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Mechanisms of Glucose Transport Regulation in Cultured Chinese Hamster Fibroblast Cell Lines

Susan Pratt Lefebvre

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ABSTRACT

Mechanisms of Glucose Transport Regulation
in Cultured Chinese Hamster Fibroblast Cell Lines

Susan Pratt Lefebvre

In this thesis, sugar transport regulation was characterized in terms of the expression and subcellular distribution of the glucose transporter (GT) in the V79 hamster fibroblast cell line, and in a respiration deficient mutant (G14) of the V79 cell line. In V79, increases in the plasma membrane GT content correlated with the stimulatory effects of insulin and glucose deprivation. In contrast, the 3-fold elevation in basal sugar transport observed in the G14 cell line did not coincide with any significant difference in either the whole cell or plasma membrane GT content when compared to V79 - suggesting an enhancement of the intrinsic activity of the GT expressed in G14. A difference in the average molecular weights of the transporters was observable between the two cell lines, however. Treatment with endoglycosidase F or tunicamycin established that the G14 cell line exhibited a hyperglycosylated form of the same core GT protein expressed in V79.

In a second study, the dramatic down-regulatory effects of D-allose were examined in both the G14 and V79 cell lines. In both cell lines, however, D-allose treatment did not alter GT content in either the whole cell or plasma membrane fractions. In this case, a negative regulatory effect on the intrinsic activity of the GT is implicated.

The regulation of sugar transport through modulation of the intrinsic activity of the GT is a rapidly evolving theory. The continued study of novel instances of sugar uptake regulation such as these could provide clues as to the factors involved in such a mechanism.

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INTRODUCTION

In most animal cells, sugars are the primary fuel for the generation of energy and subsequent survival of the cell. Because sugars are hydrophillic molecules, they do not readily diffuse across the plasma membrane, and from bacteria to yeast to man, a superfamily of related transmembrane carrier proteins are responsible for specifically binding and transporting sugars into the cell (reviewed in Baly and Horuk, 1988; Baldwin and Henderson, 1989). As a result, physiological and environmental factors are able to regulate sugar uptake through this carrier mediated process.

Regulation of sugar uptake has been shown to occur by various mechanisms, many of which involve differential expression of the carrier protein. In this thesis, I will examine the expression of the carrier, or glucose transporter, protein in a Chinese hamster lung fibroblast (V79) cell line. I will relate the effects of known regulators of sugar uptake on the glucose transporter expression in these cells, as well as investigate two novel situations shown to have dramatic effects on sugar uptake. The first situation involves the upregulation seen in a metabolic (respiration deficient) mutant of the V79 cell line (Germinario et al, 1990a), and the second concerns the down-regulation imposed by the non-metabolizable sugar, D-allose (Germinario et al, 1990b).

The glucose transporter protein(s):

With the exception of the Na+-dependent active transport of sugars in the intestine and kidney, most mammalian cells take up sugars via a passive process termed "facilitated diffusion", previously reviewed by Plagemann and Richey, 1974, and Wheeler and Hinkle, 1985. These reviews describe how the facilitated process is driven by the concentration gradient of free sugars, and is mediated by the glucose transporter (GT), a carrier protein stereospecific for D-glucose or D-glucose analogs. The transport process is further characterized as being saturable, displaying typical Michealis-Menten kinetics, and being inhibited by the fungal metabolite, cytochalasin B.

The human erythrocyte transporter is the most well characterized of the facilitative glucose transporters. This glucose transporter constitutes 4-5% of the erythrocyte membrane proteins, and on SDS-polyacrylamide gels, it migrates as a broad band with an average molecular weight of 55 kilodaltons (kD) (Baldwin et al, 1982). The diffuse nature of the band is due to the protein being heterogeneously glycosylated, and asparagine or N-linked specific deglycosylation with the enzyme endoglycosidase F removes all glycomoieties, resulting in a sharp band migrating at 46kD (Leinhard et al, 1984).

Molecular analysis of the sequence and structure of the erythrocyte GT shows that it contains 12 membrane spanning regions which make up the pore or channel structure. An

extracellular loop contains a putative asparagine-linked glycosylation site. The amino- and carboxy-terminals extend intracellularly, as well as a large cytoplasmic loop and may provide sites for covalent modification or binding of regulatory components (Mueckler et al, 1985).

It is now known that the facilitative glucose transporter exists as a multigene family of structurally related proteins. Expression of these genes is tissue dependent, and certain isoforms appear specialized to effect the unique glucose uptake properties of its tissue. Greater than 50% homology is retained across the known isoforms, and greater than 90% homology occurs within isoforms across species. The highest conservation of structure occurs in the transmembrane spanning regions, with differences occurring predominantly within the extramembranous structures.

There are five known glucose transporter isoforms to date, designated Glut1-5 (Gould and Bell, 1990). Glut1 is exemplified by the human erythrocyte glucose transporter, This isoform has been found to be expressed in almost all tissues, but is most abundant in the brain, fetal and placental tissues (Flier et al, 1987a; Charron et al, 1989). It is also the predominant isoform expressed in transformed cells - even in those cells derived from tissue types normally expressing alternate isoforms, such as hepatoma and insulinoma cells (Flier et al, 1987b; Thorens et al, 1988). Biochemical

analysis has shown the Km values of the Glut1 isoform to be 1-2mM (Baly and Horuk, 1988; Wheeler and Hinkle, 1985). This value is near the level of normal blood glucose levels, and it is felt that Glut1 is involved in the basal, or unstimulated, uptake of sugars.

The Glut2 isoform shows 55% structural identity to Glut1, and is, most notably, uniquely expressed at high levels in the liver and B-cells of the pancreas (Thorens et al, 1988; Fukumoto et al, 1988). By virtue of the high Km of Glut2 (15-20mM, Ciaraldi et al, 1986; Thorens et al, 1990), these cells are essentially permeable to glucose over a wide range of physiological concentrations. This is a significant specialization as these tissues are involved in the sensing and regulation of glucose homeostasis.

As an important regulator of sugar uptake, insulin rapidly stimulates sugar uptake in specific target tissues such as muscle and fat. These insulin target tissues are now known to express Glut4, or the insulin-regulatable GT (Birnbaum, 1989; James et al, 1988b). The Glut4 isoform has 65% identity to GLUT1 and has been shown to be expressed primarily in intracellular vesicles until insulin promotes its translocation to the plasma membrane where it dominates the insulin stimulated sugar transport of the cell (Zorzano et al, 1989; James et al, 1988a). The specialization of Glut4 for insulin responsiveness has been further demonstrated by its *de novo* expression as 3T3-fibroblasts are induced to

differentiate into adipocytes (Kaestner et al, 1989; de Herreros and Birnbaum, 1989).

The Glut3 and Glut5 isoforms are less well characterized. Glut3 was isolated from fetal skeletal muscle (Kayano et al, 1988), and, like Glut1, has been shown to be expressed at variable levels in most tissues. Glut5 has been found to be expressed in tissue from the small intestine (Davidson et al, 1992).

Mechanisms of sugar transport regulation in mammalian cells:

It has been demonstrated that the transport process is the rate limiting step in the uptake and metabolism of sugars (Plagemann and Richey, 1974), and it is through the transport step, ie. the glucose transport protein, that regulation of sugar uptake occurs. Sugar uptake regulation can be effected by a number of physiological conditions which include the growth state of the cell as well as the nutritional and hormonal state of the environment. Kinetic analyses have shown that in virtually all cases, modulation appears to be characterized by changes in the maximum transport velocity, or Vmax, with no significant changes in the Km, or the affinity of the transporter for the substrate. In some cases, the Vmax differences correlate with changes in the amount of glucose transporter expressed in the plasma membrane. In other cases, changes in plasma membrane GT expression can not fully account

for transport changes, suggesting alternative mechanisms of sugar uptake regulation (Czech et al, 1992).

In some instances, modulation of sugar uptake has been found to correlate with changes in the cellular GT content. This mode of regulation occurs as a result of altered rates of synthesis or degradation/turnover of the GT protein. For example, the long-term prolonged enhancement of sugar uptake following mitogenic stimulation by serum or growth factors (including insulin) coincides with an overall increase in GT expression. This increase is protein synthesis dependent, is characterized by a rapid elevation in Glut1 mRNA expression (Hiraki et al, 1988; Kitigawa et al, 1989; deHerreros and Birnbaum, 1989) and results in increased Glut1 synthesis (Hainque et al, 1990; Walker et al, 1990). This same mechanism appears to be responsible for the high rates of sugar uptake observed with cellular transformation (Flier et al, 1987b). This was best illustrated with the transfection of rat fibroblasts with temperature-sensitive transforming viruses (Birnbaum et al, 1987) or *src* oncogenes (White and Weber, 1988). In both cases, an increase in GT mRNA and protein synthesis was observable as transformation was induced at the permissive temperature.

The increase in sugar uptake and GT expression during glucose starvation (Charron and Kahn, 1990; Walker et al, 1989), however, is accomplished through a different mechanism.

In this case, the resultant increase has been shown to be predominantly protein synthesis independent, and is due to a decrease in the turnover or degradation of the glucose transporter protein (Haspel et al, 1986; Hara et al, 1989).

Finally, growth hormone, an insulin antagonist, has been shown to decrease sugar uptake in adipocytes (Tai et al, 1990). It has been demonstrated that with growth hormone there is a selective reduction in Glut1 mRNA and protein expression, suggesting a decline in Glut1 synthesis.

In other instances, sugar uptake regulation is not associated with altered cellular GT content. Cushman and Wardzala (1980) and Suzuki and Kono (1980) showed that insulin stimulation of fat cells, which results in a rapid increase in the sugar uptake capabilities of the cells, is effected by a recruitment, or "translocation" of glucose transporters (Cushman and Wardzala, 1980) and transport activity (Suzuki and Kono, 1980) from an intracellular microsomal storage site to the active plasma membrane site. This translocation occurs independently of *de novo* synthesis of the glucose transporter protein (Jones and Cushman, 1989), yet is dependent on ATP availability (Kono et al, 1981). This same insulin evoked event has been demonstrated in other insulin target tissues (Wardzala and Jeanrenaud, 1981; Klip et al, 1987; Wheeler, 1988), and is now known to be dominated by translocation of the Glut 4 isoform in these tissues (Zorzano et al, 1989).

A similar translocation of GT appears to be responsible for a transient, initial phase of protein synthesis independent sugar uptake stimulation observed in response to serum or growth factor stimulation (Kitigawa et al, 1989; Rollins et al, 1988). In contrast, the decrease in sugar uptake seen in human fibroblasts exposed to the glucocorticoid, dexamethasone, is accomplished via a reverse translocation of glucose transporters from the plasma membrane to the microsomes (Horner et al, 1987).

Yet, even as the translocation theory of insulin stimulation was being corroborated, evidence was being presented that this mechanism was only partially responsible for the increase in sugar uptake. Discrepancies between the magnitudes of sugar uptake enhancement versus GT increases in the plasma membrane suggest that an increase in the "intrinsic", or catalytic activity of the transporter protein may also be involved (Simpson et al, 1983; Hyslop et al, 1985; Calderhead and Leinhard, 1988; Hirschman et al, 1990). This possibility is strengthened by reports that temporally, insulin induced translocation precedes full uptake stimulation (Karnielli et al, 1981; Gibbs et al, 1988).

Further supporting the concept of glucose transporter activity regulation, other substances have been shown to alter sugar uptake, yet do not appear to have a correlative effect on total cell or plasma membrane GT expression. The

catecholamine, isoproteronol, is able to negate the uptake stimulation evoked by insulin without reversing the translocation event (Joost et al, 1986). Cholera toxin is shown to elicit an equivalent increase in sugar uptake as insulin, but accomplishes it through the translocation of much less GT (especially Glut4) to the plasma membrane (Clancy and Czech, 1990). Protein synthesis inhibitors have been shown to promote insulin-like stimulation of sugar uptake, but with no concomitant increase in plasma membrane GT in adipocytes (Baly and Horuk, 1987; Clancy et al, 1991), or in fibroblasts (Germinario et al, 1992). Phenylarsine oxide (PAO), an inhibitor of exo/endocytosis has been shown to affect both basal and insulin stimulated sugar transport in ways that further support the idea that factors other than the amount of plasma membrane integrated glucose transporter may play a role in sugar uptake regulation (Gould et al, 1989; Frost et al, 1989; Henricksen and Holloszy, 1990).

Lastly, intriguing data published by Harrison, Buxton and Czech (1991) suggests that the catalytic activity of Glut1 is suppressed when this isoform is expressed in adipocytes. When 3T3 cells were compared in the induced (adipocyte) and uninduced (fibroblast) states, it was found that the adipocytes expressed more native Glut1 and/or transfected human Glut1 in the plasma membrane than the fibroblasts, yet displayed a markedly lower rate of specific Glut1 mediated sugar transport. Calculations of the Glut1 transport:

transporter ratio indicated that Glut1 isoform transport activity may be inhibited by >90% when it is expressed in the adipocyte cell. They further suggest that in many of the examples cited above, apparent increases in intrinsic activity in adipocytes may be accomplished through the reversal of this Glut1 suppression.

Objectives:

The G14 mutant cell line is one of a number of mutants generated from the transformed V79 Chinese hamster lung fibroblast cell line and selected based on an inability to survive in a glucose-free environment (DeFrancesco et al, 1975). Many of these mutants have been characterized and shown to be unable to derive energy through oxidative metabolism, or respiration deficient (Ditta et al, 1976). Specifically, the G14 mutant has been determined to be defective in the Class I mitochondrial enzyme, NADH-Coenzyme Q reductase (Soderberg et al, 1979). As a result, these respiration deficient cell lines rely primarily on the less efficient method of glycolysis for generation of ATP and therefore require high concentrations of glucose for growth (Breen and Scheffler, 1979). In addition, these cells are characterized by a low rate of oxygen consumption and an auxotrophy for CO_2 and the amino acid, asparagine (Ditta et al, 1976; Breen and Scheffler, 1979).

Germinario et al (1990a) compared the sugar uptake characteristics of the V79 and G14 cell lines and found that in the non-proliferative, or serum starved state, G14 displays an elevated rate of sugar uptake when compared to V79. This upregulation is accomplished through a 3-fold increase in the Vmax for 2DG transport with no differences occurring in the Km, or affinity of the transport process for the substrate. In addition, the G14 cell line appeared to be virtually unresponsive to effective regulators of sugar uptake in the V79 parental cell line, such as the presence or absence of serum, insulin or glucose (Germinario et al, 1990a).

Several possibilities exist which may be responsible for the elevated rate of sugar transport in the G14 mutant, including differential synthesis and/or degradation of the glucose transporter protein, preferential expression in the plasma membrane, expression of alternate isoforms, or a combination of all, some or none of these situations. The first objective of this thesis research was to generate a polyclonal antibody which would recognize the hamster glucose transporter, thereby allowing a quantitative and qualitative comparison of GT expression in the V79 and G14 cell lines through Western analysis procedures. A reliable subcellular fractionation procedure was developed in order to further characterize the GT distribution within the cells. particularly in the plasma membrane. Through these comparative studies, we sought to determine whether a

distinction in whole cell or subcellular glucose transporter expression could be related to the differences in sugar uptake regulation observed in the respiration deficient mutant.

A second objective was to explore the effects of D-allose, a non-metabolizable sugar, which has been shown to dramatically down-regulate sugar uptake in hamster fibroblasts (Ullrey and Kalckar, 1986; Germinario et al, 1990b). In most cases, when non-metabolizable sugars, such as L-glucose or D-fructose, replace D-glucose in the incubation media, a resultant increase in sugar transport ensues due to the lack of metabolizable sugars (Germinario et al, 1985). However, when D-allose replaces D-glucose in the incubation media, up to a 60% reduction in sugar transport has been shown to result in V79 cells (Germinario et al, 1990b). This D-allose induced depression in sugar uptake coincides with a decreased Vmax, is not due to an inability of the cell to phosphorylate sugars via hexokinase, and occurs in a time and concentration dependent manner, requiring 2-4 hours and a concentration of 1mg D-allose/ml for maximal effectiveness (Germinario et al, 1990b). Suggested mechanisms of down-regulation include increased GT degradation and/or reverse translocation. In this study, subcellular fractionation and Western analysis with the GT specific polyclonal antibody were used to determine whether D-allose influences GT expression in the whole cell and/or its distribution within the cell.

MATERIALS AND METHODS

Purification of the glucose transporter:

The human erythrocyte glucose transporter (GT) was isolated from outdated packed red blood cell preparations received from the blood bank of the Jewish General Hospital (Montreal, Quebec), by the method of Baldwin and Leinhard (1986). The procedure can be broken down into three stages, and will be discussed as steps I-III. Briefly, in step I, erythrocyte membrane ghosts were produced through hypotonic lysis in a phosphate buffer (pH 8.0), and washed free of haemoglobin through a series of centrifugations. In step II, peripheral proteins were removed from the membranes in a 15mM NaOH alkaline wash (pH 11.6) and then neutralized in a Tris buffer (pH 11.6). These "stripped" membranes were then solubilized in 65mM octylglucoside detergent (Pierce) and contaminating proteins were removed on a DEAE-cellulose ion exchange column (Whatman). The flowthrough, or step III, contained the purified transporter, and was extensively dialysed against phosphate buffered saline (pH 7.4) prior to being concentrated (Amicon) to 200-500ug protein/ml.

Production and characterization of antisera:

Three male New Zealand White rabbits (2.5-3 kg) were used in the production of polyclonal antisera. Preimmune and immune sera was isolated from blood taken from the marginal

ear vein after the rabbits had been tranquillized with 100mg Ketalean (CDMV) and 20mg Atravet (Ayerst). Serum was separated from whole blood through clot retraction and centrifugation at 10,000g for 10 minutes at room temperature.

The primary immunization consisted of 250 ug of purified GT emulsified in 2.0 mls of Freund's complete adjuvant (Difco) and injected intramuscularly into the four limbs of the rabbit. On day 23 post-immunization, a half dose boost (125 ug of purified GT) in incomplete adjuvant (Difco) was administered subcutanec sly into the hip and shoulder regions. A second boost was given on day 44 in the same manner and immune sera was collected on days 62 and 71, and approximately every 2-3 weeks after that.

Where indicated, affinity purification of the antisera was accomplished using the stepII (stripped) erythrocyte ghosts as an immunoabsorbent (Schroer et al, 1986). Stripped ghosts (2 mg protein/ml) were incubated with antisera in a volume ratio of 10:1 for one hour followed by centrifugal washes in isotonic (150mM) and hypertonic (800mM) phosphate buffered saline. Elution of antibodies from the membranes was accomplished using a 200mM glycine/HCl buffer, pH 2.4, and the antibody containing supernatant was neutralized with NaOH and dialysed against phosphate buffered saline (PBS).

Evaluation of GT-specific antibody presence was done using an alkaline phosphatase enzyme-linked immunodetection (ELISA) procedure. Purified human erythrocyte GT was adsorbed

to 96-well plates by applying 50ul of 4mg GT/ml PBS to each well for 1 hour at 37°C, or 4°C overnight. Under these conditions, binding of ^{125}I-labelled GT has determined that 6.9±1.0% (range of analysis: 0.3-40ug GT/ml) of the applied protein adheres to the well (data not shown). The wells were blocked with 1% bovine serum albumin (BSA; ELISA grade, Sigma) in PBS for 1 hour at 37°C. Antisera was diluted in blocking buffer and applied at 50ul/well. After 1 hour at room temperature, unbound proteins were removed by washing wells 3 times with PBS. An alkaline phosphatase linked goat anti-rabbit secondary antibody (Promega) was applied at a concentration of 1:5000 in blocking buffer for 1 hour at room temperature. After extensive washing with PBS, an alkaline phosphatase substrate solution (p-nitrophenyl-phosphate in 1N diethanolamine, Biorad) was applied which generated a yellow color relative to the amount of secondary antibody present. Absorbance was read in a multiwell plate reader at 450nm.

Western analysis procedures:

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins follows the procedures outlined by Laemmli (1970). Unless otherwise indicated, samples were not boiled and 10% separating gels were employed under non-reducing conditions. Blotting or transfer of proteins to nitrocellulose paper (0.2um, Schleicher and Schuell) was done in a 25mM phosphate buffer, pH 6.5, for 1.5-2 hours at 300mA. The complete

transfer of proteins in the molecular weight range of interest has been substantiated by the staining of gels after transfer, and the fact that marker proteins (Biorad) greater than 100kD are transferred under these conditions. The nitrocellulose blot was blocked in 1% BSA (ELISA grade, Sigma) in Tris-buffered saline/0.5% Tween20 (TBST) for 1 hour and the primary antibody, in blocking buffer, was incubated with the blot overnight at 4°C. After several washes in TBST, .1uCi/ml ^{125}I-labelled protein A (ICN) in blocking buffer was added for 1 hour at room temperature. Following several more washes, the blot was dried and exposed to XAR film (Kodak), usually for 16-24 hours at -85°C. Quantitation of protein detection was accomplished through cutting and gamma-emission counting of labelled bands, with an appropriate matching non-labelled portion of the blot counted for background correction.

In some instances, once the specificity of the GT-directed antibodies to a single protein band had been established and the GT proteins had been fully characterized, GT quantitation was accomplished through dot blot analysis. Samples were applied directly to nitrocellulose paper using a dot blot apparatus, and then the nitrocellulose was treated in the same way as the blots for Western analysis, ie. blocked in BSA, then probed with a specific primary antibody, followed by ^{125}I-protein A. After exposure to XAR film, dots were cut and counted for a quantitative determination of GT content.

Cell culture:

Both V79 and G14 cell lines were grown in culture at 37°C in Dulbecco's modified Eagle's medium (Flow) supplemented with essential and non-essential amino acids (ICN), 5% (v/v) fetal calf serum (Flow), and in a 7% CO2/93% air environment. Cells were routinely subcultured into 75cm² tissue culture flasks (Falcon) twice weekly. The G14 cell line has a slightly longer doubling time than V79 (12 and 10 hours respectively, Andrejchyshyn, 1990), and experimental protocols involved plating such that both cell lines approached confluence simultaneously. When plates were near confluence, monolayers were rendered quiescent through serum starvation for 16-24 hours. This was done by replacing the growth media with 0%MEM which was comprised of Eagle's minimal essential media (Flow) containing no serum, 4mg/ml glucose, 1 mg/ml BSA and non-essential amino acids (ICN) to accommodate the asparagine requirements of the G14 cell line. From this basal state, monolayers were exposed to further treatments, such as insulin stimulation or glucose deprivation, which were presented in 0%MEM and paired with control conditions (0%MEM plus the treatment diluent). Specific treatment conditions will be described more completely within the text.

Measurement of sugar transport:

Sugar uptake evaluations were done using ^{3}H-2-deoxy-D-glucose (2DG), a non-metabolizable D-glucose analog (ICN).

Under the conditions employed, 2DG uptake was linear and rate limiting with no free 2DG being detected intracellularly (Germinario et al, 1989). The protocol was as follows: Confluent 35mm plates were rinsed twice with 37°C PBS and then incubated for 5 minutes with 0.8ml 50uM 2DG (specific activity: 20uCi/pmole 2DG) in PBS at 37°C. Uptake was terminated by rinsing plates 4 times with 4°C PBS containing 5uM cytochalasin B. Zero-time control plates were similarly done, except that the initial rinses consisted of 4°C PBS with cytochalasin B, and the ^{3}H-2DG was removed immediately followed by 4 rinses with 4°C PBS + cytochalasin B. Monolayers were solubilized in 1N NaOH, and aliquots were taken for protein determination and liquid scintillation counting. Uptake of ^{3}H-2DG was calculated per unit protein and zero-time uptakes were subtracted.

^{3}H-leucine and ^{3}H-mannose incorporation:

During studies employing tunicamycin, protein synthesis and glycosylation were evaluated through the incorporation of ^{3}H-leucine and ^{3}H-mannose, respectively, into TCA insoluble material. In both cases, a 10X preparation of the radiolabelled component was prepared in PBS and added to cell media to achieve a final specific activity of 2.5 uCi/ml. ^{3}H-leucine was added for the last hour of treatment, and ^{3}H-mannose incorporation was measured over the last 4 hours of treatment. At the end of the incorporation period, monolayers

were washed 4 times with PBS followed by 10% TCA (w/v) precipitation for 1 hour at 4°C. Monolayers were washed twice with 10% TCA, solubilized with 1N NaOH, and aliquots were analyzed for protein and ^{3}H content. Values were expressed in terms of dpm/mg protein.

Subcellular Fractionation:

Monolayers were lysed hypotonically by a variation of the scraping procedure outlined by Chang et al (1981). At room temperature, confluent 100mm plates were rinsed 2 times with an isotonic 1mM bicarbonate buffer, pH 8.0, followed by 2 rinses with a hypotonic preparation of the same buffer. The monolayers were then incubated for 2-5 minutes in the presence of 5mls hypotonic buffer which allowed the cells to swell and round up while remaining attached to the plate. The buffer was removed and cells were scraped into 1 ml of lysis buffer (1mM bicarbonate buffer/ 0.5mM EDTA/ 0.25M sucrose, pH 8.0) and placed on ice. Lysates were collected in a 50 ml centrifuge tube and the total lysate volume was shaken vigorously to enhance cell breakage.

Subcellular fractionation follows the protocol of Simpson et al (1983) with a few minor modifications. All operations were done at 4°C in lysis buffer. A Beckman L3-50 ultracentrifuge and a Ti60 rotor was employed for all centrifugations, with the exception of a SW-27 rotor for the gradient centrifugation. Lysates were first centrifuged at

16,000gmax for 15 minutes with the pellet (P16) representing a crude plasma membrane enriched preparation. The supernatant was centrifuged at 40,000gmax for 20 minutes, yielding a P40 pellet, and then again at 200,000 gmax for 90 minutes. This final pellet represents the microsome containing fraction and is designated P200. To further purify the plasma membrane enriched fraction, the P16 pellet was resuspended in lysis buffer and underlaid with 48% sucrose (w/v). The discontinuous gradient was centrifuged in a swing out rotor at 100,000 gmax for 75 minutes. The plasma membrane enriched 48% band was harvested, resuspended in lysis buffer and pelleted at 40,000gmax for 30 minutes.

Ouabain sensitive Na^+-ATPase was used as a marker enzyme for plasma membrane, and rotenone-insensitive NADH-cytochrome c reductase was monitored as a marker for endoplasmic reticulum (Sottocasa et al, 1967).

Whole or total membrane preparations were generated through the same monolayer lysis procedure. Lysates were centrifuged at 1000gmax for 4 minutes to remove unbroken cells, and all membranes were pelleted from the supernate at 200,000gmax for 90 minutes.

Ouabain-sensitive Na^+-ATPase assay:

Vesiculation of isolated membranes can often impair maximal Na^+-ATPase activity by limiting availability of ions and ATP to both sides of the membranes (Forbush, 1983).

Treatments with a low concentration of SDS, buffered by low concentrations of BSA, allow gentle permeabilization of the membrane vesicles, and homogeneous representation of all reagents. To achieve this, 5-10ug samples of each fraction were permeabilized for 30 minutes at room temperature in .025% SDS/.025% BSA in 30mM imidazole buffer (Forbush, 1983). The reaction was started with the addition of a reaction buffer with final concentrations of reagents being 20mM KCl/150mM NaCl/.2mM EDTA/5mM MgCl2/2mM ATP (Boehringer-Mannheim)/100mM Tris (pH 7.4), with or without 1mg/ml ouabain (Aldrich). The reaction was run at 37°C for 30-60 minutes and terminated by the addition of an equal volume of color reagent (1% sodium molybdate/ 2% ascorbic acid in 1N H_2SO4). The blue color was allowed to develop and then read in a multiwell place reader at 650nm. The amount of free phosphate generated in the reaction was determined by extrapolation from a set of standards consisting of dilutions of a 1mM monobasic phosphate solution. The ouabain-sensitive Na^+-ATPase was calculated by subtracting (+ouabain) values from (-ouabain) values.

Treatment with Endoglycosidase F:

Twenty micrograms of highly concentrated, CT rich preparations of both cells lines were treated according to the procedure of Leinhard et al (1984). Briefly, the preparations were solubilized in a .05% SDS/.5% Triton X-100/1% mercapto-

ethanol/50mM EDTA/100mM phosphate reaction buffer, pH 6.0. To this, 0.5units of endoglycosidase F (Boehringer-Mannheim) or a comparable volume of double distilled H_2O was added as a control, and the reaction was allowed to proceed at room temperature for 24 hours. Prior to analysis on SDS-PAGE, the samples were prepared in Laemelli sample buffer, however the concentration of SDS was raised to 3-4% in order to compensate for the presence of the Triton X-100. After separation of proteins by SDS-PAGE, GT detection was accomplished through Western analysis.

D-glucose inhibitable Cytochalasin B binding assays:

Cytochalasin B (CB) binding studies were done in duplicate on 25-50ug of V79 or G14 membrane preparations. All evaluations were done through the addition of a basic incubation medium to achieve final concentrations of 1%NaCl, .1uM ^{3}H-CB (specific activity: 15uCi/umole CB, DuPont), and 5uM cytochalasin E (a competitive inhibitor for CB binding to microfilaments) in a 50mM Tris buffer (pH 7.4). Specific binding conditions were met through the inclusion of 500mM D-glucose (a GT-specific CB-binding inhibitor) or 500mM L-glucose (a non-inhibitory sugar control) or 15uM unlabelled CB (to determine nonspecific binding) in the incubation media. Binding was carried out for 30 minutes at room temperature, and membranes were pelleted for 10 minutes in a Beckman airfuge (200,000gmax). The incubation media was aspirated,

pellets were solubilized in 1N NaOH, and aliquots were taken for ^{3}H-counting and protein determination.

Protein determinations:

Protein determinations were done using the Biorad preparation of the Bradford Comassie Brilliant blue assay, or by the method of Lowry et al (1951). For both assays, protein values were determined through extrapolation from a set of BSA standards.

Statistical Analyses:

All experimental conditions were evaluated simultaneously with control conditions, and statistical significance ($p<.05$) was determined using a paired student's t-test.

RESULTS

Production and characterization of the polyclonal antibody:

As a transformed fibroblast, sugar transport in the V79 cell line was predicted to be mediated by the Glut1 isoform. The human erythrocyte provides a readily available source of easily extracted Glut1 protein, and through its use as an immunogen in rabbits, it was anticipated that a polyclonal antisera would be produced that could cross-react with the hamster glucose transporter through common epitopes.

SDS-PAGE analysis of the human erythrocyte glucose transporter purification procedure demonstrated a step-wise depletion of protein which coincided with a reduced number of bands (figure 1). The final purification step (step III) yielded 1.3% of the original protein present in the membrane ghost preparation (step I), and was represented by a single broad band of protein between the 43kD and 66kD markers. This is consistent with the findings of others who have purified the glucose transporter from human erythrocytes (Baldwin et al, 1979; Sogin and Hinkle, 1980) and isolated a single polypeptide band with an average molecular weight of 55kD.

Western blotting of the purification steps was performed using MAbG3, a monoclonal antibody directed towards the human erythrocyte glucose transporter (Allard and Leinhard, 1985) as the primary antibody. The same broad band of protein between

the 43 and 66kD markers was recognized in each preparation step (figure 2). An additional second band with a molecular weight between the 21 and 31kD markers was occasionally detected in stepIII, and is considered a to be a breakdown product of the glucose transporter (Sogin and Hinkle, 1980). Quantitation of immunoreactivity demonstrated approximately a 10-fold increase in MAbG3 binding per unit protein from stepI to stepIII. Comparisons of stepIII with a purified GT preparation given to us by Dr. G.E. Leinhard (Dartmouth University, New Hampshire) showed that MAbG3 detected an equivalent protein, both qualitatively and quantitatively on a per unit protein basis (figure 3). In addition, the quantitation of MAbG3 detection was linear to at least 500ng of protein. From these results, it was ascertained that the stepIII purification product was highly enriched in MAbG3 reactive protein, was comparable to purified GT preparations obtained by others, and suitable to use as an immunogen for production of a GT specific polyclonal antibody.

Throughout the immunization program, the presence of GT directed antibodies in rabbit antisera was determined through ELISA procedures. Figure 4 demonstrates that the preimmune levels of GT-specific antibodies were not different from background values (determined through exposure of the GT-bound wells to the secondary antibody alone), and most likely reflect nonspecific binding of rabbit serum components. Post-immunization antisera, however, demonstrated a presence of GT-directed antibodies in all three rabbits which increased after

each boost. Maximal OD_{450} values were obtained at a dilution of 1:1000, and comparative titrations were done through sequential 1:2 dilutions of the antisera isolated after the second boost (figure 5). With the definition of titer being the dilution at which half-maximal absorbance is measured (after subtraction of preimmune absorbance values), titers were approximated at 1:8,000 for rabbits 1 and 3, and 1:16,000 for rabbit 2. Because the antisera from rabbit 2 demonstrated the highest titer, it was selected for affinity purification using stripped erythrocyte membranes as an immunoabsorbent (Schroer et al, 1986). After factoring in the procedural dilution of 1:12, ELISA evaluation of the affinity purified preparation indicated that a comparable concentration of specific antibody was retained after purification (ie, a titer of 1:16,000), and analysis of the unbound supernate demonstrated that no reactive antibodies remained (data not shown).

To verify specificity of the rabbit antiserum towards the glucose transporter, identical blots of SDS-PAGE resolved stepI-III proteins were simultaneously probed with either preimmune or immune sera from rabbit 2, or MAbG3. In the representative Western blots depicted in figure 6, preimmune serum showed no detection of specific protein bands (data not shown), whereas MAbG3 (figure 6a) and immune sera (figure 6b) recognized the same broad protein band located between the 43 and 66 kD markers. The immune sera yielded a greater signal

intensity due to its being a polyclonal, rather than a monoclonal, antibody. However, the relative intensities of the bands within each blot were virtually identical, indicating that MAbG3 and the rabbit antisera demonstrate comparable specificity and sensitivity towards the human GT. In a similar series of studies, identical blots containing human diploid fibroblast samples were evaluated by both the rabbit polyclonal antisera and MAbG3. In each case, GT quantitation of the samples was accomplished through extrapolation of the ^{125}I-protein A signal back to a set of similarly evaluated purified GT standards. In this way, the polyclonally derived estimate of GT content for each sample concurred with the monoclonal estimate - ie. the monoclonal:polyclonal ratio of GT content approached unity ($0.83\pm.12$; n=25; Germinario, personal communication).

The MAbG3 monoclonal antibody, however, does not recognize the rodent glucose transporter (Allard and Leinhard, 1985). When identical blots containing both human and hamster (V79) cell extracts were probed, MAbG3 detected only the human derived glucose transporters (figure 7, lanes 1 and 2). Antiserum from rabbit 2, however, demonstrated an ability to detect a protein in the V79 sample which migrated as a broad band with a molecular weight range similar to that of the erythrocyte glucose transporter (figure 7, lanes 3 and 4).

The same results are obtained with the affinity purified antibody (data not shown), demonstrating that the antibodies

which were selected by the human erythrocyte membranes are also detecting the hamster glucose transporter. To further illustrate specific crossreactivity, the addition of an excess amount of purified human erythrocyte glucose transporter to rabbit antiserum, prior to its use in Western analysis as a primary probe for hamster (and human) cell extracts, competes out all reactive antibodies, and resulted in no protein detection at all (data not shown).

These results indicate that an antibody capable of detecting the hamster glucose transporter has been generated, and serum from rabbit 2 (in most cases, an affinity purified preparation) was used throughout this study for the evaluation of glucose transporter expression in the hamster cell lines. This polyclonal antiserum will be designated as PC-2.

Subcellular fractionation:

In establishing a subcellular fractionation procedure for the V79 and G14 hamster cells, the primary goal was to isolate the two important subcellular pools of glucose transporters which have been shown to participate in sugar uptake regulation (Cushman and Wardzala, 1980). A plasma membrane (PM) enriched preparation is essential in order evaluate the quantity of GT expressed at the interface with the external environment. As the functional site of sugar uptake, plasma membrane located GT is also considered to be the site of sugar transport regulation, and its GT content is often reflective

of the transport capabilities of the cell (Walker et al, 1990). The second important fraction is the low density microsomes (LDM). Characterized as a golgi-like fraction which requires prolonged, high g-forces to pellet (Simpson et al, 1983), the LDM has been shown to contain a sequestered, interactive pool of GT proteins (James et al, 1987). These glucose transporters participate in sugar uptake regulation by shuttling to and from the plasma membrane during the process of translocation (Kono et al, 1981, Horner et al, 1987). Other important considerations in establishing a fractionation procedure were that these two fractions be as free from cross contamination as possible, and that the procedure demonstrate reliability and reproducibility across treatment conditions and cell lines.

Derived subcellular fractions were monitored for organelle content through marker enzymes. Typically, 5'-nucleotidase might be used as a marker enzyme for the evaluation of plasma membrane content and its enrichment. In these cells, however, the nucleotidase activity is very low and proved to be inadequate as a marker enzyme. Instead, a protocol was developed to evaluate another plasma membrane marker, ouabain-sensitive Na^+-ATPase. In addition, the endoplasmic reticulum marker, rotenone-insensitive NADH-cytochrome C reductase was monitored as an indicator of high density microsomes (Sottocasa et al, 1967). As an intermediate density organelle (Dallner, 1974), endoplasmic

reticulum evaluation allowed for a measure of cross contamination between the PM and LDM fractions, as well as an additional measure of cross-experimental reliability.

After numerous procedural comparisons, fractions were derived from a protocol similar to that used by Simpson et al (1983) for adipocytes. This procedure adequately isolated the PM and LDM fractions, and provided the greatest intra- and inter-experimental reproducibility. In contrast to Simpson's procedure, however, a 48% sucrose cushion was required to isolate the PM enriched fraction. Protein recoveries and ATPase enzyme profiles were not statistically different across cell lines or treatments, and were highly reproducible across experiments. Table 1 presents a composite of these evaluations across cell lines and treatments.

Characterization of the fractions shows that the first lysate pellet, P16, contains 28% of the total protein, and greater than 50% of the total plasma membrane Na^+-ATPase activity. The P16 pellet also demonstrates a high content (35% of total) of endoplasmic reticulum NADH-cytochrome c reductase activity. Enrichment and further purification of the plasma membrane was accomplished on a 48% sucrose cushion (48% band), and resulted in a reduction in the content of endoplasmic reticulum and an increase in the ATPase specific activity. The final enrichment in the plasma membrane marker

enzyme activity of the 48% band was 6.3-fold over the lysate activity.

The second pellet (P40) demonstrated the greatest enrichment in NADH-cytochrome c reductase, as well as a 2-fold enrichment in Na^+-ATPase activity. This centrifugation step was principally used as a housecleaning step - enhancing differentiation of the PM and LDM preparations through the removal of high density microsomes, as well as the PM and LDM material which would overlap during the differential centrifugation procedure.

The P200 pellet is presumed to contain the low density microsomes, and is shown to display no measurable enrichment in plasma membrane ATPase activity and only 8% of the total NADH-cytc reductase activity.

An important validation of these fractions is presented with the demonstration that a GT translocation event occurs with insulin stimulation of V79 cells. In paired studies, serum starved V79 cells were treated with media which contained either 100mU insulin/ml or its diluent (control) for 1 hour at 37°C. The Western blot in figure 8a depicts the redistribution of GT content between the 48% (PM-enriched) band and the P200 (LDM-enriched) fraction in response to insulin exposure. The results from several such experiments (figure 8b) establishes that a significant 1.44-fold increase in 2DG uptake is seen with insulin exposure (1646±289 vs 2370±328 pmoles 2DG/mg/5 min), and coincides with a

significant 1.22-fold enrichment of the GT content in the 48% band, or plasma membrane (1508±312 vs 1842±369 cpm ^{125}I-protein A). In accordance with the translocation event described by others (Klip et al, 1987), the increases observed in the PM-enriched preparation do not correlate with changes in the lysate, ie. total cellular, GT content (889±86 and 915±89 cpm ^{125}I-protein A), but are instead related to a 33% decrease in the amount of GT found in the P200, ie. LDM-enriched, fraction (1368±140 vs 909±116 cpm ^{125}I-protein A).

Similarly, D-glucose deprivation of V79 cells for 4 hours results in a 1.9-fold increase in 2DG uptake (1716±175 vs 3251±379 pmoles 2DG/mg/5 min, $p<.05$). In paired studies under these conditions, the whole cell lysate of the glucose deprived cells demonstrated a 35% increase in GT content (1331 vs. 1803 cpm ^{125}I-protein A, n=1), and a 2.5-fold increase in GT expression (478 vs. 1227 cpm ^{125}I-protein A, n=2) was observed in the 48% band (figure 9). These findings agree with other studies which have demonstrated that glucose deprivation stimulates sugar uptake through a mechanism(s) which results in an enrichment of GT content in the plasma membrane (Walker et al, 1990) that is reflective of an overall crease in cellular GT expression (Walker et al, 1989).

These results provide important information, not only concerning the validity of the fractionation procedure employed, but also regarding the V79 cell line as a model for

the study of sugar uptake regulation. The regulatory circumstances which will be explored in this thesis can now be evaluated in terms of GT expression in the whole cell and cell fractions, and can be compared to the events seen with insulin stimulation and glucose deprivation.

Comparisons of glucose transporter expression in the parental and respiration deficient cell lines:

In most cases, evaluations of glucose transporter expression in the mutant (G14) and parental (V79) cell lines were accompanied by simultaneous determinations of the 2DG uptake in the two cell lines. The results of these whole cell transport studies (figure 10b) demonstrated an average 2.92-fold enhancement in 2DG uptake in the G14 cell line under conditions of serum starvation for 16-24 hours (4145±765 vs. 1419±151 pmoles 2DG/mg/5min for G14 and V79 respectively). These results agree with the findings of Germinario et al (1990a), in which a 3-fold elevation in the Vmax of 2DG transport was found in the mutant cell line when compared to the parental.

To determine whether the enhanced sugar transport capabilities of the G14 cell is due to an increased expression of GT, whole cell lysates were analyzed by Western procedures (figure 10). Since the amount of protein per cell is equivalent in the two cell lines, comparative evaluations were done on a per unit protein basis. Two surprising observations

were made. Firstly, the G14 cell line demonstrated a slight, but insignificant, reduction in GT expression when compared to V79 lysates (805±166 and 901±237 cpm ^{125}I-protein A respectively). Secondly, an apparent difference in the electrophoretic mobilities of the glucose transporters in the two cell lines was observed on SDS-PAGE (figure 10a). The G14 cell line consistently displayed a broad protein band with a higher average molecular weight than that found in V79. The implication is that either G14 is expressing a protein which is larger in its primary structure, or, the core protein expressed is the same and post-translational processing has imposed additional mass onto the glucose transporter protein. Later in this thesis, these possibilities will be addressed, particularly in terms of the glycosylation of the protein.

Through subcellular fractionation studies, the membrane distribution of GT in the two cell lines was compared. GT content was evaluated, on a per unit protein basis, in the 48% (PM enriched) band and the P200 (LDM-enriched) fractions. The same molecular weight differences displayed in the whole cell preparations were observed (figure 10a), however, no difference was noted in the amount of GT expressed in the V79 and G14 plasma membrane preparations (2345±889 and 2369±885 cpm ^{125}I-protein A, respectively). In contrast, microsomal GT content was significantly less in the G14 cell line - displaying, on average, 49% of the GT expressed in the V79

microsomal preparation (1215±428 vs. 2501±846 cpm ^{125}I-protein A).

A similar study was done on two other respiration deficient mutants of the V79 cell line. Like G14, the G4 mutant has been shown to have a deficiency in NADH-CoQ reductase, however, the defect is in another subunit of the enzyme as shown by complementation analysis (Breen and Scheffler, 1979). Alternatively, the G7 mutant is deficient in mitochondrial protein synthesis (Soderberg et al, 1979). All three mutants present a similar phenotype in terms of sugar uptake regulation, with the Vmax of 2DG uptake being enhanced 2.2 and 2.6-fold in G4 and G7 respectively when compared to V79 (Chiasson, 1990). Interestingly, Western analysis revealed that all three respiration deficient mutants displayed a similar pattern of GT expression (figure 11). Like G14, the G4 and G7 mutants expressed a higher average molecular weight GT than that observed in the V79 parental cell. Quantitation of PM expression showed no marked difference in GT content in any of the cell lines, while the microsomal preparations of the mutants exhibited only 25-35% of the GT detected in the V79 preparation.

Based on the findings that G14 is transporting 2DG three times faster than V79 through no detectable difference in plasma membrane GT expression, the question was posed as to whether there may be an involvement of other, undetected GT

isoforms. Through a generous gift by Dr. M. Paquet (Toronto), a polyclonal antibody directed towards the carboxy terminal of the Glut4 isoform was made available. Western evaluations using this specific antibody, however, revealed no detectable Glut4 in either the V79 or G14 cell lines (data not shown).

To examine the possibility that other isoforms may play a role in the regulation of sugar transport in these cells (Kayano et al, 1988; Gould and Bell, 1990), D-glucose inhibitable CB-binding studies were done. This assay provides an alternate, biochemical measure of facilitative GT content by determining the amount of CB bound to the GT through its specific, competitive displacement with D-glucose. Total membrane fractions from both V79 and G14 were evaluated and directly compared to PC-2 Western analysis of the same fractions. Table 2 demonstrates that non-specific 3H-CB binding (ie. in the presence of high concentrations of unlabelled CB) is virtually identical in both cell lines, yet comprises 62% and 77% of the total (L-glucose present) binding in V79 and G14, respectively. D-glucose is able to inhibit total CB binding in both cell lines, and as an indicator of GT content, establishes that the G14 preparations contains 54% of the D-glucose inhibitable CB-binding measured in the V79 samples. Western analysis of these same preparations depicted G14 as expressing 45% of the GT content detected in the V79 samples. These results allow for certain statements to be made: 1) PC-2 closely approximates the values of GT detection

derived from CB-binding studies, and 2) both assays demonstrate that G14 expresses less GT protein than V79 in total membrane preparations.

In order to determine if the molecular weight differences of the glucose transporters expressed in the parental and mutant cells were due to pre- or post-translational differences, it was proposed that examination of the glycosylation characteristics might provide some information. Leinhard et al (1984) demonstrated that heterogeneous asparagine-, or N-linked, glycosylation is the basis for the range of molecular weights seen in the expression of the native erythrocyte glucose transporter. This was accomplished through treatment of the glucose transporter protein with the specific N-linked deglycosylating enzyme, endoglycosidase F; the resultant (core) protein migrated as a sharp band with a molecular weight of 46kD.

When GT-rich membrane preparations of the G14 and V79 cells were treated with endoglycosidase F and evaluated by Western analysis, it was found that in both cell lines, the glucose transporter proteins were reduced to a sharp band migrating at the same molecular weight, with approximately the same mobility as the 43kD marker (figure 12). This indicates that the difference in the migratory patterns on SDS-PAGE are not due to the expression of another GT protein, per se, but

rather due to an overall increase in the glycosylation of the same core protein when expressed in the mutant cell.

To further test this finding, cells in culture were exposed to the glycosylation inhibitor, tunicamycin. Because tunicamycin can inhibit protein synthesis as well as glycosylation, the concentration of tunicamycin was titrated to maximally inhibit glycosylation (ie., ^{3}H-mannose incorporation) while keeping protein synthesis (^{3}H-leucine incorporation) inhibition to a minimum. A concentration of 0.5ug tunicamycin/ml was utilized in four separate studies, and achieved a 66$\pm$12% and 64$\pm$11% inhibition of mannose incorporation in V79 and G14 respectively, and leucine incorporation inhibitions of 29$\pm$9% and 8$\pm$6% respectively. At least 24 hours of exposure to tunicamycin was required in order to obtain an appreciable accumulation of a non-glycosylated form of the glucose transporter in both cell lines. As can be seen in figure 13 (lanes 1-4), only a percentage of the transporter is expressed in the non-glycosylated state, however the results confirm those found with endoglycosidase F - the nonglycosylated glucose transporter protein migrates at the same molecular weight in both cell lines, suggesting that V79 and G14 are expressing "glycoforms" of the same core protein.

During these studies, 2DG transport was monitored, and in the representative experiment depicted in figure 13, the protein synthesis inhibitor cycloheximide (CHX) was included

as a third condition at a concentration (0.05ug/ml) selected to best match the inhibition of leucine incorporation seen with tunicamycin. Within this study, CHX and tunicamycin inhibited ^{3}H-leucine incorporation 18 and 28% respectively in V79 cells, while G14 demonstrated a 9% increase with both treatments. ^{3}H-mannose incorporation was inhibited to a similar extent in both cell lines - 10% (CHX treatment) and 50% (tunicamycin treatment). The two treatments had similar effects on sugar transport - resulting in an 18% and 20% increase in 2DG uptake in tunicamycin and CHX treated V79 cells compared to untreated cells, and a 6% and 18% decrease in G14 cells. In contrast, however, no difference in total GT content was observed with CHX treatment in either cell line, while tunicamycin treated V79 and G14 cells demonstrated a 1.5 and 2-fold increase in total GT expression respectively. This same increase in Glut1 expression, with no observable change in 2DG uptake, has been observed in tunicamycin treated L6 muscle cells (Maher and Harrison, 1991).

Finally, it has been previously shown in this thesis that the GT detected in the V79 cell line is responsive to regulators of sugar uptake, such as insulin exposure or glucose deprivation. Stimulation of V79 with 100mU/ml insulin for 1 hour resulted in an increase in 2DG uptake, which coincided with an apparent translocation of glucose transporters from the LDM-enriched to the PM-enriched

fractions (figure 8). Germinario et al (1990a) demonstrated that unlike V79, 2DG uptake in the G14 cell line was insulin unresponsive. Using the same protocol as that used for V79, these findings were confirmed - insulin stimulation of the G14 cell line resulted in no significant increase in 2DG uptake (figure 14). As well, no significant changes in whole cell, plasma membrane or microsomal glucose transporter expression occurs in G14 after insulin exposure, suggesting that G14 may be incapable of evoking a translocation of glucose transporters in response to insulin.

Effects of D-allose on GT expression:

Germinario et al (1990b) and Ullrey and Kalckar (1986) demonstrated that in hamster fibroblasts, media replacement of D-glucose by D-allose induces a dramatic downregulation of sugar transport. In addition, D-allose has been shown to act as effectively as D-glucose in the reversal of glucose deprivation enhancement of sugar transport (Ullrey and Kalckar, 1986). It has been suggested that the mechanism of uptake suppression may be due to increased degradation or turnover of the glucose transporter protein, or due to a reverse translocation of transporter protein out of the plasma membrane (Germinario et al, 1990b). Through the use of PC-2 and the subcellular fractionation procedures developed herein, a determination of the effects of D-allose on GT expression and distribution in both the V79 and G14 cell lines were done.

After 24 hours of serum starvation in glucose-containing media, cells were exposed to media containing either 4mg D-glucose/ml (control) or 4mg D-allose/ml as the sole sugar source. Exposure to these conditions was for 4 hours prior to determination of 2DG uptake and subcellular fractionation. Figure 15 demonstrates that D-allose treated V79 cells displayed only 62% of the 2DG uptake observed in D-glucose treated control cells (962±200 vs 1538±262 pmoles 2DG/mg/5min) and concurs with the data presented by Germinario et al (1990b). Western analysis comparisons of GT content in the D-glucose and D-allose treated whole cell lysates showed no significant difference (931±101 vs 851±60 cpm ^{125}I-protein A, respectively), and analysis of subcellular fractions showed that GT expression in the plasma membrane (2000±607 vs 2132±252 cpm ^{125}I-protein A) and the microsomal preparations (1261±252 vs 1241±214 cpm ^{125}I-protein A) was similarly unaffected by exposure to D-allose (figure 15). Based on these evaluations, it appears that the diminished uptake seen in the presence of D-allose is not due to a decline in GT expression, nor is it the result of a redistribution of GT out of the plasma membrane.

Exposure of G14 cells to D-allose resulted in a similar profile. The 2DG uptake of D-allose treated cells was 41% of that measure in G14 control cells (3930±642 vs 1625±706 pmoles 2DG/mg/5min for D-glucose and D-allose treated cells respectively), and in experiments where the effects of D-

allose was measured simultaneously in both V79 and G14 cells, the 3-fold difference in 2DG uptake was retained in the allose treated cells (data not shown). Yet again, no apparent differences in GT content were observable in whole cell lysate (1313±92 vs 1247±41 cpm ^{125}I-protein A), plasma membrane (1109±70 vs 1162±56), or microsomal membrane (1298±85 vs 1438±353) preparations of D-glucose vs. D-allose treated G14 cells (figure 16).

More interesting, however, was the ability of insulin to reverse the allose induced depression of 2DG uptake in V79 cells. Experiments were done in which four separate conditions were simultaneously evaluated: a D-glucose treated (control) state; an insulin-stimulated control state; a D-allose treated state; and an insulin-stimulated D-allose state. Within this series of experiments (figure 17), the effects of insulin and allose exposure on 2DG uptake reflected the results presented previously. The addition of insulin to allose treated cells elicited a "reversal", or stimulation of 2DG uptake such that values were reinstated to control levels (1762±535 vs 1884±341 pmoles 2DG/mg/5min for D-glucose and D-allose+insulin treated cells respectively).

No differences in the GT content of whole cell lysate preparations were observed under any of the conditions (ie. the mean ± s.e.m. across all conditions was 690±34 cpm ^{125}I-protein A and 1008±21 cpm ^{125}I-protein A for two independent

experiments). In contrast, however, changes in PM and LDM GT expression were observed to occur under the various treatment conditions (figure 17). Again, D-allose suppression of uptake was not apparently related to any decrease in PM GT expression. On the contrary, this series of determinations demonstrated an increase in PM expression which may be reflective of the fact that these cells are actually in a state of glucose deprivation. When control and D-allose treated cells were insulin stimulated, a translocation of GTs from the LDM to the PM fractions was seen to occur in both conditions. If a transport:PM-GT ratio is calculated, it can be seen that control and insulin stimulated control cells generate values approaching 2:1. D-allose treated cells and insulin stimulated D-allose treated cells generate ratios of .65:1 and 1:1 respectively, however, and clearly indicate that D-allose is reducing the activity of the PM expressed glucose transporter protein by approximately 50%. Thus, it would seem that the reversal of the D-allose suppression of 2DG uptake seen with insulin stimulation is more of a compensatory response, requiring an 80% increase in PM-located GTs in order to return to control levels of sugar transport.

From another perspective, however, insulin has promoted the translocation of roughly the same amount of GT into the plasma membrane in both the control and D-allose-treated cells (245$\pm$155 vs 288$\pm$165 cpm ^{125}I-protein A), and has resulted in similar gains in sugar transport (757$\pm$200 and 852$\pm$164 pmoles

2DG/mg/5 min respectively). The transport:PM-GT ratios for these net increases are approximately 3:1 under both conditions. This might indicate that insulin is reversing the allose induced activity depression as well as promoting translocation. Alternatively, it could be considered that the D-allose effect is specific to PM located GT, thereby leaving the internally (LDM) expressed GTs unaffected by the allose inhibition.

An identical study with G14 cells provides further insight into the mechanisms of action of D-allose, and the ability of insulin to reverse the D-allose effect on sugar transport in the V79 cell line. In contrast to the V79 cells, insulin stimulation of D-allose treated G14 cells did not reverse the suppression of sugar transport (311 vs 389 pmoles 2DG/mg/5 min), nor did it induce any translocation of GTs into the plasma membrane (1082 vs 979 cpm ^{125}I-protein A). This lends additional strength to the idea that the G14 cells are insulin unresponsive due to an inability to translocate glucose transporters. These findings also support the argument that, in V79, translocation is the primary mechanism responsible for the insulin induced reversal of the allose effect.

The inclusion of D-allose in the 2-DG uptake incubation media did not alter the sugar transport capabilities of V79 cells, indicating that D-allose itself was not acting as a

competitive inhibitor at extracellular sugar binding sites (Germinario et al,1990b). Cytochalasin B is able to block sugar transport, however, through an ability to bind to the glucose transporter at an intracellular site, and can be specifically displaced with high concentrations of D-glucose. The possibility that D-allose may exert its suppressive effects through an ability to bind to an intracellular site on the GT was evaluated through the measurement of 3H-CB binding in the presence of 500mM concentrations of D-allose, D-glucose (a competitive inhibitor of CB-binding), or L-glucose (a non-competitive control sugar). Table 3 indicates that, unlike D-glucose, D-allose does not act as an inhibitor of CB binding, and therefore is not likely to interact at an intracellular site on the glucose transporter.

Figure 1. SDS-PAGE analysis of human erythrocyte GT purification steps. Approximately 0.15% of the recovered protein from steps I-III were resolved on a 10% gel and the protein bands were visualized through Comassie brilliant blue staining. Lane 1 contains molecular weight standards, and lanes 2-4 contain 210ug step I protein, 105ug step II protein and 7ug step III protein respectively.

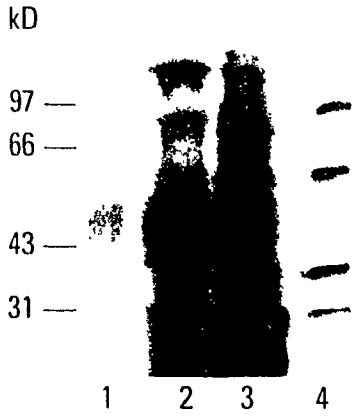

kD
97
66
43
31
1
2
3
4

Figure 2. MAbG3 detection of Glut1 protein in human erythrocyte GT purification steps. SDS-PAGE resolved proteins from steps I-III were transferred to nitrocellulose paper, as described in Materials and Methods, and then probed with the human Glut1 specific MAbG3 monoclonal antibody. Immunodetected proteins were visualized through exposure to ^{125}I-protein A and autoradiography. Lanes 1-3 contain 2ug step I protein, 1ug step II protein, and 500ng step III protein, respectively.

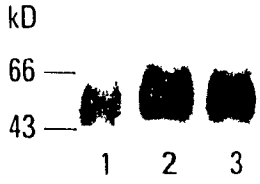
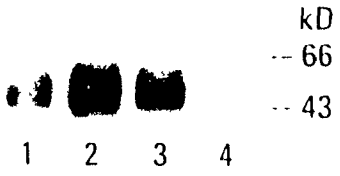

kD
66
43
1
2
3

Figure 3. Comparison of step III GT content with a purified GT preparation from another laboratory. A. 250 and 500ng of step III protein (lanes 1 and 2, respectively) and 400 and 200 ng of an isolated human erythrocyte GT preparation donated by G.E. Leinhard (Dartmouth University, lanes 3 and 4, respectively) were Western blotted using MAbG3 as the primary antibody. B. The ^{125}I-protein A detected bands were excised and counted in a gamma-emission counter. After an appropriate, unlabelled portion of the nitrocellulose blot was counted for background and subtracted from all values, the cpm generated by each band was plotted against the amount of protein loaded onto the gel. ▲, Leinhard GT; O, stepIII.

A.


kD
66
43
1
2
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4

B.

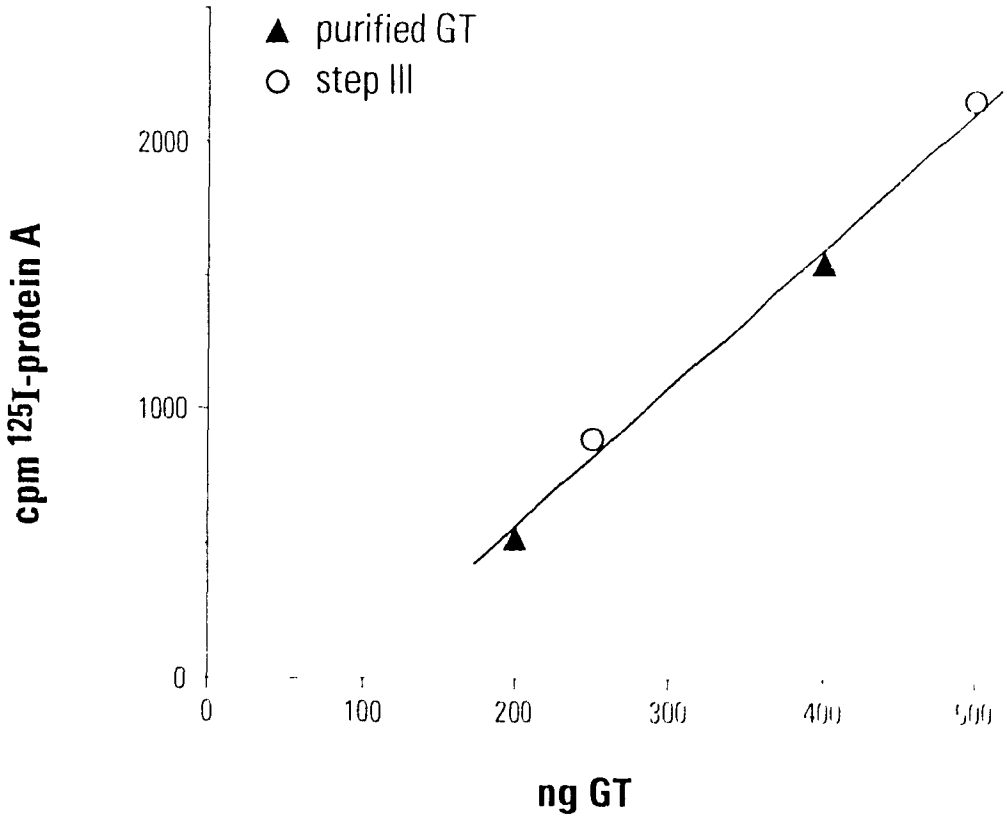

purified GT
step III
2000
1000
0
0
100
200
300
400
500
cpm 125I-protein A
ng GT

Figure 4. Evaluation of rabbit antisera for GT specific antibodies. Throughout the immunization protocol, rabbit sera were tested for GT-specific antibodies using ELISA procedures. Preimmune sera were tested as well as sera collected 35 and 62 days after the primary immunization. The values presented are from a single assay in which all serum samples from rabbits 1-3 were evaluated at a 1:1000 dilution, and are representative of several similar determinations. Background values (ie. nonspecific binding of the alkaline phosphatase conjugated secondary antibody to GT adsorbed wells) were subtracted.

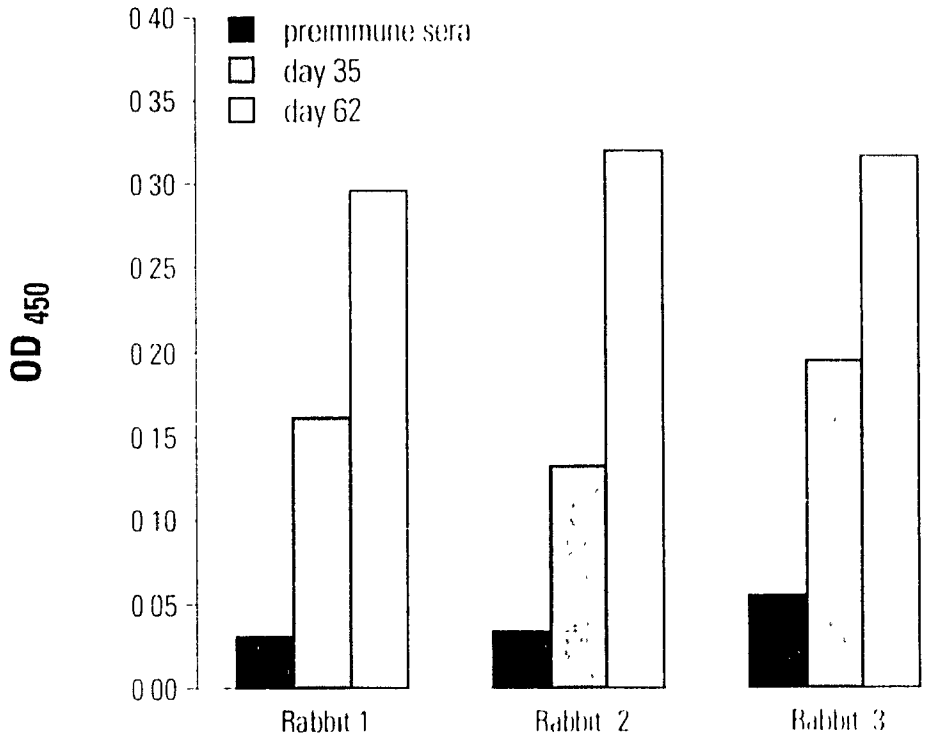

0 40
0 35
0 30
0 25
0 20
0 15
0 10
0 05
0 00
OD 450
preimmune sera
day 35
day 62
Rabbit 1
Rabbit 2
Rabbit 3

Figure 5. ELISA determinations of GT-specific antibody titer in rabbits 1-3. Preimmune (open symbols) and immune (closed symbols) sera collected at day 62 were sequentially diluted and GT-specific antibody presence was evaluated through ELISA procedures.

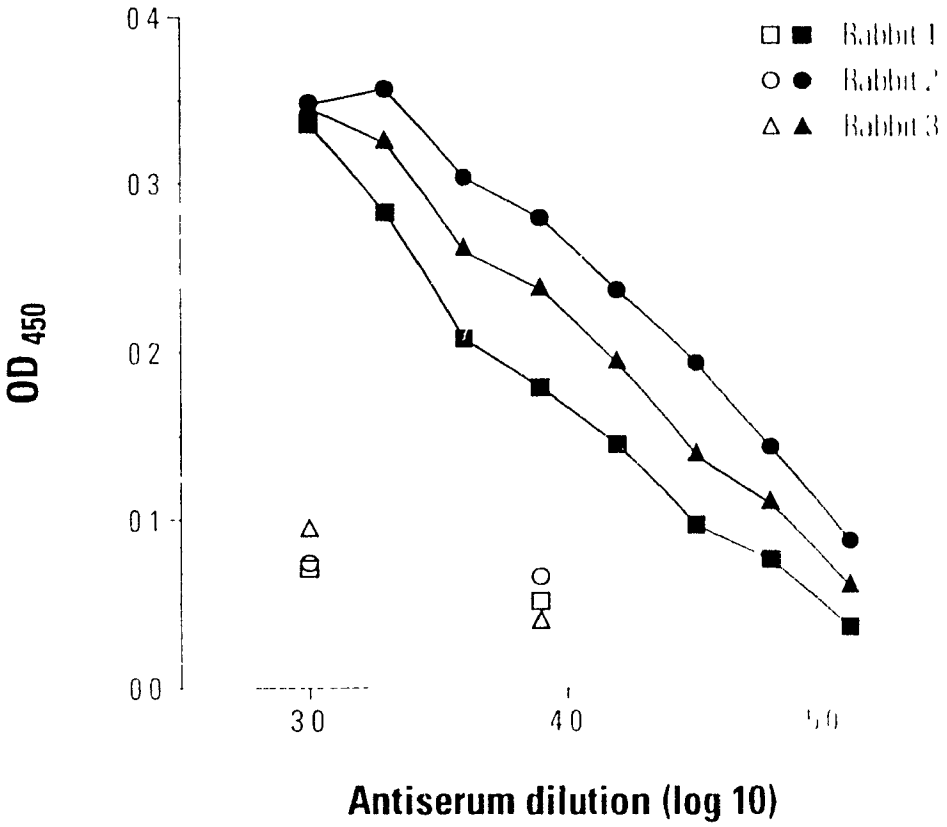
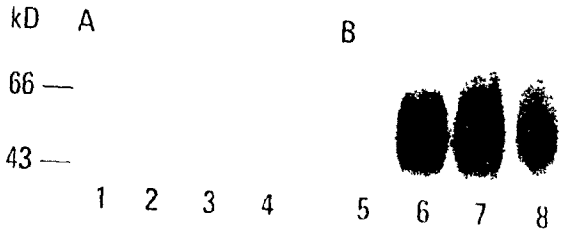

0 4
0 3
0 2
0 1
0 0
OD 450
Rabbit 1
Rabbit 2
Rabbit 3
30
40
Antiserum dilution (log 10)

Figure 6. Comparison of GT detection by MAbG3 and rabbit antisera through Western analysis of human GT. Identical blots of human erythrocyte preparations (stepsI-III) were probed with A) MAbG3 or B) antisera from rabbit 2, and then exposed to ^{125}I-protein A and autoradiographed. For both blots, lane 1 contains 500ng stepI protein, lane 2 contains 500ng stepII protein, and lanes 3 and 4 contain 250 and 125ng of stepIII protein respectively. Labelled bands were cut and counted for ^{125}I-protein A binding with an appropriately sized unlabelled portion of the blot counted for background and subtracted from all values. Values obtained for lanes 1-4 were 485, 1810, 1423 and 906 cpm ^{125}I-protein A for the MAbG3 immunoblot (A); 691, 4551, 3952 and 2170 for the rabbit antisera immunoblot (B).


kD
A
B
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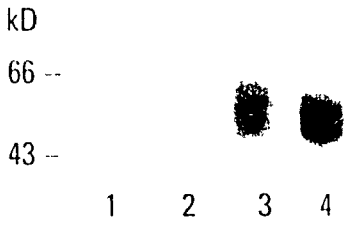
7
8

Figure 7. Comparison of GT detection by MAbG3 and rabbit antisera through Western analysis of human and hamster GT. Identical blots of human (100ng stepIII protein, lanes 1 and 3) and hamster (50ug V79 lysate protein, lanes 2 and 4) samples were probed with MAbG3 (lanes 1 and 2) or antisera from rabbit 2 (lanes 3 and 4), and then exposed to ^{125}I-protein A and autoradiographed.


kD
66 --
43 --
1
2
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4

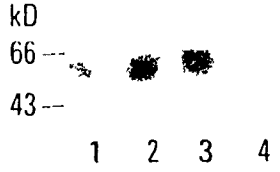
Table 1. Protein and Marker Enzyme Analysis of Subcellular Fractions

						GRADIENT FRACTIONS	
		LYSATE	P16	P20	P200	48%band	PG
PROTEIN RECOVERY	%	(100)	28.4±2.0	3.6±0.3	5.6±0.6	3.5±0.3	8.4±0.7
	(n)	(25)	(25)	(25)	(20)	(18)	(11)
PLASMA MEMBRANE	S.A.	.49±.11	.95±.14	1.04±.17	.56±.10	3.04±.37	.64±.18
(Ouabain-sens.	-fold	1.0	2.0	2.1	1.1	6.3	1.3
Na^+-K^+ATPase)	%	100	56	8	6	22	11
	(n)	(12)	(14)	(15)	(14)	(15)	(8)
ENDOPLASMIC RET.	S.A.	1.81±.06	2.27±.18	4.50±.60	2.83±.95	3.50±.45	2.30±43
(NADH-CytC	-fold	1.0	1.3	2.5	1.6	1.9	1.3
reductase)	%	100	35	9	8	6	11
	(n)	(7)	(7)	(7)	(7)	(7)	(6)

Subcellular fractions were evaluated for protein content and marker enzyme activity.
Specific activities (S.A.) are expressed as nmoles free phosphate generated/mg protein/hour for ATPase, and nmoles cytochrome C reduced/mg protein/hour for NADH-CytC reductase.
Values are expressed as the mean ± s.e.m.
n=number of determinations.

Figure 8. Effects of insulin stimulation on 2DG uptake and GT expression in V79 cells. V79 cells were serum starved for 16-24 hours and then exposed to treatment media containing 100mU insulin/ml, or its diluent, for one hour. A) A representative Western blot, transferred from an 11.5% SDS-polyacrylimide gel and probed with PC-2, depicting GT expression in the PM-enriched 48% band (lanes 1 and 3) and in the LDM-enriched P200 fractions (lanes 2 and 4). Lanes 1 and 2 represent the control or untreated cell preparations, and lanes 3 and 4 represent preparations from insulin treated cells. 10ug of protein were loaded per lane. B) Compiled data from a number of paired experiments (n=number of determinations) in which 2DG uptake determinations (n=8) were done concurrently with subcellular fractionation. GT expression was evaluated through Western and/or dot blot analysis of equivalent amounts of protein using PC-2 and quantitated by cutting and counting as described in figure 6. Values are presented for the whole cell lysate (n=4), the 48% (PM-enriched) band (n=11) and the P200 (LDM-enriched) preparations (n=10).
*p<.05

A.


kD
66
43
1 2 3 4

B.

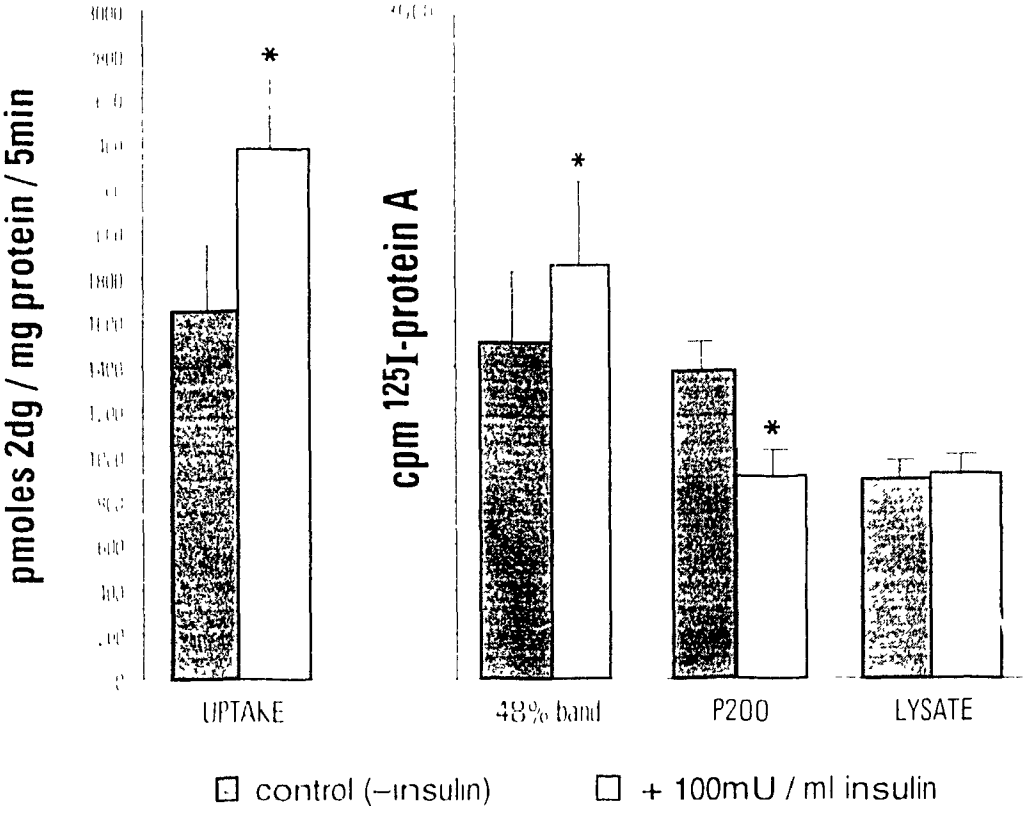

pmoles 2dg / mg protein / 5min
cpm 125I-protein A
*
*
*
UPTAKE
48% band
P200
LYSATE
control (–insulin)
+ 100mU / ml insulin

Figure 9. Effects of glucose deprivation on GT expression in V79 cells. V79 cells were serum starved for 16-24 hours and then exposed to treatment media containing 4mg D-glucose/ml, or no glucose, for 4 hours. 7.5ug of protein from the 48% band preparations (lanes 1 and 2), and 15ug of the P200 (lanes 3 and 4) preparations were loaded onto the gel. Lanes 1 and 3 contain samples from the control (glucose-fed) cells, and lanes 2 and 4 represent the glucose deprived cell preparations.


kD
66
43
1
2
3
4

Figure 10. Comparison of 2DG uptake and GT expression in V79 and G14 cells. In paired experiments, serum starved V79 and G14 cell preparations were compared. A) Representative Western blot depicting GT expression in V79 (lanes 1,3 and 5) and G14 (lanes 2, 4, and 6) preparations. Lanes 1 and 2 contain 25ug of whole cell lysate protein; lanes 3 and 4 contain 10ug of 48%band protein; lanes 5 and 6 contain 10ug of P200 protein. B) 2DG uptake (n=10), and GT expression in the 48% band (n=10), P200 (n=12) and whole cell lysate (n=8) preparations are presented, as determined through quantitation from Western and dot blot analyses.
*p<.05

A.


kD
66
43
1 2 3 4 5 6

B.

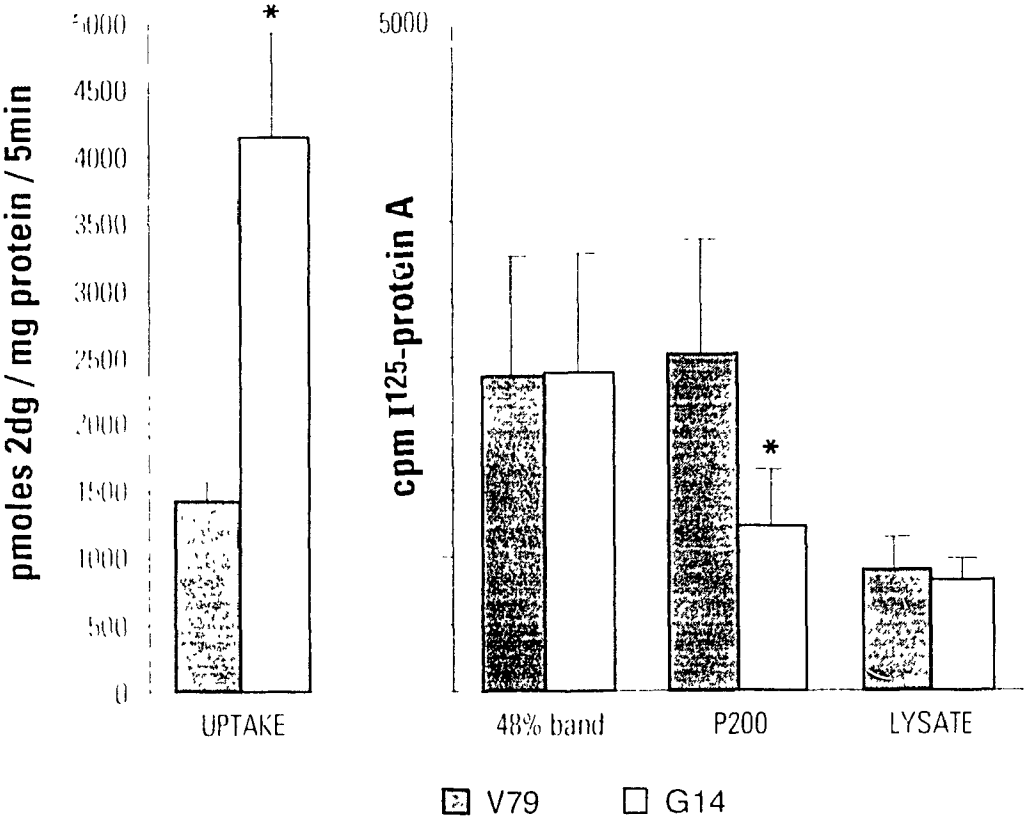

pmoles 2dg / mg protein / 5min
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
UPTAKE
cpm I125-protein A
5000
*
48% band
P200
LYSATE
V79
G14

Figure 11. Comparison of GT expression in subcellular fractions of respiration deficient cells. From a single experiment, lanes 1-4 depict GT expression in 10ug of the 48% band from V79, G14, G4 and G7 respectively, and lanes 5-8 contain 10ug from the P200 preparation in the same order. The excised ^{125}I-protein A counts for lanes 1-8 were 997, 1117, 1055, 634, 1023, 294, 371, and 266 cpm respectively.


kD
66
43
1
2
3
4
5
6
7
8

Table 2. Cytochalasin B Binding vs. Immunological evaluation of GT in V79 and G14 Membrane Preparations

	CB binding (pmoles CB/ug protein)			Western Quantitation
	Nonspecific	Total	D-Glu.Inh.	(cpm ^{125}I-Prot.A/ug protein)
V79	6.8±0.8	11.0±1.0	2.2±0.4	40.7±12.8
G14	7.0±0.6	9.0±0.6	1.2±0.3	19.5±5.5
(n)	(3)	(5)	(5)	(4)

Total membrane preparations from each cell line were evaluated for ^{3}H-CB binding in the presence of 15uM unlabelled CB (nonspecific) or 500mM L-glucose (total) or 500mM D-glucose. D-Glucose inhibitable binding represents the GT specific portion of total CB binding. These same preparations were evaluated through Western blotting procedures as described in Materials and Methods.
Values are expressed as the mean ± s.e.m.
n=number of determinations.

Figure 12. Endoglycosidase F treatment of V79 and G14 membrane preparations. Total membrane preparations were treated with (lanes 3 and 4) and without (lanes 1 and 2) endoglycosidase F as described in Materials and Methods, and then analyzed on 12% polyacrylimide gels. Lanes 1 and 3 contain 20ug V79 membrane protein, and lanes 2 and 4 contain 20ug of G14 membrane protein.


kD
66
43
31
1
2
3
4

Figure 13. Western analysis of V79 and G14 preparations following tunicamycin or cycloheximide treatment. After 30 hours of treatment, 50ug of V79 (lanes 1, 3, and 5) and G14 (lanes 2, 4, and 6) cell lysate preparations were evaluated on a 12% gel as follows: control, or untreated cells (lanes 1 and 2), tunicamycin (0.5ug/ml) treated cells (lanes 3 and 4), and CHX (0.05ug/ml) treated cells (lanes 5 and 6). The total cpm ^{125}I-protein A for lanes 1-6 are 1163, 597, 1797, 1249, 1129, and 510 respectively.


kD
66
43
1
2
3
4
5
6

Figure 14. Effects of insulin stimulation on 2DG uptake and GT expression in G14 cells. In paired experiments, serum starved G14 cells were treated for 1 hour with media containing 100mU/ml insulin or its diluent. 2DG uptake determinations (n=5) and GT expression in the 48% band (n=4), P200 fraction (n=5) and whole cell lysate (n=3) were determined as previously described.

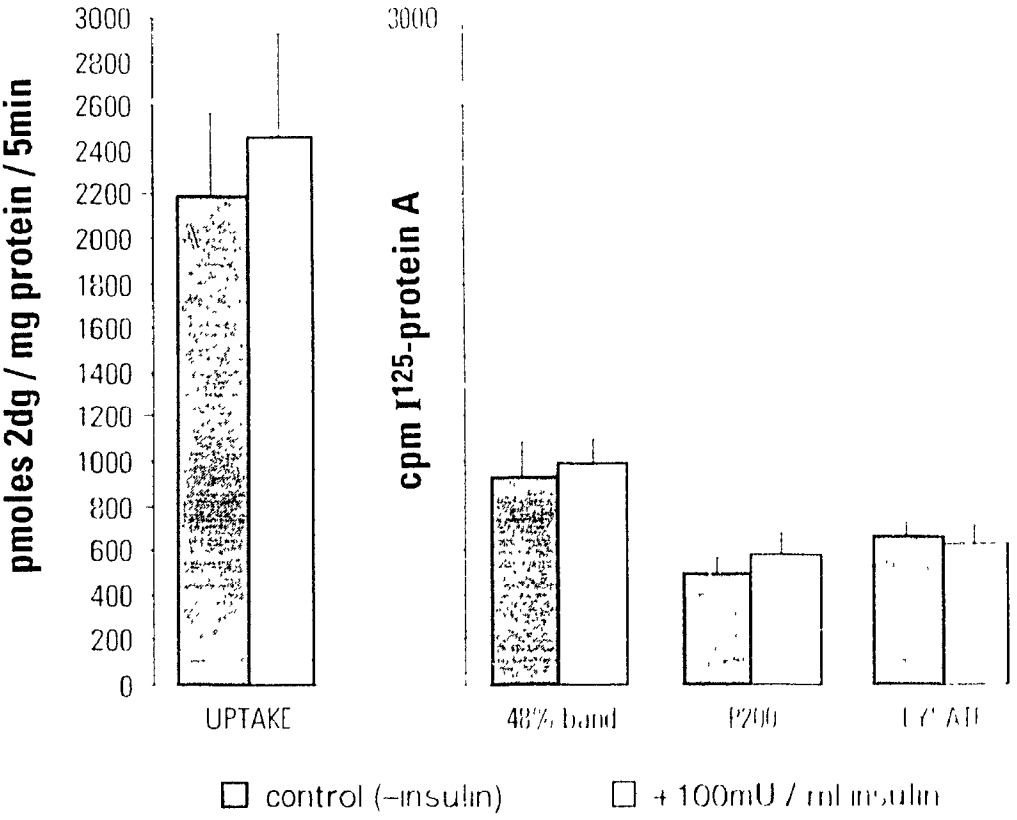
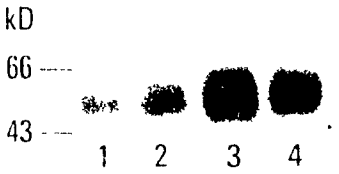

pmoles 2dg / mg protein / 5min
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
UPTAKE
cpm I125-protein A
3000
48% band
P200
control (-insulin)
+ 100mU / ml insulin

Figure 15. Comparison of 2DG uptake and GT expression in D-Allose treated V79 cells. In paired experiments, serum starved V79 cells were treated for 4 hours with media containing 4mg D-glucose/ml (control) or 4 mg D-allose/ml. A) Representative Western blot depicting GT expression in the 48%band (lanes 1 and 2, 5ug protein/lane) and P200 (lanes 3 and 4, 10ug protein/lane) preparations of control (lanes 1 and 3) and D-allose treated cells (lanes 2 and 4). B) 2DG uptake (n=7), and GT expression in the 48% band (n=7), P200 (n=7) and whole cell lysate (n=4) preparations are presented, as determined through Western and dot blot analysis.

A.


kD
66
43
1
2
3
4

B.

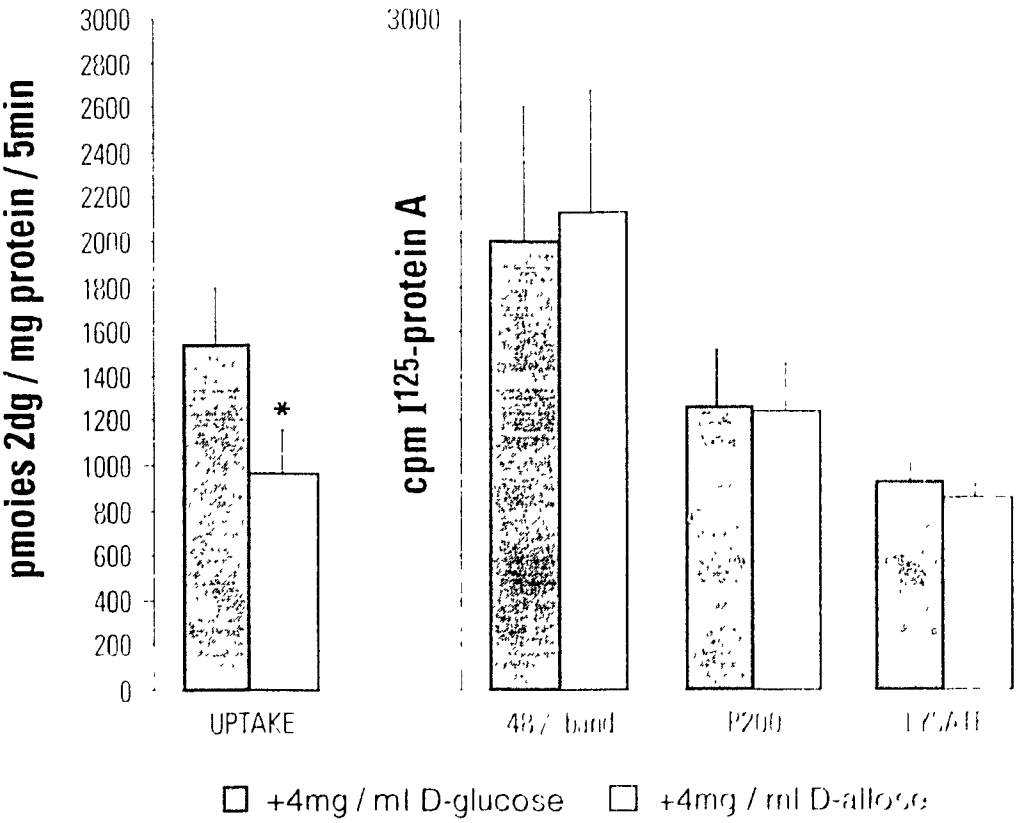

pmoies 2dg / mg protein / 5min
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
UPTAKE
cpm I125-protein A
3000
48 / band
P200
LYSATE
+4mg / ml D-glucose
+4mg / ml D-allose

Figure 16. Comparison of 2DG uptake and GT expression in D-Allose treated G14 cells. In paired experiments, serum starved G14 cells were treated for 4 hours with media containing 4mg D-glucose/ml (control) or 4 mg D-allose/ml. 2DG uptake (n=3), and GT expression in the 48% band (n=2), P200 (n=2) and whole cell lysate (n=2) preparations are presented, as determined through Western and dot blot analysis.
*p<.05

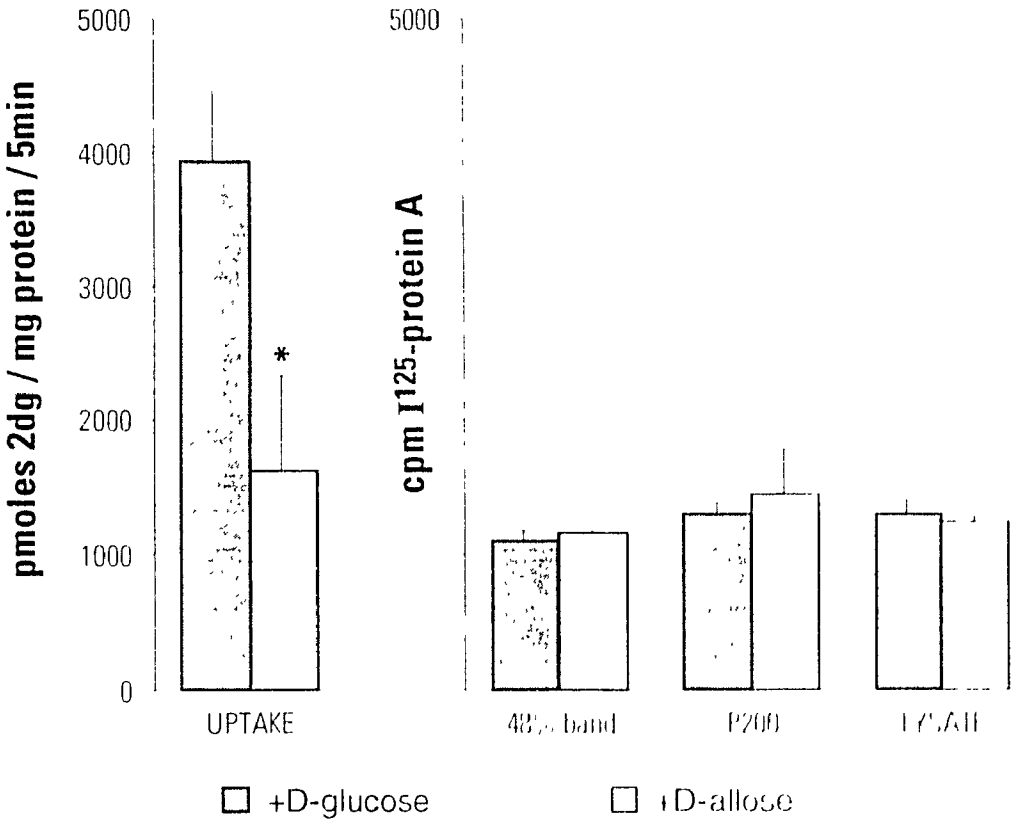

pmoles 2dg / mg protein / 5min
5000
4000
3000
2000
1000
0
*
UPTAKE
cpm I125-protein A
5000
48% band
P200
TCA/AH
+D-glucose
+D-allose

Figure 17. Insulin reversal of D-allose effects in the V79 cell line. Control (D-glucose treated) and D-allose treated cells were stimulated with 100mU insulin/ml during the last hour of treatment. Data depicts the results of 4 separate experiments in which all four conditions were evaluated simultaneously. 2DG uptake determinations and GT expression in the cell fractions were determined as previously described.
*$p<.05$ for D-glucose treated $\pm$ insulin paired evaluations;
•$p<.05$ for D-allose treated $\pm$ insulin paired evaluations.

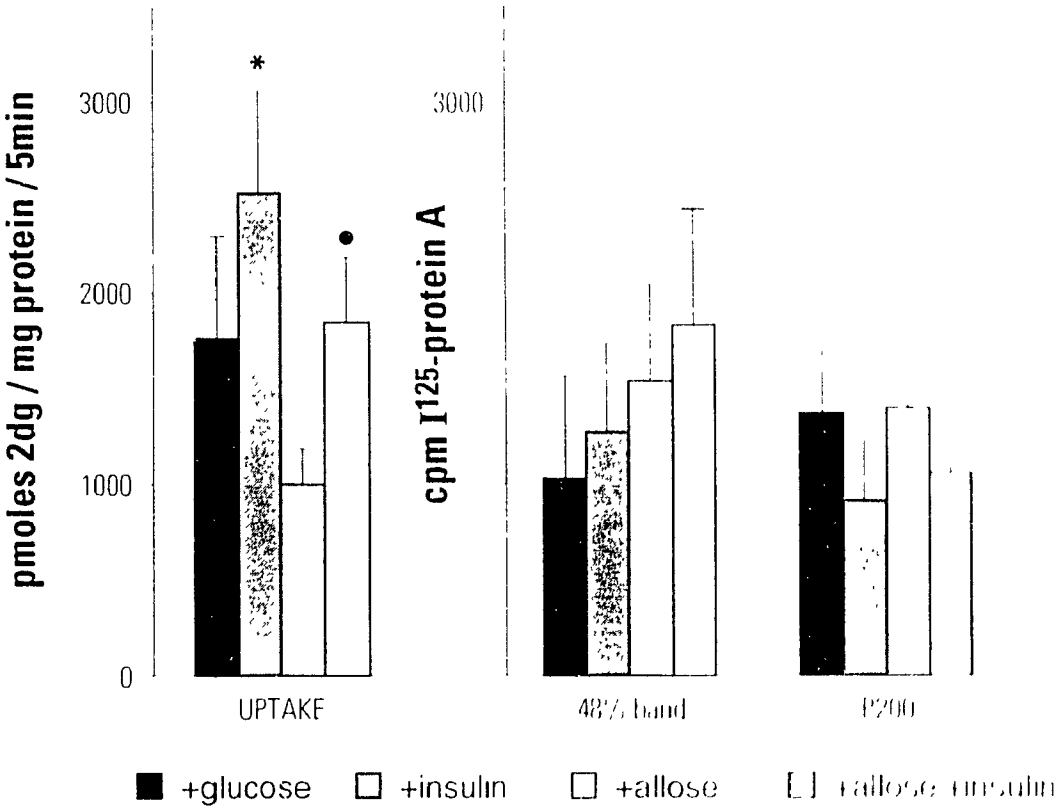

pmoles 2dg / mg protein / 5min
3000
2000
1000
0
*
•
UPTAKE
cpm I125-protein A
3000
48% band
P200
+glucose
+insulin
+allose
allose insulin

Table 3. Cytochalasin B Binding to V79 Membranes in the Presence of D-Allose

	+L-glucose	+D-glucose	+D-allose
pmoles CB/ug protein	12.7±2.0	10.1±1.8	14.2±1.9
(n)	(6)	(6)	(6)

Total membrane preparations from the V79 cell line were evaluated for ^{3}H-CB binding (as described in Materials and Methods) in the presence of 500mM L-glucose, 500mM D-glucose or 500mM D-allose.
Values are expressed as the mean ± s.e.m.
n=number of determinations.

DISCUSSION

This thesis follows two papers published by Germinario et al (1990a and b), in which novel instances of sugar uptake regulation were presented. In one paper, a dramatic upregulation of sugar transport was demonstrated in a respiration deficient mutant (G14) Chinese hamster lung fibroblast cell line when it was compared to its parental (V79) cell line (Germinario et al, 1990a). The second paper described the ability of a non-metabolizable sugar, D-allose, to suppress sugar transport in V79 cells (Germinario et al, 1990b). By producing a hamster glucose transporter reactive polyclonal antibody, and a reliable subcellular fractionation procedure for these cells, we have been able to characterize the effects of these and other known regulators of sugar transport in terms of the expression and distribution of the GT protein in these two cell lines.

The polyclonal antibody preparation used in this study (PC-2) demonstrated a specificity towards the human glucose transporter comparable to that observed with the established Glut1 monoclonal antibody, MAbG3 (Allard and Leinhard, 1985) (figure 6). In addition, PC-2 demonstrated cross reactivity to a homologous protein expressed in the V79 and G14 hamster cells (figure 7). Verification that this was the hamster glucose transporter protein was achieved by showing that the

specific antibodies involved detected both the human and hamster glucose transporters.

In order to further verify the specificity of PC-2 towards the hamster glucose transporter, and to validate the fractionation procedure adopted, V79 cells were exposed to well characterized regulators of sugar uptake. Both insulin exposure and glucose deprivation resulted in a stimulation of 2DG uptake (1.4 and 1.9-fold respectively over control transport levels) which corresponded with increased GT content in the 48% (PM enriched) band (1.2 and 2.5-fold over control, respectively) (figures 8 and 9). In both of these situations, sugar transport regulation is apparently mediated through GT expression at the active plasma membrane site, however the mechanisms involved are different. The observation that glucose deprivation of the V79 cell line also resulted in an increase in whole cell GT expression agrees with the events described in other similarly treated cells, ie. a net increase in cellular GT content (Haspel et al, 1986) which is reflected in the plasma membrane (Walker et al, 1989 and 1990). On the other hand, insulin stimulation did not alter whole cell GT content, but rather displayed an increase in plasma membrane expressed GT accompanied by decreased GT expression in the LDM-enriched (P200) fraction. This event is typical of the translocation response observed in other insulin responsive cells (Simpson et al, 1983; Wardzala and Jeanrenaud, 1981).

Through these evaluations, several important points have been established. Firstly, the GT protein detected in V79 demonstrates characteristic responsiveness to sugar uptake regulators which is readily observed in the context of the fractionation procedure employed. Secondly, a relationship is established which correlates the 2DG uptake capabilities of the V79 cell with the amount of GT expressed in the plasma membrane preparation. Finally, an interactive relationship has been demonstrated between the LDM and the PM enriched fractions, thereby allowing comparative evaluations of the intracellular GT pools and their potential roles in sugar uptake regulation.

GT regulation in respiration deficient mutants:

It has long been known that, in most cell types, blocking the aerobic generation of ATP will result in an increase in glycolytic (anaerobic) metabolism (Elbrink and Bihler, 1975; Mercado et al, 1989). This metabolic shift results in an increased demand for glucose, and is accompanied by a stimulation in sugar transport. In a respiration deficient human fibroblast cell strain (Andrejchyshyn et al, 1991), and in L6 muscle cells exposed to a hypoxic environment (Bashan et al, 1992), increased sugar transport capabilities are accompanied by an increase in whole cell GT expression. Heart cells (Wheeler, 1988) and skeletal muscle (Cartee et al, 1991)

have been shown to respond to hypoxic conditions with an insulin-like translocation of GT to the plasma membrane.

Based on these studies, it was expected that a comparison of GT expression in V79 and G14 cells would reveal, at least, an increase in the PM enriched fraction of the respiration deficient cell. However, when the G14 mutant was quantitatively evaluated through Western analysis, it was found that the 3-fold enhancement of 2DG uptake was not apparently related to any difference in the amount of GT expressed in the whole cell, or in the plasma membrane, when compared to V79 (figure 10).

What was apparent, was a difference in the SDS-PAGE migration of the glucose transporter expressed in the two cell lines (figure 10). The reduced mobility of the GT in the G14 cell line indicates that a higher molecular weight protein is being recognized. The G4 and G7 cell lines are two other genetically distinct respiration deficient mutants of the V79 cell line (Soderberg et al, 1979; Breen and Scheffler, 1979), and exhibit a similar enhancement in 2DG uptake as that seen in G14 (Chiasson, 1990). When subcellular fractions of the G4 and G7 cells were analyzed, it was found that they demonstrated the same quantitative and qualitative pattern of GT expression as that seen in G14 (figure 11).

It was considered that sugar transport in G14 may be regulated through the expression of an alternate, undetectable glucose transporter isoform. It has been shown that events

such as transformation (Thorens et al, 1988) and the differentiation of 3T3-adipocytes (de Herreros and Birnbaum, 1989) can induce the expression of GT isoforms not normally expressed in the precursor cell, thereby altering the sugar transport characteristics of the cell. When V79 and G14 total membrane preparations were probed for expression of the Glut4 isoform, none was detected in either cell line. In addition, D-glucose inhibitable CB binding evaluation of GT expression in the two cell lines closely approximated the relative amounts of GT detected by PC-2 Western analyses of the same samples (Table 2), indicating that PC-2 is sufficiently able to portray the GT expression of both cell lines.

These findings further suggest that the molecular weight differences of the GT observed in the two cell lines are the result of pre- or post-translational modifications. Comparisons of the unglycosylated forms of the V79 and G14 transporters, either through the *in vitro* enzymatic removal of N-linked oligosaccharides with endoglycosidase F (figure 12), or through *in vivo* inhibition of N-linked glycosylation by tunicamycin (figure 13), demonstrated that the G14 mutant cell line is expressing a hyperglycosylated form of the same core protein found in the parental cell line.

Several approaches have been taken in an effort to determine the functional role of GT glycosylation. These studies looked at sugar transport in: 1) glycosylation deficient mutants (Haspel et al, 1988); 2) CHO cells

transfected with a mutated, glycosylation-defective GT (Asano et al, 1991); 3) tunicamycin treated cells (Olden et al, 1979; Wertheimer et al, 1990); 4) deglycosylated, liposome integrated glucose transporters (Feugeas et al, 1990 and 1991), and 5) sialic acid free (ie. neuraminidase treated) intact adipocytes (Ciaraldi, 1989). These studies suggest that reduced glycosylation results in diminished sugar transport capabilities. Of particular interest, Ciaraldi (1989) has provided evidence that glycosylation may play a role in the modulation of GT intrinsic activity - ie. neuraminidase treatment of insulin-stimulated fat cells led to a decline in the Vmax of sugar transport, with no concomitant change in the GT content of the plasma membrane. Of further interest is a study (Bramwell et al, 1990) which demonstrated that tumorigenic cells express a more glycosylated GT when compared to non-tumorigenic cells. The authors related these findings to a previous report in which it had been shown that tumorigenic cells demonstrated a reduced transport Km, or increased affinity of the GT for the sugar substrate when compared to their non-tumorigenic counterparts (White et al, 1983). While no significant difference in the Km was found in the V79 and G14 cell lines (Germinario et al, 1990), it is clear that the respiration deficient cell line exhibits a more efficient mode of sugar transport. The glycosylation differences observed in the two cell lines present a potential site of activity modulation.

An attempt was made to discern whether the differential glycosylation of the GT in the two cell lines could be related to the sugar transport properties of the cells through glycosylation inhibition with tunicamycin. A large increase in GT expression occurred with tunicamycin treatment in both cell lines (figure 13), yet sugar uptake was only slightly affected by the treatment. Since tunicamycin also inhibited protein synthesis at the concentration employed, the effects the protein synthesis inhibitor, CHX, were examined. When compared at concentrations which inhibited leucine incorporation equally, both CHX and tunicamycin produced similar, yet modest, effects on 2DG uptake. From these findings, it would appear that the influence of tunicamycin on sugar transport could be related to its protein synthesis inhibitory effects. In contrast, however, CHX had no effect on GT expression, thereby presenting the possibility that tunicamycin treated cells have compensated for reduced GT activity through increased expression. Similar increases in Glut1 were observed in glycosylation inhibited L6 muscle cells (Maher and Harrison, 1991), due to an enhanced stability of both Glut1 message and protein.

The role of the hyperglycosylation of the GT in the respiration deficient mutants, however, remains to be determined. In general, it has been shown that variations in the extent of glycosylation can influence protein targeting or secretion, catalytic efficiency, and/or rates of degradation

(Rademacher et al, 1988; Paulson, 1989). As an adaptive response, increased glycosylation can be envisioned as affecting GT regulation at a number of levels which might be beneficial to the respiration deficient mutant cells - eg, 1) by enhancing the activity of the GT; 2) by influencing the targeting or rate of expression in the PM; or 3) by increasing the half-life of the protein. By examining the role of glycosylation through the use of inhibitors, such as tunicamycin, all of these possibilities must be considered, as well as the additional metabolic effects (Elbein, 1981) of the inhibitors themselves (eg. protein synthesis inhibition) which might influence GT regulation. However, by continuing these studies, post-translational processing may be indicated as yet another mechanism by which sugar transport can be regulated.

When the G14 cell line was evaluated for insulin responsiveness, it was found that neither the increase in 2DG uptake nor the translocation of GT seen in V79 was demonstrable in the mutant (figure 14). Germinario et al (1990a) showed that V79 and G14 possess comparable insulin binding properties, and that insulin is able to evoke a growth response in both cell lines, indicating that the lack of response is not likely to be due to differences in receptor binding of insulin. Through quantitation of the subcellular glucose transporter expression (figure 10), we have shown that one possible explanation is that there is a lack of

translocatable transporters in the G14 microsomes. A lack of translocatable glucose transporters has been considered to be at least partially responsible for the insulin resistance of liver cells (Ciaraldi et al, 1986) and some diabetic cells (Sivitz et al, 1989). This same situation may be the reason for the lack of serum stimulation of sugar transport seen in the G14 cell line (Germinario et al, 1990a), since sugar transport stimulation by serum is considered to initially be due to translocation of glucose transporters (Kitigawa et al, 1989).

On the other hand, depressed ATP levels have been correlated with a lack of insulin responsiveness (Kono et al, 1977) due to an inability to effect GT translocation (Kono et al, 1981). The respiration deficient mutants have been characterized as having reduced ATP levels (Sodereberg et al, 1980), and this property may regulate insulin responsiveness as well.

In summary, it appears that the increased uptake capabilities of these respiration deficient mutants of the V79 cell line are accomplished through an enhancement in the activity of the glucose transporter protein, rather than through an enrichment in its PM expression. To tease out the specific regulatory factors involved is a difficult process, however with the methodologies available to date, an idea as to whether the structural differences observed are related to

the GT activity and/or involved in its regulation of expression could be explored.

One method which might be used to determine the relative activities of the GT proteins involves the evaluation of sugar transport in isolated PM vesicles. This has been done in other studies (Buchanan et al, 1985; Cheung and Lo, 1984) and has the advantage of removing the protein from cytosolic components such as ATP (Carruthers and Helgerson, 1989) or second messengers (Segal, 1987) which may be regulating transport activity. On the other hand, if the GT activity is structurally regulated, eg, through glycosylation (Asano et al, 1991), phosphorylation (Gibbs et al, 1986) or other covalent modifications, the regulatory differences would be expected to be retained.

To further explore this line of thinking, transfection of allotypic GT cDNA has been done by some investigators (Thorens et al, 1988; Vera and Rosen, 1990) in order to understand whether transport activity is structurally or environmentally determined. If, for example, the V79 and G14 cell lines were transfected with the CDNA encoding the human Glut1 isoform, its expression could be readily differentiated from the hamster GT through the use of the MAbG3 antibody (Allard and Leinhard, 1985). Questions which might be asked and answered through this type of study could include: Is the human GT also hyperglycosylated in the respiration deficient mutants, or is this event in some way specific to the hamster

GT? Can the additional expression of GT reduce the differences in sugar transport between the mutant and parental cells and/or alter the responsiveness of G14 to sugar uptake regulators? If the human GT does appear to play an active role in the regulation of sugar transport in the cells, is its subcellular distribution differentially regulated in the two cell lines?

Finally, in other instances where sugar transport is modulated through no detectable change in plasma membrane GT content (Clancy et al, 1991; Germinario et al, 1992), regulatory mechanisms which involve "cryptic carriers" have been proposed and may have relevance to the respiration deficient mutant. One hypothesis is that PM integrated GT proteins can be rendered inactive through their sequestration into invaginations until a regulatory event (possibly ATP-related) allows for their exocytotic-like exposure to the extracellular environment (Hyslop et al, 1985). Another hypothesis outlines how active GTs could be located at the plasma membrane, yet only loosely associated and thereby lost during the fractionation procedure (Wang, 1987; Gould et al, 1989). Either of these mechanisms could be seen to account for the discrepancies observed between sugar transport and GT expression in the PM fraction of the V79 and G14 cells. One way to evaluate this possibility would be through the use of GT-directed antibodies which would bind to extracellular epitopes on intact cells. These types of studies have been

done by others (Burdett and Klip, 1988; Harrison et al, 1990), and whether sequestered are loosely associated, if a glucose transporter was active (ie. available to participate in sugar transport), it would be detectable through its accessibility to the external environment, and therefore to these antibodies.

GT regulation in D-allose treated cells:

By investigating the effects of the non-metabolizable sugar, D-allose, on GT expression, we had hoped to elucidate the mechanisms by which the depression in sugar transport occurs. When the effects of allose were evaluated in the V79 and G14 cell lines, it was found that 2DG transport was significantly reduced in both cell lines (38% and 59%, respectively). In both cell lines, however, the downregulation by allose did not relate to a decrease in either whole cell or plasma membrane GT expression (figures 15 and 16). These findings rule against the possibilities that allose is able to either enhance the degradation of the GT protein, or to evoke a reverse translocation of GT out of the plasma membrane. What these findings do suggest is that the allose effect is respiration independent, and is able to depress sugar transport at the activity level of the transport protein - possibly by reducing the activity level of each protein, or by completely inhibiting sugar uptake in a percentage of the transporters.

The reversal of the allose effects on sugar transport, by the replacement of D-allose with D-glucose, requires at least 6 hours for complete recovery (Germinario et al, 1990b). It has been demonstrated here, however, that in V79, insulin (in the continued presence of allose) is able to reinstate glucose-fed levels of 2DG uptake in less than one hour (figure 17). This insulin-induced reversal is apparently accomplished through the translocation of transporters to the plasma membrane, which is substantiated by the fact that neither the reversal in uptake nor the increase in plasma membrane GT expression is demonstrated in similarly treated G14 cells.

From the results presented in this study, the possibility has been proposed that allose acts principally to inhibit the transport capabilities of PM expressed glucose transporters. With this in mind, it would be relevant to review the reported actions of D-allose on sugar transport under other conditions. For example, D-allose has been described as being at least as effective as D-glucose in the reversal of the glucose starvation effect, ie. reducing sugar transport by approximately 60% (Ullrey and Kalckar, 1986). D-allose has also been described as potentiating the effect of tunicamycin inhibition on sugar transport (Ullrey and Kalckar, 1989), but also can be viewed as a 40% of inhibition of the tunicamycin treated cell. In both of these circumstances, and under other readily testable conditions, it would be pertinent to verify whether D-allose is influencing the expression of Gts in the

plasma membrane (which would be dictated by the primary treatment), or whether it is simply inhibiting the transport activity of those transporters expressed in the plasma membrane at the time of allose exposure. The results of these studies may provide a common thread in the determination of the effects of allose, and may provide further information as to whether allose is able to influence internally expressed Gts.

Originally, D-allose was described as a non-metabolizable hexose analog which was taken up by a GT-mediated process in adipocytes, and therefore had the potential to be labelled and used for sugar transport studies (Loten et al, 1976). In hamster fibroblasts, however, it was found that D-allose does not enter the cell via the GT, but is only taken up diffusionally and, as such, does not show any appreciable intracellular accumulation (Germinario, personal communication). Furthermore, Germinario (unpublished data) has observed that D-allose treatment reduces 2DG uptake in uninduced 3T3-fibroblasts, but no such reduction is observable in induced 3T3-adipocytes. It is now known that these two cell types differ through the additional expression of Glut4 in adipocytes (Birnbaum, 1989), and recently, an apparent repression of Glut1 transport activity has been reported when this isoform is expressed in adipocytes (Harrison et al, 1991). Based on these findings, it would appear that the D-

allose suppressive effects on sugar transport could be either tissue specific and/or Glut1 specific.

Through CB-binding studies (Table 3), it has been shown that D-allose does not apparently mediate its effects by directly binding to an intracellular site on the GT molecule, nor does it inhibit 2DG uptake by competing for transport at the extracellular interface (Germinario, 1990b). It is possible however, that a metabolite generated in association with D-allose exposure may interact at such a site. With this in mind, it might be useful to perform membrane vesicle studies from D-allose treated an untreated cells such as those described for the respiration deficient mutants. However, it might be even more interesting to explore the effects of D-allose on sugar transport in human erythrocytes. As a cell rich in membrane expressed Glut1 transporters, it would be expected that inhibition of sugar uptake would be dramatic if the D-allose effect was Glut1 specific. If so, these cells offer the advantages of being well characterized, metabolically simple, and readily prepared as membrane vesicles. In the preparation of erythrocyte membrane vesicles, procedures are available which can be used to dictate the orientation or "sidedness" of the vesicles. With these types of preparations, it can be determined whether allose itself is the repressor of sugar transport, or whether metabolism is required for its effects. Whether the regulator is allose or a metabolite, its site of action could then be

determined through the use of right-side or inside out vesicles and exposure to allose or cytosolic preparations of allose exposed cells.

Summary:

It has been well documented that sugar transport can be modulated through the expression and distribution of the GT isoform family. An increasing body of evidence is now being presented which indicates that regulation may also occur through the modulation of the catalytic or "intrinsic" activity of the glucose transporter protein(s) (Czech et al, 1992). As such, disruption of this mode of sugar uptake regulation has been implicated in the pathogenicity of diseases such as diabetes (Kahn, 1992).

In this thesis, two instances have been presented in which dramatic positive and negative regulation of sugar transport apparently occurs through changes in GT activity rather than GT expression or distribution. The G14 respiration deficient model suggests that the manner in which glucose is metabolized may play a role GT activity regulation and responsiveness, and presents an intriguing paradigm for the study of the relationship between GT glycosylation and sugar transport. The suppressive effects of D-allose on GT activity presents an equally intriguing model, and may also have pertinence in deciphering the roles of metabolism and/or metabolites in sugar transport regulation.

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