the CB-binding sites.

The following pieces of evidence suggest that two specific CB-binding sites are present in undifferentiated rat L6 myoblasts. (i) Scatchard analysis of the binding data revealed the presence of two specific CB-binding sites in glucose-grown L6 cells (Fig. 1). The K_d values of CB_H and CB_L were 0.11 ± 0.02 and 1.01 ± 0.03 µM respectively. Binding studies with purified plasma membrane also yielded K_d values of 0.09 ± 0.01 and 1.63 ± 0.29 µM for CB_H and CB_L respectively (Fig. 3). (ii) The two CB-binding sites can be differentially inhibited by specific sugar analogues. CB_H is preferentially inhibited by MeGlc, whereas CB_L is specifically inhibited by dGlc (Fig. 1). Fig. 5 shows that D-glucose decreases the B_{max} value of CB_H by 63 %, without altering its K_d value. On the other hand, D-glucose decreases only the binding affinity of CB_L , without diminishing the binding capacity. (iii) Glucose starvation increased the level of CB_L without affecting CB_H (Fig. 1). (iv) Since only CB_H , but not CB_L , can be detected in transport mutant D23 (Figs. 2 and 3), the two CB-binding sites are therefore likely coded for, or regulated by, different genetic elements. In conclusion, the two CB-binding sites not only differ in their binding affinity, but their levels can also be differentially altered by various biochemical, physiological and genetic manipulations. Since both whole-cell and plasma-membrane studies yielded similar data, and since each type of binding assay was performed and analysed under identical conditions, it was unlikely that our observations were artefacts arising from the methods of analysis. It can be calculated from the B_{max} values that there are 2.4×10^5 CB_H and 6.4×10^5 CB_L binding sites per glucose-grown L6 myoblast respectively. It is noteworthy that about 3.3×10^5 binding sites/cell are found in human erythrocytes (Klip *et al.*, 1982).

The next question concerns the identity of these two specific CB-binding sites. Both biochemical and genetic evidence indicate that CB_L is associated with the high-affinity hexose-transport system. (i) CB_L can be inhibited by the preferred substrates of the high-affinity transport system, but not by the preferred substrate of the low-affinity system (Figs. 1 and 5). (ii) Glucose starvation activates not only the high-affinity transport system, but also CB_L (Fig. 1). (iii) A mutant defective in the high-affinity hexose-transport system is also defective in CB_L (Figs. 2 and 3; D'Amore & Lo, 1986c). (iv) Glucose starvation fails to generate the expected increase in either CB_L or the high-affinity transport activity in this mutant. The above correlations strongly suggest that CB_L is

associated with the high-affinity hexose-transport system.

One of the advantages of using mutant D23 is that it allows one to examine the properties of the low-affinity hexose-transport system without the interference of the high-affinity transport system. Both whole-cell and plasma-membrane studies show that CB_L can hardly be detected in this mutant, and yet the properties of its CB_H are quite similar to those of glucose-grown L6 cells (Figs. 2 and 3). It is noteworthy that K_m and V_{max} values for the low-affinity hexose-transport system in this mutant are also similar to the corresponding values in L6 cells. In common with L6 cells, the levels of CB_H and the low-affinity transport system remain unaltered in both glucose-grown and glucose-starved mutant D23. Furthermore, the mutant-D23 CB-binding site on this mutant is similar to the L6 CB_H in its insensitivity to dGlc. The above observations, together with the fact that these two sites can be inhibited by the substrate of the low-affinity hexose-transport system, serve to demonstrate the close correlation between CB_H and the low-affinity hexose-transport system.

Membrane preparations are useful in determining the location of the CB-binding sites. Whole-cell studies indicate that two specific CB-binding sites are present in rat myoblasts. If one site is associated with the plasma membrane, and the other is with another membrane, then the ratio of the B_{max} values of the two sites should vary considerably in different membrane preparations. Figs. 3 and 4 indicate that both CB binding sites can be detected in plasma membrane, LDMF and LDMF; the ratio of their B_{max} values of CB_L and CB_H are around 3.80. Since both plasma-membrane and LDMF preparations are relatively free of cross-contamination, both CB_H and CB_L are likely to be present in similar proportions in these two preparations. It should be noted that whereas LDMF contains not more than 2–5% contamination by the plasma membrane (as judged by the level of Na,K-ATPase), the specific activities of both CB-binding sites in LDMF are about 15% of those in the plasma membrane. This is different from rat adipocytes, in which there are ten times more CB-binding sites on the microsomes (microsomal fractions) than on the plasma membrane (Simpson & Cushman, 1986). It is noteworthy that undifferentiated rat myoblasts also differ from adipocytes in their lack of response to insulin treatment (Klip *et al.*, 1983).

Plasma-membrane vesicles are also useful in determining the orientation of specific sites. Whereas trypsin treatment has no effect on CB binding to whole cells and sealed right-side-out vesicles, it can abolish CB binding to membrane fragments or inside-out vesicles (Fig. 6). This suggests a cytoplasmic location for CB binding sites. A similar observation has also been made by other workers (Deziel & Rothstein, 1984; Shanahan & D'Artell-Ellis, 1984; Krupka & Deves, 1986). It should be noted that the above observation can also be explained by the possibility that trypsin treatment of a component on the membrane inner surface may lead to the release of externally bound CB. If this were the case, then the present study serves to illustrate the orientation of the site of trypsin attack. Plasma membrane is also useful in determining the effect of specific substrate metabolites. Table 2 shows that D-glucose 6-phosphate, but not its deoxyglucose counterpart, is very effective in activating CB binding. Whole-cell studies suggest that this glucose

6-phosphate-binding site is not accessible on the cell surface. The finding that CB binding can be activated by a sugar analogue suggests that a regulatory mechanism may be involved in CB binding.

Several interesting conclusions can be drawn on the regulatory mechanism of CB binding. Two models can be proposed for the regulation of CB binding. Model A depicts that transport substrates inhibit CB binding by competitive inhibition. Model B predicts CB binding may be regulated by an allosteric mechanism. Both whole-cell and plasma-membrane binding studies tend to support the second model. (i) If the ligands are competing for the same site, their K_d and K_i values should be quite similar, as seen in the case of different sugars competing for the same transporter (D'Amore & Lo, 1986a). Figs. 1 and 3 show that the K_d values for CB binding differ significantly from the K_i values of CB for both transport processes (D'Amore & Lo, 1986c). For example, the K_d value for CB_L is $1.32 \mu M$, whereas the K_i of CB for the corresponding transport system is $0.16 \mu M$. (ii) If competitive inhibition is involved, 50% inhibition of CB binding should be observed when the substrate/CB ratio has a value about that of the affinity ratios. Both whole-cell and plasma-membrane CB binding studies indicate that at least several hundred thousand times higher concentrations of transport substrates are required to bring about 50% inhibition of CB binding. (iii) The K_i value of dGlc for the low-affinity hexose transporter is 1 mM (D'Amore & Lo, 1986a). Model A would predict that 400 mM-dGlc should be able to inhibit CB binding to this transporter. However, this is clearly not the case (Fig. 1). (iv) Similarly, the lack of effect of MeGlc on CB_L also suggests that CB cannot be competing with MeGlc for the high-affinity hexose transporter. (v) Fig. 5 shows that D-glucose decreases the B_{max} , but not the K_d , values of CB_H . This diminution in binding capacity suggests that D-glucose is not competing for the CB binding site on the low-affinity hexose transporter. (vi) Table 2 indicates that CB binding is activated by D-glucose 6-phosphate. The only evidence that may support Model A comes from the effect of D-glucose on CB_L (Fig. 5). However, this effect is observed only at very high concentration of D-glucose (400 mM). So this decrease in binding affinity is not likely to be due to competitive inhibition. The above findings suggest that CB binding may be regulated in an allosteric manner; it is inhibited by specific hexose-transport substrates and activated by glucose 6-phosphate. In agreement with this notion, it was recently demonstrated that D-glucose and CB may be binding to different sites on the hexose transporter (Jung *et al.*, 1986).

In conclusion, we have shown in the present study that two CB-binding sites are present in the undifferentiated rat myoblasts. Both biochemical and genetic studies demonstrate the close correlation between the CB-binding sites and the hexose-transport systems. Finally, binding of CB to these sites may be regulated by various sugar analogues in an allosteric manner.

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