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# Identification of the Human Erythrocyte Glucose Transporter (GLUT1) ATP Binding Domain: A Dissertation

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# IDENTIFICATION OF THE HUMAN ERYTHROCYTE GLUCOSE TRANSPORTER (GLUT1) ATP BINDING DOMAIN

A Dissertation Presented

By

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A Dissertation presented

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# Abstract

The human erythrocyte glucose transport protein (GLUT1) interacts with, and is regulated by, cytosolic ATP. This study asks the following questions concerning ATP modulation of GLUT1 mediated sugar transport. 1) Which region(s) of GLUT1 form the adenine nucleotide-binding domain? 2) What factors influence ATP modulation of sugar transport? 3) Is ATP interaction with GLUT1 sufficient for sugar transport regulation?

The first question was addressed through peptide mapping, n-terminal sequencing, and alanine scanning mutagenesis of GLUT1 using [$^{32}$P]-azidoATP, a photoactivatable ATP analog. We then used a combination of transport measurements and photolabeling strategies to examine how glycolytic intermediates, pH, and transporter oligomeric structure affect ATP regulation of sugar transport. Finally, GLUT1 was reconstituted into proteoliposomes to determine whether ATP is sufficient for the modulation of GLUT1 function in-vitro.

This thesis presents data supporting the hypothesis that residues 332-335 contribute to the efficiency of adenine nucleotide binding to GLUT1. In addition, we show that AMP, acidification, and conversion of the transporter to its dimeric form antagonize ATP regulation of sugar transport. Finally, we present results that support the proposal that ATP interaction with GLUT1 is sufficient for transport modulation.

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# Abbreviations

| | |
|---|---|
| 2DOG | 2-Deoxyglucose |
| 3OMG | 3-O-methyl-D-glucose |
| ATP | adenosine 5′-triphosphate |
| C-Ab | GLUT1 c-terminal antibody |
| CCB | cytochalasin-b |
| Cos-7 | simian kidney cell line |
| δ-antibody | tetrameric GLUT1 exofacial antibody |
| DTT | dithiothreitol |
| Ghosts | sealed erythrocyte membranes lacking cytosol |
| GLUT1 | human erythrocyte glucose transport protein |
| $His_6$ | 6 histidines |
| HA | hemagglutinin |
| HA-Ab | anti-HA antibody |
| N-Ab | GLUT1 N-terminal antibody |
| Ni-NTA | nickel column media |

| | |
|---|---|
| PMSF | phenylmethylsulfonylfluoride (serine protease inhibitor) |
| PFK | phosphofructokinase |
| RE700A | S. Cerevisiae URA 52 mutant with seven of its glucose transporters (HXT 1-7) deleted |
| SDS | sodium dodecyl sulfate |
| Stripped Ghosts | red blood cell ghosts stripped of peripheral membrane proteins |

# Chapter I

## Introduction

A selectively permeable barrier, the cell membrane, surrounds all mammalian cells. This barrier is composed primarily of lipids and proteins. The hydrophobic nature of this cell membrane allows non-polar molecules, and only the very smallest polar molecules to pass through by simple diffusion. In contrast, polar molecules larger than water such as ions, sugars, amino acids, nucleotides, and cell metabolites must be transported across the cell membrane by specialized integral membrane proteins which function as channels or carriers. The channels are water filled pores that allow substances (selected on the basis of charge and size) to diffuse down an electrochemical gradient across membranes.

In contrast, carrier proteins transfer bound solutes across membranes by way of conformational changes. Two types of mediated transport have been described, facilitated and active. Facilitated transport is characterized by rapid, protein mediated, bidirectional transport of solutes across the membrane, down an

electrochemical gradient. Active transport couples a metabolic energy source (i.e. ATP) to the rapid protein mediated movement of solute against a concentration gradient (Carruthers, 1990).

Glucose, a hexose monosaccharide, is an essential source of energy in mammalian and non-mammalian cells. Cellular metabolic machinery requires D-glucose for the production of ATP (Darnell, 1990). A family of facilitative glucose transporters selectively pump D-glucose across cell membranes with a net movement of sugar from areas of high concentration to areas of low concentration (Stein, 1986). Stereoselectivity, saturation kinetics, and inhibition by the fungal metabolite cytochalasin B (CCB) characterize these facilitative glucose transporters.

Five mammalian glucose transporter isoforms have been identified in the facilitative glucose transporter family. These five isoforms are highly homologous (50-80%) yet exhibit distinct tissue distributions (Gould and Bell, 1990). GLUT1, the erythrocyte glucose transporter, is found in virtually all mammalian tissues including red blood cells, brain microvessels, kidney, colon, and placenta (Mueckler et al., 1985; Birnbaum et al., 1986; Gould and Bell, 1990).

GLUT2, the liver type transporter, is located in liver, kidney, small intestine, and pancreatic β-cells (Thorens et al., 1988). The presence of GLUT3 in fibroblasts and blood vessels suggests a ubiquitous tissue distribution (Kayano et al., 1988). GLUT4, the insulin sensitive transporter, is found in skeletal and cardiac muscle as well as adipocytes (Birnbaum, 1989; Charron et al., 1989; Fukumoto et al., 1989; James et al., 1989; Kaestner et al., 1989). GLUT5, a fructose transporter, is found primarily in the small intestine with lower levels of expression identified in kidney, skeletal muscle, and subcutaneous fat (Kayano et al., 1990).

The goal of this laboratory is to understand all aspects of facilitative glucose transport. To accomplish this, we have chosen to study GLUT1, the human erythrocyte form of the facilitative glucose transporter. Specifically, our interest lies in understanding how GLUT1-mediated sugar transport is regulated. Certain characteristics of GLUT1 make it ideal for studying transport regulation. First, GLUT1 comprises 6% of total human red blood cell protein. The ease with which these erythrocytes can be acquired facilitates the purification of large quantities (10-20 mg/unit of blood) of the

transporter for use in experimental protocols. In addition, the cytosolic environment of red blood cells can be readily controlled by reversible hypotonic lysis, thereby permitting analysis of cytosolic determinants of transport.

*Sugar Transport Regulation*

Certain cell types, such as skeletal and cardiac muscle, adipocytes, and nucleated avian erythrocytes can metabolize sugar at rates that exceed transport capacity. Under these conditions glucose transport becomes the rate-limiting step in sugar utilization, and cells become susceptible to acute transport regulation (Elbrink and Bihler, 1975; Carruthers, 1990).

Compounds that regulate glucose carrier function can be divided into two groups: hormones and metabolic factors. The primary hormone to which GLUTs respond is insulin, which is secreted by the pancreatic islets in response to changing blood nutrient levels (Vander, 1990). Cells that respond to insulin include adipocytes and muscle. In contrast, metabolic factors such as anoxia, ischemia, exercise, and metabolic poisons, have been shown to affect skeletal muscle, cardiac muscle, and nucleated avian erythrocytes. In either

case, exposure to these substances and conditions stimulates glucose uptake (Carruthers, 1990).

Two hypotheses exist which explain the mechanisms governing transport acceleration. Transport capacity is defined by the following equation: $V_{max} = K_{cat}[E]_t$ where $K_{cat}$ represents the catalytic turnover of an enzyme, and $[E]_t$ is equal to total enzyme concentration. It is apparent from this relationship that an increase in either transporter catalytic turnover or transporter number would alter transport capacity ($V_{max}$). The recruitment hypothesis suggests that transport stimulation is a direct result of transporter redistribution to the plasma membrane from a subcellular pool (increased $[E]_t$) (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). In contrast, the activation hypothesis implies that transport acceleration is the result of an increase in the intrinsic activity of transporters already present in the membrane (increased $k_{cat}$) (Carruthers, 1990). Individually, carrier recruitment or activation could result in sugar transport stimulation. However, these two mechanisms could also function coordinately in cells. Under these

conditions, inactivation and reinternalization would result in transport inhibition (Carruthers, 1990).

The following sections present four model cell types that exhibit acute regulation of glucose transport, skeletal and cardiac muscle, avian erythrocytes, and human red blood cells.

*Skeletal Muscle*

Skeletal muscle is the primary site of glucose disposal *in-vivo* (Douen et al., 1990; Klip and Paquet, 1990). Sugar transport in this tissue is maximally stimulated by insulin, as well as exercise, anoxia/hypoxia, ischemia, and inhibition of oxidative phosphorylation, all of which result in an increased demand for ATP (Clausen, 1975; Elbrink and Bihler, 1975). Subcellular fractionation and cytochalasin B binding experiments have shown that stimulation is characterized by a two-fold increase in plasma membrane and t-tubule carrier levels (Hirshman et al., 1988; Wheeler, 1988; Douen et al., 1990; Cartee et al., 1991; Goodyear et al., 1991).

Experimentally, striated muscle has been found to contain two transporter isoforms: GLUT1 (Mueckler et al., 1985; Birnbaum et al., 1986; Gould and Bell, 1990) and GLUT4 (Cushman and Wardzala, 1980; Birnbaum, 1989; Charron et al., 1989; James et al., 1989). GLUT1, detected primarily at the cell surface, is proposed to be responsible for basal transport rates in unstimulated skeletal muscle (James et al., 1988). In contrast, transport stimulation due to anoxia and exercise appears to result from GLUT4 translocation to the cell membrane from an intracellular compartment (Douen et al., 1990; Gao et al., 1994). More recent evidence has suggested that skeletal muscle contains a pool of conformationally masked GLUT4 that becomes exposed during transport stimulation (Wang et al., 1996). Supplementary experiments have indicated that GLUT4 translocation in skeletal muscle is not sufficient to account for the level of transport stimulation observed in response to anoxia and exercise (Morgan et al., 1959; Morgan et al., 1961; Morgan et al., 1965; Sternlicht et al., 1988; Wheeler, 1988; Klip and Paquet, 1990). The implication is that transport stimulation in skeletal muscle may be due to both an increase in the turnover rate of existing carriers

as well as the total number of transporters present (Sternlicht et al., 1988; Klip and Paquet, 1990).

The degree to which recruitment and activation contribute to exercise, anoxia, and metabolic-depletion induced transport stimulation in skeletal muscle has not yet been determined. Carruthers suggests that this question might be answered by measuring transport kinetics and carrier capacity in sarcoplasmic vesicles isolated from basal and stimulated muscle (Carruthers, 1990). The structural complexity of skeletal muscle and the lack of definitive markers for subcellular fractions make this a technically difficult task that has hindered analysis (Klip and Paquet, 1990)

*Cardiac Muscle*

Two major glucose transporter isoforms – GLUT1 and GLUT4 - are expressed in cardiac muscle. Levels of GLUT1 (Calderhead et al., 1990; Kraegen et al., 1993) were found to be 3 to 4 fold greater in heart muscle than in skeletal muscle (Doria et al., 1993). In the absence of insulin, GLUT4 is localized to tubulovesicular elements adjacent to the sarcolemma and transverse tubules (Fischer et al.,

1997). Immunohistochemical techniques have detected GLUT1 in cardiomyocyte plasma membranes and intercalated discs (Doria et al., 1993). Subcellular fractionation has revealed that a significant quantity of GLUT1 is also present in intracellular membranes (Fischer et al., 1995; Fischer et al., 1997). Both carriers were found to undergo translocation from an intracellular compartment to the cell surface in response to ischemia, hypoxia or contractile activity (Kolter et al., 1992; Doria et al., 1993; Sun et al., 1994; Wheeler et al., 1994; Zorzano et al., 1997). This is contrary to what is proposed to occur in skeletal muscle, where GLUT1 is detected only at the plasma membrane (James et al., 1988). Fischer et al (1997) identified two intracellular pools of carrier in cardiomyocytes that are available for translocation, one of which contained significant quantities of GLUT1. The evidence suggests that this pool of carriers is insulin insensitive (Fischer et al., 1997). It is not yet certain whether transport stimulation in heart muscle results from transporter recruitment alone, or occurs in combination with cell surface transporter activation. Wheeler found that glucose transporters in the heart show a 50% increase in membrane levels in

response to anoxia compared to a 150% increase in glucose uptake (Wheeler, 1988). In contrast, studies undertaken by Haworth et al showed that anoxia increases glucose transport in rat cardiomyocytes 10-20 fold (Haworth and Berkoff 1986). In heart, as in skeletal muscle, it appears that translocation may not account for the degree of transport stimulation seen in response to anoxia, ischemia, and exercise. This provides additional evidence that carrier activation may constitute a necessary part of the process of transport stimulation.

*Avian/Nucleated Erythrocytes*

In contrast to human erythrocytes, avian erythrocytes contain cytosolic structures, primarily mitochondria and a nucleus (Harris and Brown, 1971; Brown, 1975). Sugar transport in avian erythrocytes is subject to regulation by cellular metabolic status. Under basal conditions, protein-mediated net sugar uptake is barely detectable. Exposure to anoxia or to inhibitors of oxidative metabolism results in a robust stimulation of net sugar uptake (Cheung et al., 1977; Simons, 1983). Recent studies have

demonstrated that avian erythrocyte GLUT1, which is present at the cell surface at all times (Diamond and Carruthers, 1993), functions as an antiporter under normoxic conditions (Cloherty et al., 1996). This means that sugar uptake is coupled to sugar exit. Following metabolic disruption, avian red cell GLUT1 can catalyze sugar uniport (Cloherty et al., 1996) allowing net sugar uptake in the absence of intracellular sugar. Antiport function appears unaffected by cellular metabolic status. The mediator of this antiport/uniport switching mechanism is currently unknown. Cloherty et al has proposed that regulatory factors exist in the avian erythrocyte which allosterically inhibit GLUT1 uniport. Cloherty further proposes that AMP dependent protein kinase is involved in this activation/deactivation pathway (Cloherty et al., 1996).

While avian erythrocyte GLUT1-mediated sugar uniport is controlled more completely by cellular metabolic status than is human erythrocyte sugar uniport, the similarity between transport regulation in both tissues is striking. Human erythrocyte sugar transport is also mediated by GLUT1 (Mueckler et al., 1985; Wheeler and Hinkle, 1985) which is assembled and functions as an allosteric

homotetramer (Hebert and Carruthers, 1992; Zottola et al., 1995). In freshly isolated erythrocytes, net uptake of 3-O-methylglucose (a transported but nonmetabolized sugar) is slower by as much as 150-fold than 3OMG exchange transport (Cloherty et al., 1996). Following hypotonic cell lysis and cellular resealing in ATP-free medium, 3OMG net uptake (but not exchange transport) is stimulated by 3 to 10-fold (Helgerson et al., 1989). When lysed erythrocytes are resealed in ATP-containing saline, 3OMG net uptake and exchange are indistinguishable from transport observed in intact cells (Helgerson et al., 1989). This suggests that as with avian red cell sugar transport, uptake stimulation in human erythrocytes results from accelerated uniport function and that cytoplasmic ATP may regulate cellular glucose antiport/uniport switching (Levine et al., 1998).

*ATP Regulation of GLUT1 in Human Erythrocytes*

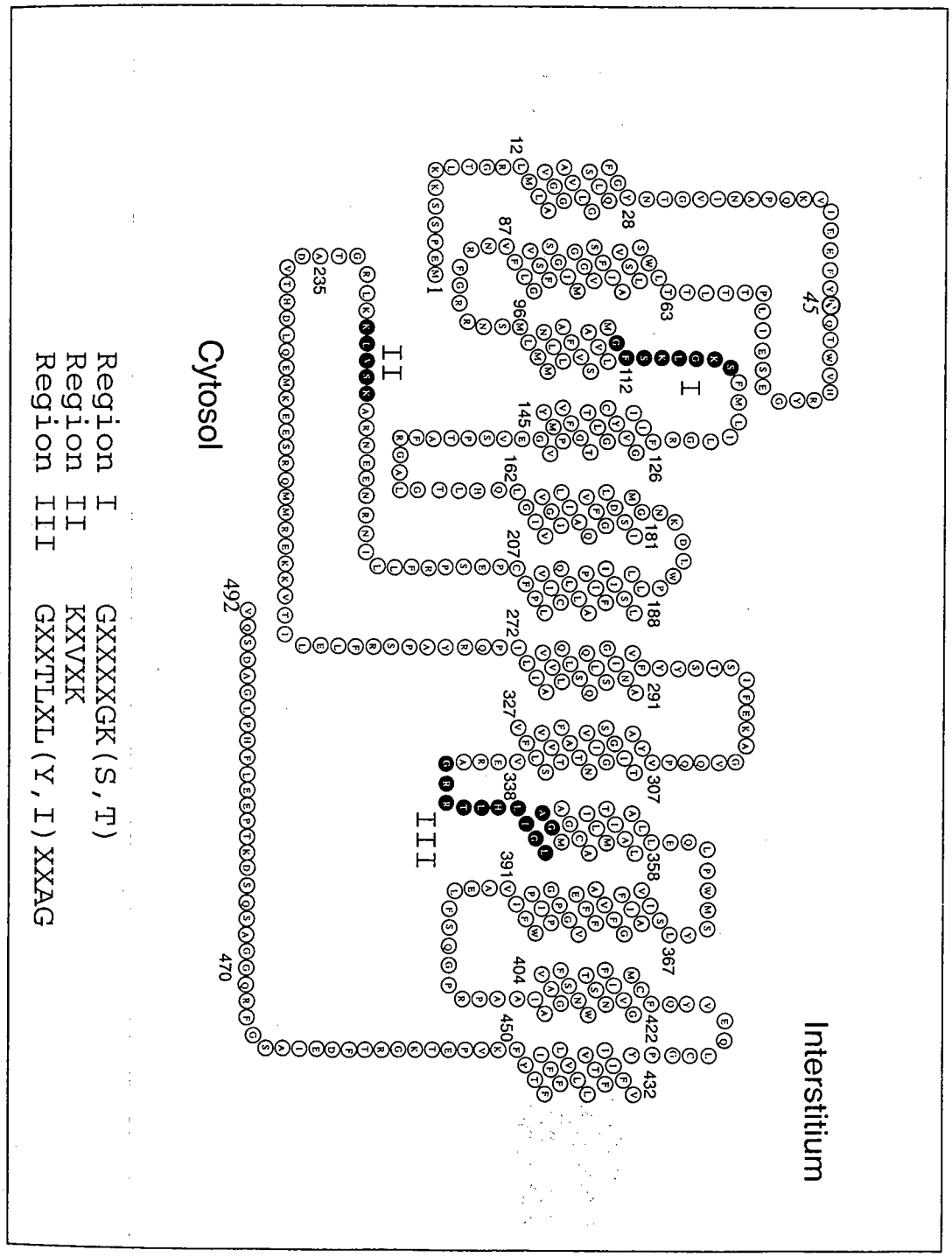
Human erythrocyte GLUT1 is subject to allosteric inhibition by ATP (Carruthers and Helgerson, 1989; Helgerson et al., 1989) which converts low affinity high capacity net sugar uptake to high affinity low capacity uptake. Maximum rates of exchange transport and net

sugar exit appear unaffected by cellular ATP levels (Helgerson et al., 1989). The net effect of ATP on 3OMG uptake at subsaturating sugar levels (50 to 100 $\mu$M) is to stimulate transport by a factor proportional to the increase in the ratio $V_{max}/K_{m(app)}$ while transport at 3OMG levels greater than $K_{m(app)}$ (saturated transport) is inhibited.

We have demonstrated that the isolated human erythrocyte glucose transport protein is also a nucleotide binding protein (Carruthers and Helgerson, 1989). ATP binding to GLUT1 promotes a GLUT1 conformational change that leaves the GLUT1 carboxyl-terminus less accessible to peptide-directed antibodies. This ATP-dependent conformational change is antagonized by AMP and ADP which appear to function as competitive inhibitors of ATP binding to GLUT1. Initial peptide mapping studies identified a range of residues from 270-380 as a likely region where ATP attaches (Carruthers and Helgerson, 1989). The primary sequence of GLUT1 indicates the presence of three potential consensus ATP binding sequences (Figure 1). Sequence I is a Walker A motif located between residues 111-118, sequence II is a hydrophobic loop motif which spans residues 225-229, and sequence III, located at residues

***Figure 1.*** **GLUT1 putative topography and consensus ATP binding domains.** The three proposed consensus ATP binding sequences located in GLUT1 are highlighted in black. Sequence I is a Walker A motif located between G111 and S117. Sequence II is referred to as a loop motif and stretches from K225-K229. Sequence III, a Walker B motif, extends from G332-G343.

Figure 1


Interstitium
Cytosol
I
II
III
Region I GXXXXGK(S,T)
Region II KXVXK
Region III GXXTLXL(Y,I)XXAG

332-343, is a Walker B motif which overlaps with the proposed region of ATP binding on GLUT1 [Walker, 1982 #4492]. It is unclear whether ATP binding to GLUT1 is sufficient to mediate sugar transport regulation in human red blood cells or whether other molecular species are required.

Wheeler has demonstrated that ATP does not modulate the activity of reconstituted human GLUT1 protein (Wheeler, 1989). A number of explanations could account for these results. 1) Required cellular components could be missing or dysfunctional in these reconstituted vesicles or 2) the GLUT1 used in these experiments may exhibit an abnormal ATP response or capacity to transport sugar (Carruthers and Helgerson, 1989). It is more probable, however, that the problem lies with Wheeler's use of reduced (DTT-treated) GLUT1 which we have shown to exist as a GLUT1 dimer (Hebert and Carruthers, 1992). Work in our laboratory has shown that conversion of tetrameric GLUT1 to its dimeric form ablates ATP binding and ATP modulation of transport (Levine et al., 1998). These results imply that ATP modulation of GLUT1 is dependent upon transporter quaternary structure. In contrast to Wheeler's

work, our studies employ the physiologic, non-reduced form of GLUT1 [ a GLUT1 tetramer (Hebert and Carruthers, 1992)] that responds to ATP.

*Strategy*

In the present study we ask: 1) What physiologic factors influence ATP-modulation of sugar transport? 2) Is ATP interaction with GLUT1 sufficient for sugar transport regulation? 3) Where is the GLUT1 nucleotide-binding domain? To address these questions, we use a combination of transport measurements and azidoATP photolabeling strategies to examine the effects of glycolytic intermediates, pH changes, and transporter oligomeric structure on ATP modulation of GLUT1. In addition, reconstitution assays using purified erythrocyte GLUT1 are carried out to determine if ATP is sufficient for modulation of GLUT1 mediated sugar transport. Finally, peptide mapping, n-terminal sequencing, and alanine scanning mutagenesis are used to locate the GLUT1 ATP binding domain.

Our findings suggest that intracellular AMP and acidification antagonize ATP regulation of sugar transport, and that ATP-

interaction with GLUT1 is sufficient for transport modulation. The data also support the hypothesis that tetrameric, not dimeric GLUT1, is regulated by ATP. In addition we show that residues 301-364 form an integral part of the ATP binding domain on GLUT1, and that epitope tagged human GLUT1 expressed in Cos-7 cells is normal with respect to sugar transport and azidoATP binding. Finally, alanine substitution at residues 338-340 ablates sugar transport, while alanine substitution at residues 225-229, and 332-334 increases [$^{32}$P]ATP binding.

# Chapter II

## Materials and Methods

***Materials.*** 8-Azido [$\gamma$-$^{32}$P]ATP was purchased from ICN, Costa Mesa, CA. [$^{3}$H]-3-*O*-Methylglucose was purchased from Dupont NEN, Wilmington, DE. Rabbit antisera (C-Ab) raised against a synthetic carboxyl-terminal peptide of the rat brain glucose transport protein (residues 480-492) was obtained from East Acres Biologicals, Southbridge, MA. Glycoprotein Detection Kits and ECL detection reagents were purchased from Amersham Pharmacia Biotech, Piscataway, NJ. Gradient SDS PAGE gels were purchased from Owl Scientific, Portsmouth, NH. Nitrocellulose and Immobilon-P were obtained from Fisher Scientific, Pittsburg, PA. Dulbecco's Modified Eagle Medium, FBS, Penicillin/Streptomycin, and G418 (Geneticin) were purchased from Gibco BRL, Grand Island, NY. The QuickChange Site-Directed Mutagenesis Kit was acquired from Stratagene, Marco Island, FL. pcDNA3.1+ vector was supplied by Invitrogen, Carlsbad, CA. Fugene 6 Transfection Reagent, anti-HA antibody (mouse monoclonal HA-ab), Endoproteinase Lys-C, and N-

dodecyl-β-D-maltoside were obtained from Roche, Indianapolis, IN. Nickel-NTA (Ni-NTA) was purchased from Qiagen, Valencia, CA. Centricon concentrators were acquired from Millipore, Bedford, MA. All remaining materials were purchased from Sigma unless stated otherwise.

***Solutions***. Tris medium consisted of 50 mM Tris-HCl, 5 mM $MgCl_2$ pH 7.4. Sample buffer (2X) contained 0.125M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, and 50 mM DTT. KCl medium consisted of 150 mM KCl, 10 mM Tris-HCl, 5 mM $MgCl_2$, 4 mM EGTA, pH 7.4. Stopper consisted of ice cold KCl medium plus 10 $\mu$M cytochalasin B, 100 $\mu$M phloretin, and 10 $\mu$M $HgCl_2$, pH 7.4. Lysis buffer contained 10 mM Tris HCl, 1 mM EGTA, pH 7.2. Phosphate buffered saline (PBS) contained 171 mM NaCl, 10 mM $Na_2HPO_4$, 3.4 mM KCl, 1.84 mM $KH_2PO_4$, pH 7.3. Buffer A included 300 mM NaCl, 10 mM imidazole, 20% Glycerol, and 50 mM $NaH_2PO_4$. Buffer B was composed of Buffer A + 0.1% N-dodecyl β-D maltoside. Buffer C contained 10 mM Tris-HCl (pH) 7.5, 300 mM NaCl, 25 mM imidazole, and 0.1% N-dodecyl β-D maltoside. Elution buffer consisted of 10 mM Tris-HCl (pH 7.5),

500 mM NaCl, 300 mM imidazole, and 0.1% N-dodecyl β-D maltoside. Krebs Ringer Phosphate (KRP) contained 4.5% NaCl, 5.75% KCl, 7.85% $CaCl_2 \cdot H_20$, 19.1% $MgSO_4 \cdot 7H_2O$, and 0.1 M $Na_2HPO_4$.

***N-Terminal Antisera.*** A peptide corresponding to GLUT1 residues 88-95 was synthesized by the University of Massachusetts Medical School Peptide Synthesis facility. This peptide was conjugated to keyhole limpet hemocyanin using a kit purchased from Pierce. Rabbit antisera (N-Ab) against this GLUT1 peptide were obtained from Animal Pharm Services Inc of Healdsburg, CA. Whole IgGs were purified from crude serum by ammonium sulfate precipitation (Sogin and Hinkle, 1980).

***Red Cells***. Erythrocytes were isolated from whole human blood by repeated wash/centrifugation cycles in ice-cold KCl medium. One volume of whole blood was mixed with 3 volumes of KCl medium and centrifuged at 10,000 x g for 5 minutes at 4 °C. Serum and the buffy coat were aspirated, and the wash/centrifugation cycle was repeated until the supernatant was clear and the buffy coat was no

longer visible. Cells were resuspended in 20 volumes of KCl medium and, if used in subsequent sugar transport assays, were incubated at room temperature for 30 minutes in order to deplete intracellular sugar levels.

***Erythrocyte Membrane Ghosts.*** Washed red cells were lysed in 40 volumes lysis medium, incubated on ice for 10 minutes, then centrifuged at 22,000 x g for 20 minutes at 4 °C. The supernatant was aspirated, and the lysis/centrifugation/aspiration cycle was repeated. The resulting membranes were resealed by incubation in KCl medium at 37 °C for 60 minutes and harvested by centrifugation at 22,000 x g for 20 minutes at 4 °C.

***Glucose Transport Protein.*** Glucose transport protein was purified from human erythrocytes as described previously (Hebert and Carruthers, 1992). The carrier co-purifies with red cell lipid and is incorporated into unsealed proteoliposomes upon removal of detergent by dialysis (Carruthers, 1986).

***Glucose Carrier Labeling with 8-Azido[$\gamma^{32}$P]ATP.*** Labeling was as described previously (Carruthers and Helgerson, 1989). Briefly, a methanolic solution of 8-azido[$\gamma$-$^{32}$P]ATP is dried under nitrogen and resuspended in 500 $\mu$L Tris medium pH 8.5 (90 $\mu$Ci $^{32}$P, 10 $\mu$M final [azidoATP]). Purified GLUT1 proteoliposomes (500 $\mu$g protein) are sedimented by centrifugation at 14,000 x g for 20 minutes and combined with the methanol-free 8-azido[$\gamma$-$^{32}$P]ATP solution. The suspension is incubated on ice for 30 minutes by which time ATP binding to GLUT1 achieves equilibrium. Samples are placed in a plastic weigh boat on ice and irradiated for 90 seconds at 280 nm in a Rayonet Photochemical Reactor. Following *uv* irradiation (photolabeling), GLUT1 is washed three times to remove unbound 8-azido[$\gamma$-$^{32}$P]ATP by centrifugation (14,000 x g for 15 minutes) and resuspended in 500 $\mu$L Tris medium pH 8.5. Photolabeling of Cos-7 recombinant hGLUT1 is identical to that described above except for the following 1) samples eluted from Ni-NTA were combined directly with the methanol-free 8-azido[$\gamma$-$^{32}$P]ATP at pH 7.4, and 2) irradiation was carried out in separate wells of a plastic ELISA dish on ice. The presence of detergent precludes washing the sample to

remove unbound azidoATP. The effects of pH on azidoATP binding to GLUT1 were examined by varying the pH of the Tris medium over the range 5.5 - 8.5 during photolabeling. $MgCl_2$ was present at 5 mM in all experiments. Photolabeling of GLUT1 was carried out at concentrations of 8-azido[$\gamma$-$^{32}$P]ATP that were lower than the $K_m$ for ATP binding to the transporter. In this instance, the amount of bound 8-azido[$\gamma$-$^{32}$P]ATP is given as:

$$bound = \frac{B_{max}}{K_d}[azidoATP]$$

This method makes it possible to detect changes in both the $B_{max}$ and the $K_d$ for 8-azido[$\gamma$-$^{32}$P]ATP binding to GLUT1.

***Glycoprotein Detection.*** 8-Azido[$\gamma$-$^{32}$P]ATP photolabeled GLUT1 carbohydrate was detected using the Amersham ECL Glycoprotein Detection Kit. Briefly, peptide oligosaccharides are oxidized using 10 mM sodium metaperiodate then labeled using 0.55 mM biotin hydrazide. Subsequent detection of biotinylated glycoprotein employs standard western blotting techniques using streptavidin-horseradish peroxidase (1:5,000) and ECL chemiluminescence.

***Proteolysis of Labeled Carrier.*** 8-Azido[$\gamma$-$^{32}$P]ATP- and/or biotin-labeled GLUT1 was sedimented by centrifugation at 14,000 x g for 20 minutes and resuspended in sample buffer ± chymotrypsin, endoproteinase Lys-C or endoproteinase Glu-C (protein/enzyme 20:1 by weight). Proteolysis was allowed to proceed for 30 minutes at 37 °C and was arrested by addition of phenylmethylsulfonylfluoride (PMSF, 0.2 mM). The resulting GLUT1 fragments were separated by gradient gel electrophoresis (10-20% polyacrylamide SDS gels) and transferred to nitrocellulose membranes for autoradiography and western blotting.

***Polyacrylamide Gel Electrophoresis.*** Proteins were resolved on 10% acrylamide gels as described previously ***(Laemmli, 1970)*** or, where separation of proteolytic fragments was required, gradient gel electrophoresis (10-20% polyacrylamide gradients) was employed.

***Western Blotting.*** Proteolytic fragments containing GLUT1 carboxyl-terminal residues 480–492, GLUT1 N-terminal residues

88-95 and/or a hemagglutinin tag (HA) were detected by western blot analysis. Peptides separated by SDS PAGE were transferred electrophoretically to nitrocellulose or immobilon membranes, which were subsequently blocked for 1 hour in PBS containing 15% Carnation™ nonfat dry milk. Following three five-minute washes in PBS, membranes were incubated for 1 hour in primary antibody (C-Ab and N-Ab) diluted 1:1000 or (HA-ab) 1:400 in PBS containing 2% nonfat dry milk. Following three wash cycles to remove primary antibody, membranes were exposed for 45 minutes to secondary antibody (protein-A or goat anti-mouse Horseradish Peroxidase conjugate) diluted 1:3000 in PBS containing 2% nonfat dry milk. Detection of antigen/antibody/protein-A complexes was by chemiluminescence using Amersham ECL reagents.

***Protein Sequencing***. 8-Azido[$\gamma$-$^{32}$P]ATP-labeled GLUT1 (100 $\mu$g) was sedimented by centrifugation at 14,000 x g for 20 minutes then resuspended in 0.1% SDS/Tris-HCl pH 7.4 ± 5 $\mu$g endoproteinase Lys-C. Digestion was carried out for 18-24 hours at 4 °C. GLUT1 fragments were separated using 1 mM thioglycolate pre-equilibrated

gradient gels. Peptides were transferred electrophoretically to PVDF membranes and following detection by autoradiography and Coomassie blue staining, bands of interest were excised from the membrane, washed extensively in distilled water and subjected to microsequence analysis by Dr. John Leczyk of the University of Massachusetts Medical School Protein Chemistry facility.

***Net 3-0-Methylglucose Uptake.*** Sugar-depleted cells or ghosts at ice temperature were exposed to 5 volumes of ice cold KCl medium containing [$^3$H]-3-*O*-methylglucose and variable concentrations of unlabeled 3-*O*-methylglucose. Uptake was measured over intervals of 15 seconds to 1 minute, then 50 volumes (relative to cell volume) of stopper solution were added to the cell/ghost suspension. Cells/ghosts were sedimented by centrifugation (14,000 x g for 30 seconds), washed once in stopper, collected by centrifugation, and extracted in 500 $\mu$L of 3% perchloric acid. The acid extract was centrifuged, and duplicate samples of the clear supernatant were counted. Zero-time uptake points were prepared by addition of stopper to cells/ghosts prior to addition of medium containing

sugar and radiolabel. Radioactivity associated with cells/ghosts at zero-time was subtracted from the activity associated with cells/ghosts following the uptake period. All uptakes were normalized to equilibrium uptake where cells/ghosts were exposed to sugar medium at 37 °C for 60 minutes prior to addition of stopper. Uptake assays were performed using solutions and tubes pre-equilibrated to 4 °C.

***Effects of Glycolytic Intermediates on 3-0-Methylglucose Uptake.*** Glycolytic intermediates were introduced into human erythrocyte ghosts by addition to the ice-cold resealing medium prior to membrane resealing. The membranes were warmed to 37 °C for 60 min then resealed ghosts were harvested by centrifugation (22,000 x g for 20 minutes at 4 °C), and washed once in 50 volumes KCl medium to remove extracellular glycolytic intermediates. Ghosts were then processed for 3-*O*-methylglucose uptake measurements as described above.

***Effects of pH on ATP-modulation of 3-O-methylglucose uptake.*** Membrane ghosts were resealed in KCl medium containing 5 mM $MgCl_2$, varying [ATP] and adjusted to a pH ranging from 5.5 to 8.0. All other solutions (uptake media and stopper) were also adjusted to the same pH. Ghosts were then processed for 3-*O*-methylglucose uptake measurements as described above.

***Reconstituted GLUT1-mediated sugar transport.*** EggPC (40 mg in hexane) and cholesterol (10 mg) (≈ 80:20 molar ratio) were dissolved in hexane. The organic phase was evaporated under a stream of $N_2$ and remaining trace quantities of hexane removed *in vacuo* for 3 hr. The resulting lipid film was dissolved in Tris medium containing 5% (by weight) sodium cholate and mixed with 100 μg GLUT1 solubilized in 50 mM sodium cholate. This mixture (4 mL) was dialyzed overnight against 6L detergent-free Tris medium and the resulting suspension of small unilamellar proteoliposomes distributed into 0.5 mL fractions in microcentrifuge tubes. Each tube was frozen in a dry ice/isopropyl alcohol mixture, thawed at room temperature then subjected to 2 additional rounds of

freeze/thawing. The resulting large unilammelar proteoliposomes (mean diameter = 2.5 $\mu$m by oil immersion phase contrast microscopy) sedimented readily at 14,000 x g and were used for sugar transport determinations at 24 °C. 3OMG uptake (0.05 mM) was measured into 100 $\mu$L proteoliposome suspension (2 $\mu$g GLUT1) at 0, 10, 20, 30 and 60 sec in Tris medium ± 10 $\mu$M CCB (a transport inhibitor; $K_{i(app)}$ = 0.2 $\mu$M). Equilibrium uptake was measured by incubation for 2 hr at 24 °C. Uptake was arrested by addition of 1 mL ice cold Tris medium containing 10 $\mu$M CCB. Proteoliposomes were sedimented by centrifugation at 14,000 x g for 5 min at 4 °C, the supernatant was aspirated and the liposomes were washed again in Tris medium containing CCB. Uptake, v, was computed as

$$v = [3OMG]\frac{(cpm_t - cpm_0)}{(cpm_\infty - cpm_0)t}$$

where t is time, $cpm_0$, $cpm_t$, and $cpm_\infty$ correspond to radioactivity associated with proteoliposomes at time zero, time t and at equilibrium.

***Tissue Culture.*** Cos-7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal

bovine serum (FBS), 100 units/mL penicillin, and 100 ug/mL streptomycin in a 37° C humidified 5% $CO_2$ incubator.

*Construction of Wild Type & Mutant GLUT1-HA $His_6$.* A 1.7 Kbp fragment of GLUT1 cDNA was previously subcloned into the BamH1 cloning site of pGEM3Z (pGEM3Z-GT1) (Zottola, 1995). Site directed mutagenesis via a Stratagene QuickChange$^{tm}$ Kit was used to introduce a unique Mfe1 restriction site at residue 1685 in pGEM3Z-GT1. An HA-$His_6$ epitope tag was inserted into the coding region of GLUT1 at this location. The presence and orientation of the HA $His_6$ tag in the GLUT1 coding region was confirmed by sequencing. The tagged GLUT1 was then further subcloned into the BamH1 site of the pcDNA3.1+ mammalian expression vector. Scanning alanine mutagenesis was undertaken through the three conserved ATP binding domains found in GLUT1. Mutants were again engineered with the Stratagene QuickChange$^{tm}$ Kit, and their presence was confirmed by sequencing.

***Transient Expression of Wild-type and Mutant GLUT in Cos-7 cells.*** Subconfluent Cos-7 cells were transfected with wild type and mutant GLUT1 HA 6His cDNA using the Fugene 6 transfection reagent system. Following transfection, cells were maintained in DMEM for 24 hours, at which time 0.72 mM G418 (Geneticin) was added to the media and the cells were allowed to continue growing for an additional 48 hours. For harvesting, 6 culture plates of cells (150 mm) were washed twice with PBS + 5 mM EDTA, then scraped into 20 mL lysis buffer mixed with 1 mM eukaryotic cell protease inhibitor cocktail, and PMSF. Membranes were prepared according to Loo & Clark, 1993 (Loo et al., 1993). Briefly, cells were homogenized for five minutes with a Fisher Dyna-Mix, then spun at 5000 x g for 10 minutes at 4°C. The supernatant was decanted and centrifuged at 100,000 x g for 1 hour to pellet the crude membrane fraction. Membranes were resuspended in 300 µL of Buffer A with 1 mM protease inhibitor cocktail and frozen at -70°C until use.

***Purification of Wildtype and Mutant GLUT1.*** Purification of recombinant GLUT1 HA $His_6$ was described in Loo and Clark, 1995

(Loo and Clarke, 1995). Briefly, extracts were solubilized in 1.7 mL solubilization buffer (Buffer A + 1% N-dodecyl β-D maltoside) then spun at 15,000 x g for 15 minutes to remove insoluble material. The supernatant was allowed to react for 1 hour at 4°C with 2 mls of Ni-NTA column media which was pre-equilibrated with 4 mL buffer B (buffer A + 0.1% N-dodecyl β-D maltoside). The column was washed twice with 8 mL buffer B, and twice with 8 mL buffer C. Samples were eluted from the column with 1.5 mL elution buffer and concentrated in centricon-10 concentrators.

***D-glucose Uptake in Cos-7 Cells with Wild Type & Mutant GLUT1-HA 6His.*** Subconfluent Cos-7 cells in 12 well culture dishes were transfected with wild-type and mutant GLUT1-HA $His_6$ as described previously. D-glucose uptake was measured at 72 hours post transfection. Prior to uptake, cells were serum starved in DME for 2 hours at 37°C. Next, cells were washed twice with KRP then incubated at 37 °C for 15 minutes in KRP +/- 10 μM CCB. Cells were exposed to 400 μL [$^3$H]2-deoxyglucose diluted 1:40 in KRP (10 μCi)

containing 0.1 mM unlabeled 2-deoxyglucose. Uptake was measured at various times, from 5 minutes to 30 minutes and stopped by washing twice with 1 mL ice cold KRP. Duplicate samples were counted following extraction with 450 µl 0.1% Triton-X100. Time zero uptake points were prepared by adding ice cold KRP to each well before the addition of medium containing sugar and radiolabel. Radioactivity associated with cell extracts at time zero was subtracted from the activity associated with cell extracts following the uptake period. All uptakes were normalized for protein. Uptake assays were performed using solutions and dishes pre-equilibrated to 37 °C.

***δ-Antibody Binding.*** Cos-7 cells were grown and transfected in 12 well culture dishes as previously described. δ-antibody binding was measured 72 hours post transfection. δ-antisera reacts exclusively with exofacial epitopes of tetrameric GLUT1. Unless noted, all solutions were pre-warmed to 37°C. Cells were incubated for 1 hour at 37°C with sheep δ-antibody diluted 1:1000 in DMEM +10% fetal bovine serum. Following two washes in KRP pH 7.4, cells were

incubated for 30 minutes with [$^{125}$ I]-protein G diluted 1:1200 in KRP (protein G recognizes and interacts with antibodies raised in sheep). After two additional washes in KRP, duplicate samples were counted following extraction in 500 μL of 1% triton in KRP pH 7.4. Protein G binding to Cos-7 cells in the absence of primary antibody was used to quantitate background radioactivity and was subtracted from each sample. All measurements were normalized to the quantity of protein present in each well.

***Cytochalasin B Binding.*** Membranes from Cos-7 cells transfected with GLUT1 HA $His_6$ and mutant 338A were isolated as described previously. Samples were resuspended in 700 μL 6.25 μM [$^3$H]-cytochalasin B (CCB), 100 μM phloretin, and 10 μM cytochalasin D in PBS at pH 7.4 and allowed to equilibrate on ice for 10 minutes. This suspension was placed in a plastic weigh boat on ice and irradiated for 4 minutes at 280 nM in a Rayonet Photochemical Reactor. Following UV irradiation membranes were washed with PBS to remove unbound CCB and sedimented by centrifugation (100,000 x g for 15 min). Proteins were resolved on 10% SDS PAGE

gels and stained for protein or transferred to nitrocellulose for C-Ab and HA-Ab western blotting. Gel slices (0.2 cm) were extracted from stained gels and incubated overnight at 50° C in scintillation fluid containing 300 μL 30% $H_2O_2$ before being counted. All samples were corrected for protein concentration.

***Calculation of $Mg^{2+}$ and ATP levels.*** In some GLUT1 photolabeling experiments, pH and [ATP] were varied systematically at fixed EGTA (4 mM) and $MgCl_2$ (5 mM) levels. Exogenous $CaCl_2$ was not added, thus total Ca levels were limited to Ca-contamination (approx. 15 $\mu$M; (Helgerson et al., 1989)). Computation of $Mg^{2+}$, $Mg_2$.ATP, $Mg_2$H.ATP, ATP, $Mg_2$.EGTA, $Mg_2$HEGTA and EGTA levels was carried out using the WinMaxC program (Bers et al., 1994) and equilibrium constants cited by Smith and Martell (Martell and Smith, 1974) for $H^+$, $Ca^{2+}$, $Mg^{2+}$ and $K^+$ interaction with EGTA and ATP adjusted for pH and temperature (4 °C). The apparent dissociation constants (-log) calculated for EGTA and ATP at pH 5.5 are: $K_{EGTACa}$ = 2.91, $K_{EGTAMg}$ = 2.91, $K_{ATPCa}$ = 3.30, $K_{ATPMg}$ = -0.31; at pH 6 are: $K_{EGTACa}$ = 3.32, $K_{EGTAMg}$ = 3.33, $K_{ATPCa}$ = 4.30, $K_{ATPMg}$ = 0.19; at pH 7

are: $K_{EGTACa}$ = 3.88, $K_{EGTAMg}$ = 3.89, $K_{ATPCa}$ = 6.29, $K_{ATPMg}$ = 1.22; at pH 8 are: $K_{EGTACa}$ = 4.02, $K_{EGTAMg}$ = 4.03, $K_{ATPCa}$ = 8.27, $K_{ATPMg}$ = 2.39 and at pH 8.5 are: $K_{EGTACa}$ = 4.03, $K_{EGTAMg}$ = 4.04, $K_{ATPCa}$ = 9.22, $K_{ATPMg}$ = 3.10. Our calculations confirm that $Mg^{2+}$ levels decline linearly over the pH range 5.5 to 8.5 (from 2.4 to 0.5 mM) due to increasing chelation by EGTA and variable association with ATP. $Mg_2ATP$ plus $Mg_2HATP$ levels increase from 2.85 mM at pH 5.5 to a peak of 3.5 mM at pH 7.0 and thereafter decline to 3.2 mM at pH 8.5. Free ATP levels are greatest at pH 5.5 (1.4 mM), are lowest at pH 7 (0.4 mM) and thereafter increase to 0.8 mM at pH 8.5.

***Computation of transport and labeling constants.*** In some experiments, transport rates and GLUT1 labeling by ATP were shown to change in a nonlinear (saturable) manner with increasing [ATP] (e.g. Fig. 3). In these instances, best fit curves were generated by nonlinear regression using the software package KaleidaGraph™ 3.08d (Synergy Software, Reading, PA) and assuming the following relationships:

Fig 3A pH 8, uptake = $k1+\frac{k2[ATP]}{K_{dATP}+[ATP]}$ where k1 = basal transport in the absence of ATP, k2 is the increment in transport produced by saturating ATP levels and $K_{dATP}$ is the apparent $K_d$ for ATP interaction with the transport system.

Fig 3A pH 5.5, uptake = $k1+\frac{k2[ATP]}{K_{dATP1}+[ATP]}-\frac{k3[ATP]}{K_{dATP2}+[ATP]}$ where k1, k2 and $K_{dATP1}$ correspond to those parameters for Fig 3A pH 8 but where k3 and $K_{dATP2}$ describe an additional interaction with ATP that reduces net uptake.

Fig 3B pH 5.5 and 8.0, labeling = $k4-\frac{k5[ATP]}{K_{iATP}+[ATP]}$ where k4 is labeling in the absence of unlabeled ATP, k5 is the decrease in labeling produced by saturating ATP levels and $K_{iATP}$ is the concentration of ATP that inhibits labeling half-maximally.

# Chapter III

## Factors Influencing ATP Modulation of Erythrocyte Sugar Transport

The ATP-sensitivity of human erythrocyte sugar transport suggests that regulation of glycolysis and sugar transport in nucleated erythrocytes (Wood and Morgan, 1969; Whitfield and Morgan, 1983; Diamond and Carruthers, 1993) and cardiac tissue (Mansford, 1968; Haworth and Berkoff, 1986) is coordinated through a common regulatory principle. The major site of glycolytic regulation is phosphofructokinase (PFK) which catalyzes the committed step in glycolysis. PFK is subject to allosteric inhibition by ATP and by citrate and to stimulation by fructose-2,6-bisphosphate and by AMP and ADP (Carpenter and Hand, 1986). Pyruvate kinase, the enzyme controlling the outflow from glycolysis is subject to allosteric inhibition by ATP and to stimulation by fructose-1,6-bisphosphate (Waygood et al., 1976; Rosevear et al., 1987).

Work in this laboratory has shown that ATP is an allosteric inhibitor of human erythrocyte GLUT1 (Carruthers and Helgerson,

1989; Helgerson et al., 1989). The net effect of ATP on 30MG uptake in red blood cell ghosts at subsaturating sugar levels (50-100 μM) is to stimulate transport. In contrast, transport at saturating sugar levels (>100 μM) is inhibited (Helgerson et al., 1989).

The sugar transport experiments that defined this ATP effect were measured in red blood cell ghosts (human red blood cells which have been hypotonically lysed) or inside out red blood cell vesicles (IOV's) resealed with and without 4 mM ATP at pH 7.4 (Helgerson et al., 1989). Under these circumstances, normal parameters such as pH and cellular redox state are artificially controlled. This is not however the case under normal physiologic conditions. For example, sugar transport in cardiac and skeletal muscle as well as nucleated erythrocytes responds to external stimuli such as muscle contraction, anoxia, and inhibition of oxidative metabolism (Cheung et al., 1977; Simons, 1983; Whitfield and Morgan, 1983; Haworth and Berkoff, 1986; Hansen et al., 1995; Lee et al., 1995). We were concerned that our experimental system lacked the types of intercellular communication (i.e. local

environmental conditions) that might exist in intact tissues and organs. This led us to ask 1) What metabolic factors affect ATP modulation of GLUT1? 2) Does the oligomeric structure of GLUT1 contribute to the ATP effect?

To answer these questions, glycolytic intermediates, oligomeric structure, and changing pH were tested for their ability to alter 30MG uptake into red blood cell ghosts resealed with 4 mM ATP. In addition, these factors were examined for their capacity to affect azidoATP photolabeling of purified GLUT1. Our findings suggest that intracellular AMP and acidification antagonize ATP regulation of sugar transport, and that ATP interaction with GLUT1 is sufficient for transport modulation. Our findings also support the hypothesis that ATP regulates only tetrameric GLUT1.

## *Results*

### *Glycolytic Intermediates*

Glycolysis and GLUT1-mediated transport appear to be regulated coordinately in certain tissues (Cloherty et al., 1996). We wanted to investigate whether GLUT1-mediated sugar transport might be

subject to regulatory feedback mechanisms similar to those found in the glycolytic pathway.

Intracellular (but not extracellular; see (Helgerson et al., 1989) Mg.ATP converts 3OMG transport by resealed erythrocyte ghosts from a high capacity, low affinity transport system ($V_{max}$ =1069 ± 84 $\mu$M/min, $K_{m(app)}$ = 12.3 ± 0.8 mM) to a low capacity, high affinity system ($V_{max}$ = 220 ± 19 $\mu$M/min, $K_{m(app)}$ = 386 ± 24 $\mu$M; n=4). The net effect of ATP is to increase 3OMG uptake at 0.1 mM sugar by 2 to 5 fold (see also (Carruthers and Helgerson, 1989)). We measured 3OMG uptake at 0.1 mM sugar to determine how resealing erythrocyte ghosts with or without glycolytic intermediates affects basal and ATP-modulated sugar transport.

Table 1 shows that intracellular AMP (2 mM) inhibits ATP-modulation of transport. Extracellular AMP has no measurable effect on basal or ATP-modulated 3OMG transport. This result is not unexpected because previous work in this laboratory has shown that $AMP_i$, though unable to mimic the action of $ATP_i$ on transport, competes with $ATP_i$ for access to the transport system (Carruthers and Helgerson, 1989). Resealing ghosts with 2 mM ADP stimulates

**Table 1: Effects of glycolytic intermediates on erythrocyte 3OMG uptake**

| | 3OMG uptake in μmol/L cell water/min | |
|---|---|---|
| [1]Addition | 0 Mg.ATP | 4 mM Mg.ATP |
| no addition | 19 ± 2 | 49 ± 3 |
| AMP (2 mM) | 4 ± 1 | 7 ± 1 |
| ADP ( 2 mM) | 39 ± 3 | no Δ |
| NAD (2 mM) | [2]no Δ | no Δ |
| NADH (2 mM) | no Δ | no Δ |
| Pyruvate (4 mM) | no Δ | no Δ |
| Citrate (2 mM) | no Δ | no Δ |
| Lactate (2 mM) | no Δ | no Δ |
| Fructose 1,6 bisphosphate (4 mM) | no Δ | no Δ |
| Fructose 2,6 bisphosphate (4 mM) | no Δ | no Δ |
| $Na_2HPO_4$ (4 mM) | no Δ | no Δ |
| 2,3-Diphosphoglycerate (2 mM) | no Δ | no Δ |
| Phosphocreatine (1 mM) | no Δ | no Δ |
| Phosphoenolpyruvate (0.1 mM) | no Δ | no Δ |
| Fructose-6-phosphate (0.1 mM) | no Δ | no Δ |
| Glucose-6-phosphate (0.1 mM) | no Δ | no Δ |
| Glyceraldehyde-3-phosphate (0.1 mM) | no Δ | no Δ |
| 2-Phosphoglycerate (0.1 mM) | no Δ | no Δ |
| Adenosine (50 μM) | no Δ | no Δ |

[1]Erythrocyte ghosts were resealed in KCl medium containing 5 mM $MgCl_2$ plus the addition shown and adjusted to pH 7.4.

[2]no Δ indicates that there is no significant difference between the test measurement and the measurement observed with no addition. The number of measurements per condition was 3 or more. Results are shown as mean ± SEM.

basal transport. This result was unexpected because ADP, like AMP, competes with ATP for binding to the transporter. We hypothesize that residual adenylate kinase present in red cell ghosts converts ADP to ATP causing transport stimulation (Carruthers and Helgerson, 1989). The remaining glycolytic intermediates and ATP analogs (including adenosine) are without effect on basal or ATP-modulated transport whether applied at the cytoplasmic or extracellular (not shown) surface of the erythrocyte membrane.

*pH*

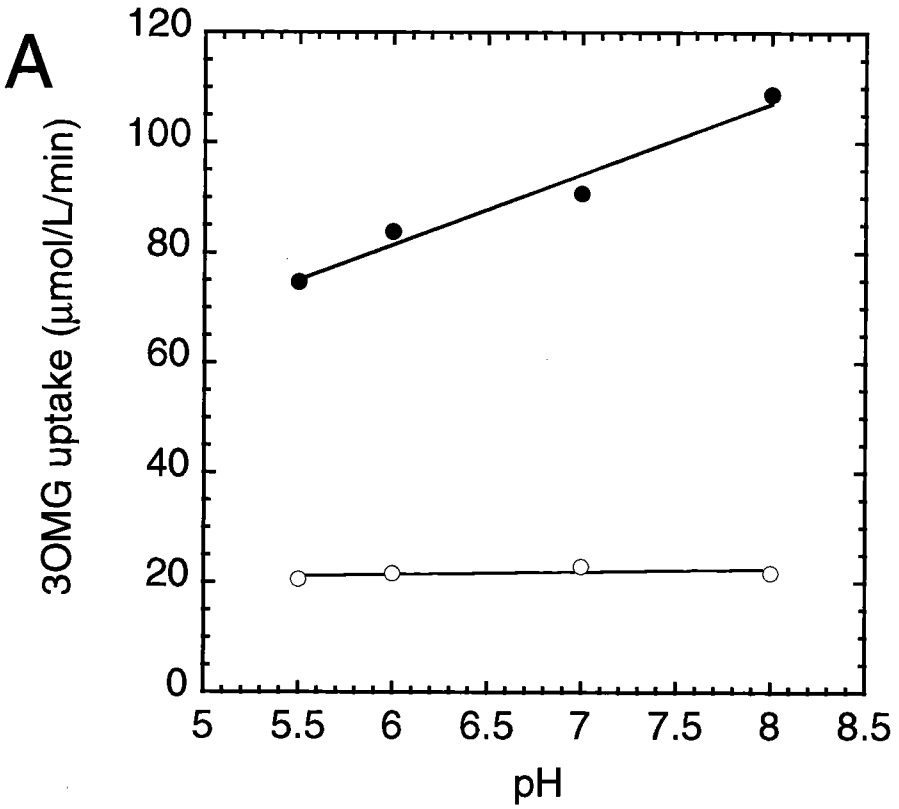
The experiments describing effects of glycolytic intermediates on basal and ATP-modulated 3OMG uptake were performed at pH 7.4. Under physiologic conditions in muscle and erythrocytes, increased glycolysis elevates cellular lactate levels resulting in acidosis (Cairns et al., 1993). We therefore examined the ability of altered $pH_i$ to modulate ATP-regulation of GLUT1-mediated sugar transport. Red blood cell ghosts were resealed in KCl medium containing or lacking 4 mM Mg.ATP adjusted to pH 5.5, 6.0, 7.0 or 8.0. Both extra- and intracellular media were adjusted to the same pH to avoid pH

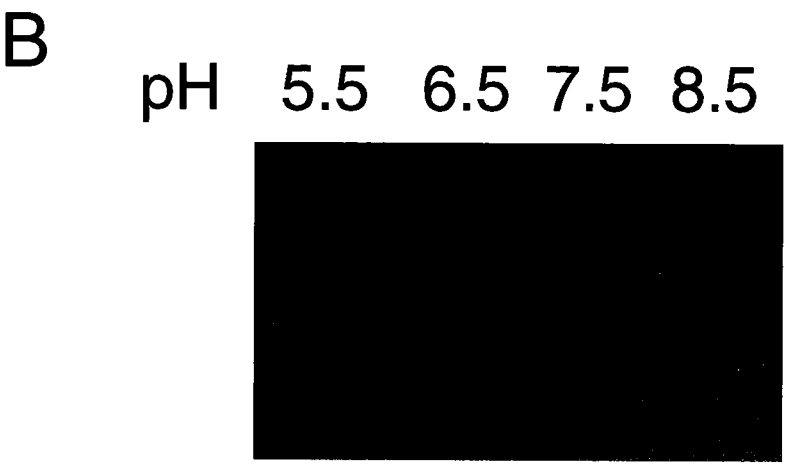
changes due to transmembrane proton flow. Earlier studies have demonstrated that the extracellular sugar uptake site is remarkably insensitive to alterations in external pH (Sen and Widdas, 1962). KCl medium was employed in both extra- and intracellular media to avoid transmembrane KCl gradients and $K^+$ transport-induced volume changes.

Figure 2A shows that as pH decreases from 8.0 to 5.5, ATP modulation of 100 $\mu$M 3OMG uptake is reduced. Subsequent experiments (see Fig 3A) show even stronger inhibition of ATP-modulated transport. Basal transport rates are unaffected. Calculation of free Mg, free ATP and Mg.ATP levels indicates that this inhibition is unrelated to systematic (pH-dependent) alterations in the ionization state of ATP (see Materials and Methods). The loss of ATP-modulation of transport at lower pH could result from altered binding kinetics (affinity or capacity of GLUT1 for ATP) or from the pH dependent binding of a regulatory molecule to the ATP-GLUT1 complex. To examine these possibilities, purified GLUT1 was photolabeled at pH 5.5, 6.5, 7.5, and 8.5 with 10$\mu$M azidoATP. Previous studies from this laboratory have demonstrated that

***Figure 2.*** **Effects of pH on ATP-modulation of sugar transport (A) and GLUT1-labeling by azido-[$\gamma$-$^{32}$P]ATP (B). A** Erythrocytes were lyzed and resealed in KCl medium containing (●) or lacking (○) 2 mM exogenous ATP. The pH of the resealing and extracellular media is shown on the abscissa. Ordinate: initial rates of 100 $\mu$M 3OMG uptake ($\mu$mol/L cell water/ min). These results summarize triplicate measurements. Lines drawn through the data points were computed by the method of least squares. **B** Purified GLUT1 was incubated in Tris-HCl medium containing 10 $\mu$M azido-[$\gamma$-$^{32}$P]ATP at the pH indicated for 15 minutes at 4 ˚C. Each sample was *uv* irradiated for 1 min and 30 $\mu$g of labeled protein resolved on 10% SDS PAGE minigels. Proteins were transferred to nitrocellulose and labeled bands containing azido[$\gamma$-$^{32}$P]ATP were identified by autoradiography.

Figure 2


A
120
100
80
60
40
20
0
3OMG uptake (µmol/L/min)
5
5.5
6
6.5
7
7.5
8
8.5
pH


B
pH 5.5 6.5 7.5 8.5

GLUT1 and azidoATP associate reversibly to form a binary complex and that azidoATP binding to GLUT1 is inhibited competitively by ATP, ADP, and AMP (Carruthers and Helgerson, 1989). Proteins were resolved on minigels and labeling was quantitated by autoradiography. Figure 2B shows that contrary to our expectations, ATP incorporation into GLUT1 is *reduced* as the pH increases from 5.5 to 8.0.

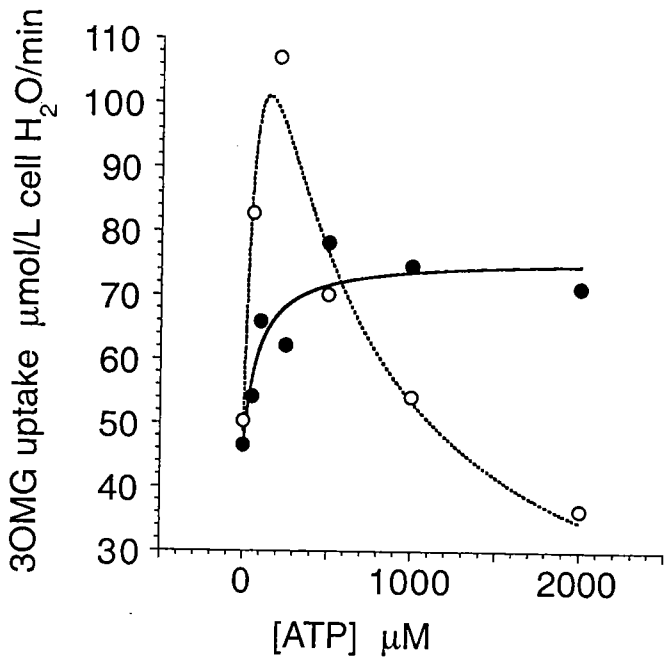
To better understand this response to pH, we measured the ATP-dependence of 30MG uptake in resealed red cell ghosts at pH 5.5 and 8.0. Figure 3A shows that 100$\mu$M 30MG uptake increases in a saturable manner with ATP concentration at pH 8.0. At pH 5.5, however, transport is maximally stimulated at 200 $\mu$M ATP, but is inhibited at 2 mM ATP. Figure 3B summarizes three separate experiments in which azido[$\gamma$-$^{32}$P]ATP labeling of GLUT1 was carried out at pH 5.5 and 8.0 in the presence of increasing concentrations of unlabeled ATP. ATP inhibition of GLUT1 labeling by azidoATP shows simple competition kinetics at both pH 5.5 and 8.0 but maximum labeling by azidoATP and $K_{i(app)}$ for ATP inhibition of labeling are reduced at pH 8.0. The ATP content of erythrocyte ghosts resealed

***Figure 3.*** **Effects of pH on the concentration-dependence of ATP-modulation of sugar transport (A) and ATP-inhibition of GLUT1-labeling by azido-[$\gamma^{32}$P]ATP (B). A**. Erythrocytes were lysed and resealed in KCl medium containing 5 mM $MgCl_2$ plus increasing concentrations of ATP at pH 8.0 (●) or at pH 5.5 (○). Ordinate: initial rates of 100 $\mu$M 3OMG uptake ($\mu$mol/L cell water/ min). Abscissa: ATP content of the resealing medium in $\mu$M. The results summarize three separate experiments each made in triplicate. Lines drawn through the data points were computed by nonlinear regression (see Materials and Methods). These analyses assume that ATP-modulation of transport at pH 8 is comprised of basal transport (*k1* seen at 0 ATP) plus a simple, ATP-dependent Michaelis-Menten component where *k2* is the increment in transport produced by saturating ATP levels and $K_{dATP}$ is the apparent $K_d$ for ATP interaction with the transport system. The computed constants are: *k1* = 46 $\mu$M/min, *k2* = 30 $\mu$M/min; $K_{dATP}$ = 89 $\mu$M ATP. ATP-modulation of transport at pH 5.5 is similar to that at pH 8 but is characterized by an additional interaction with ATP that reduces net uptake where *k3* is the decrement in transport produced by saturating ATP levels and $K_{dATP2}$ is the apparent $K_i$ for ATP inhibition of transport. The computed constants are: *k1* = 48 $\mu$M/min, *k2* = 259 $\mu$M/min; $K_{dATP1}$ = 189 $\mu$M ATP, *k3* = 259 $\mu$M/min; $K_{dATP2}$ = 199 $\mu$M ATP. **B**. ATP-inhibition of GLUT1-photolabeling by azido-[$\gamma$–$^{32}$P]ATP at pH 5.5 (○) and pH 8.0 (●). Purified GLUT1 was incubated for 15 minutes at 4 °C in Tris-HCl medium containing 5 mM $MgCl_2$, 15 $\mu$M azido-

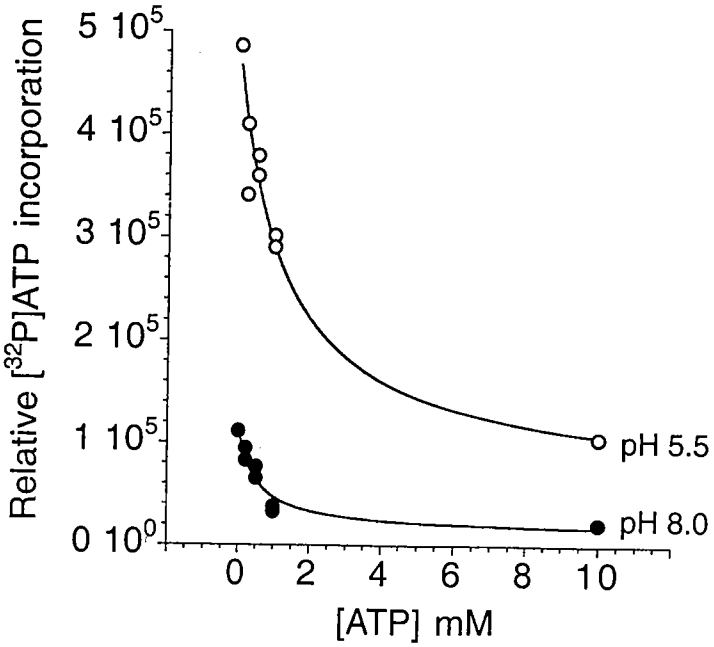
[$\gamma$-$^{32}$P]ATP at pH 5.5 or pH 8.0 in the presence of increasing concentrations of ATP. Each sample was *uv* irradiated for 1 min and 30 $\mu$g of labeled protein resolved on 10% SDS PAGE minigels. Peptides were transferred to nitrocellulose and labeled bands containing [$\gamma$-$^{32}$P]ATP were identified by autoradiography. Ordinate: relative [$\gamma$-$^{32}$P]ATP incorporation into GLUT1 as judged by densitometric analysis of resulting autoradiograms. Abscissa: [ATP] present during photolabeling (mM). These data summarize 2 separate experiments. Lines drawn through the points were computed by nonlinear regression (see Materials and Methods). This analysis assumes that *k4* is the extent of labeling in the absence of ATP and that ATP competitively inhibits labeling (*k5* is the decrease in labeling produced by saturating ATP levels and $K_{iATP}$ is the concentration of ATP that inhibits labeling half-maximally). The computed constants are: pH 8.0, $k4 = (1.1 \pm 0.1) \times 10^5$, $k5 = (1.0 \pm 0.1) \times 10^5$, $K_{iATP} = 0.5 \pm 0.2$ mM ATP; pH 5.5, $k4 = (4.7 \pm 0.3) \times 10^5$, $k5 = (4.1 \pm 0.5) \times 10^5$, $K_{iATP} = 1.3 \pm 0.5$ mM ATP.

Figure 3

A


3OMG uptake µmol/L cell $H_2O$/min
110
100
90
80
70
60
50
40
30
0
1000
2000
[ATP] µM

B


Relative [$^{32}$P]ATP incorporation
5 $10^5$
4 $10^5$
3 $10^5$
2 $10^5$
1 $10^5$
0 $10^0$
pH 5.5
pH 8.0
0
2
4
6
8
10
[ATP] mM

with 4 mM ATP at pH 5.5 or 8.0 is not significantly different (pH 5.5, [ATP] = 3.1 mM; pH 8.0, [ATP] = 3.1 mM; ATP content of ghosts resealed in the absence of exogenous ATP: pH 5.5, [ATP] = 0.07 mM; pH 8.0, [ATP] = 0.069 mM).

*Oligomeric Structure*

Erythrocyte-resident GLUT1 exists as a homotetramer but dissociates to form a homodimer upon erythrocyte exposure to extracellular reductant (Hebert and Carruthers, 1992; Zottola et al., 1995). In the present study we ask: does ATP modulate both dimeric and tetrameric GLUT1-mediated sugar transport? Table 2 shows that erythrocyte ghost exposure to reductant (2 mM DTT) inhibits ATP-modulation of 3OMG uptake but is without effect on basal uptake measured in the absence of ATP. Reduced capacity to modulate GLUT1-mediated sugar transport in reductant-treated erythrocyte ghosts could result from reduced ATP binding to GLUT1 or from altered interaction of ATP-liganded GLUT1 with regulatory cellular components. Figure 4 shows that incorporation of 8-azido[$\gamma$-$^{32}$P]ATP into reduced GLUT1 is significantly impaired.

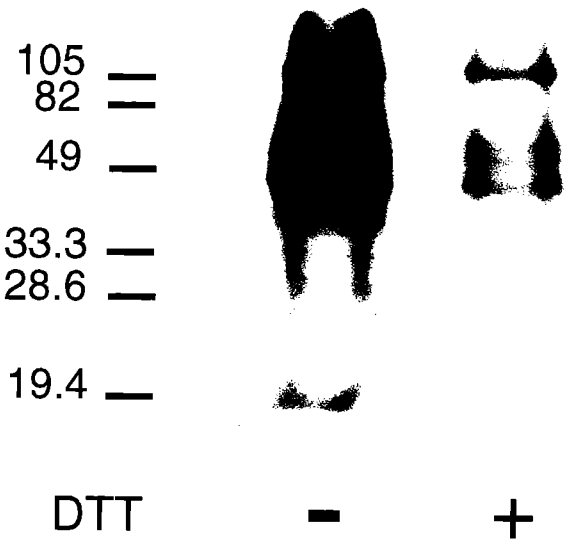
**Table 2: Effect of reductant on ATP-modulation of erythrocyte 3OMG uptake**

| Condition | 3OMG uptake<br>μmol/L cell water/min |
|---|---|
| Control | 41.2 ± 7.4 |
| 2 mM DTT | 57.3 ± 7.1 |
| 2 mM ATP | 97.7 ± 11.6 |
| 2 mM ATP + 2 mM DTT | 48.1 ± 3.4 |

Hypotonically lyzed erythrocytes were resealed ± 2 mM ATP then incubated ± 2 mM DTT for 30 min at 37 °C. 3OMG uptake at 100 μM 3OMG was measured at 4 °C. The table summarizes results as mean ± SEM of 3 separate experiments made in triplicate.

***Figure 4.*** **Azido-[$\gamma$-$^{32}$P]ATP labeling of dimeric and tetrameric GLUT1.** Purified GLUT1 was incubated in Tris-HCl medium + 5 mM $MgCl_2$ ± 4 mM DTT for 30 minutes at 4 °C. Following labeling with azidoATP, 30 $\mu$g samples of protein were resolved on 10% SDS PAGE minigels. Peptides were transferred to nitrocellulose and bands containing [$\gamma$-$^{32}$P]ATP were identified by autoradiography. The presence (+) or omission (-) of DTT treatment is indicated above each lane.

Figure 4



*Does ATP modulate GLUT1 mediated sugar transport directly?*

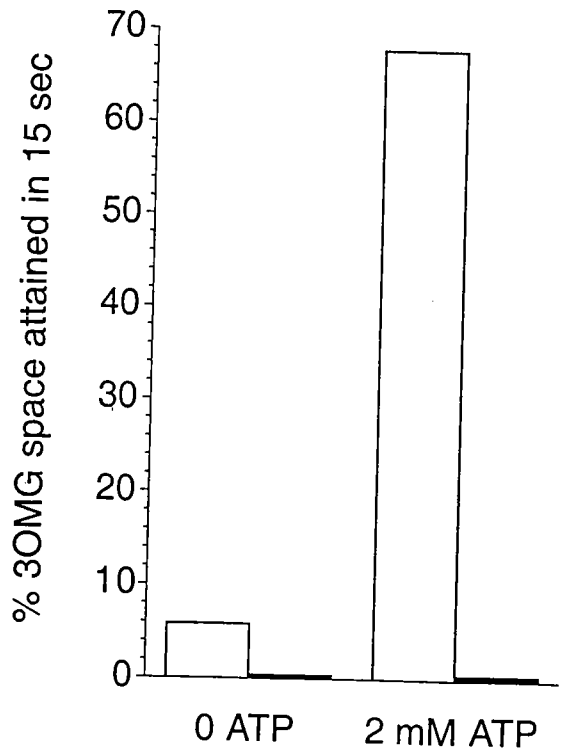
Reconstitution of purified erythrocyte GLUT1 into liposomes (EggPC and cholesterol in an 80:20 molar ratio) containing and lacking 2 mM Mg.ATP provides an in-vitro system that consists solely of GLUT1, ATP, and the lipid bilayer. Inhibition of sugar transport into GLUT1 proteoliposomes following treatment with cytochalasin B (10 μM CCB) insures that we are measuring protein mediated solute movement across the lipid bilayer as opposed to non-specific leakage. Cytochalasin B (CCB) inhibitable 3-*O*-methylglucose uptake by reconstituted GLUT1 proteoliposomes is robustly stimulated by 2 mM Mg.ATP (Figure 5).

## *Discussion*

We examined whether ATP regulation of transport in resealed ghosts is subject to modulation by glycolytic intermediates known to regulate glycolysis. Only AMP antagonizes the action of ATP on 3OMG uptake. Citrate, fructose-1,6-bisphosphate and fructose-2,6-bisphosphate (key regulators of PFK or pyruvate kinase) are without effect on basal or ATP stimulated sugar uptake. Other intermediates

***Figure 5.*** **ATP stimulates 3OMG uptake into reconstituted, purified GLUT1 proteoliposomes.** GLUT1 (100 $\mu$g) was reconstituted into eggPC:cholesterol proteoliposomes and uptake of 50 $\mu$M 3OMG was measured at 20 ˚C in the presence of 5 mM $MgCl_2$ ± 2 mM ATP, and in the presence or absence of 20 $\mu$M cytochalasin B (CCB). Ordinate: 3OMG uptake expressed as % of the equilibrium 3OMG space of proteoliposomes accessed during 15 sec of uptake; Abscissa: [ATP] of medium during reconstitution and uptake determination (0 or 2 mM ATP). Uptake in the presence of CCB is shown by the solid bars. This figure is representative of three separate experiments.

Figure 5



were similarly inactive. Adenosine also failed to modulate GLUT1 mediated sugar transport. This suggests that ATP is not interacting with the adenosine receptor, nor is the adenine ring responsible for the effects of ATP on GLUT1. ADP, however, mimics the ability of ATP to modulate transport. These effects of AMP and ADP on sugar transport in erythrocyte ghosts are similar to those reported previously (Carruthers and Helgerson, 1989). External application of ADP inhibits ATP modulation of sugar efflux from inside out erythrocyte membranes vesicles and, like AMP, antagonizes the action of ATP which reduces C-Ab binding to purified GLUT1 (Carruthers and Helgerson, 1989). This suggests that residual adenylate kinase present in erythrocyte ghosts converts ADP to ATP. This hypothesis is supported by ATP assays of ADP-loaded ghosts (Carruthers and Helgerson, 1989). We propose, therefore, that ATP regulation of sugar transport is subject to inhibition by AMP and possibly by ADP. The implication is that the glucose transporter – an enzyme of glycolysis – is characteristically sensitive to the energy charge of the cell.

All experiments investigating the action of glycolytic intermediates on ATP modulation of sugar transport were performed at fixed pH (7.4). Under normal circumstances, increased glycolytic flux gives rise to elevated lactic acid levels and reduced pH (Elliott et al., 1992; Cairns et al., 1993; Smith et al., 1993). We therefore examined the effect of altered pH on basal and ATP regulated sugar transport. To our surprise, we observed that while reduced pH inhibited ATP-modulation of sugar transport, azido[$\gamma$-$^{32}$P]ATP-labeling of GLUT1 was increased. Reduced pH serves to increase both maximum photoincorporation of [$\gamma$-$^{32}$P]ATP and $K_{i(app)}$ for ATP-inhibition of photolabeling. ATP stimulation of transport at pH 8.0 increases in a saturable manner with nucleotide concentration. However, ATP stimulation of transport at pH 5.5 increases with nucleotide concentrations up to 200 $\mu$M and thereafter falls with increasing ATP. ATP assays indicate that intracellular ATP is not degraded to a significantly greater extent at low pH. Computation of free $Mg^{2+}$, ATP and Mg.ATP levels indicates that these observations are not explained by systematic, pH-dependent changes in these species. We conclude that intracellular

ATP exerts antagonistic actions on sugar transport at pH 5.5. The nature of the inhibitory action is presently unknown. Increased GLUT1 labeling by azidoATP at reduced pH raises the possibility that a unique, transport-antagonistic ATP binding site is revealed at acidic pH. Future studies must address this possibility.

Isolated GLUT1 is an ATP binding protein. Resealing erythrocyte ghosts with Mg.ATP results in sugar uptake stimulation at subsaturating [3OMG] and produces uptake inhibition at saturating [3OMG] (Carruthers and Helgerson, 1989). Addition of 2 mM Mg.ATP to reconstituted GLUT1 proteoliposomes results in stimulation of 3OMG uptake at subsaturating (50 $\mu$M) 3OMG levels. ATP modulations of purified GLUT1 and erythrocyte resident GLUT1 mediated sugar transport are, therefore, fundamentally similar. We conclude that ATP, GLUT1, and the lipid bilayer, are sufficient for ATP modulation of GLUT1 function.

This conclusion contrasts with that of Wheeler who demonstrated that ATP does not modulate the activity of reconstituted human GLUT1 protein (Wheeler, 1989). However, Wheeler's studies employed reduced (DTT-treated) GLUT1 which we

have shown to exist as a GLUT1 dimer (Hebert and Carruthers, 1992). Our studies employ the physiologic, non-reduced form of GLUT1 (a GLUT1 tetramer, see (Hebert and Carruthers, 1992). We therefore asked two questions. 1) Is reduced GLUT1 susceptible to ATP-regulation? 2) Does reduced GLUT1 bind ATP? Our studies have shown that reductant treatment of erythrocytes converts resident tetrameric GLUT1 to dimeric GLUT1 (Zottola et al., 1995) and our present findings indicate that reductant also ablates ATP-modulation of 3OMG transport. Reductant (DTT) treatment of purified GLUT1 results in the loss of azidoATP labeling. These results provide a rational explanation for Wheeler's findings and suggest strongly that dimeric GLUT1 does not present binding sites to nucleotides. They further reinforce the view that red cell-resident GLUT1 exists as the ATP sensitive, GLUT1 tetramer (Zottola et al., 1995).

# Chapter IV

## Peptide Mapping the GLUT1 ATP Binding Domain

Isolated human erythrocyte glucose transporter (GLUT1) functions as an ATP binding protein (Carruthers and Helgerson, 1989). Work in this laboratory has shown that the GLUT1 carboxyl terminus becomes less accessible to peptide-directed antibodies following ATP exposure. This ATP dependent conformational change is antagonized by AMP and ADP that appear to function as competitive inhibitors of ATP binding to GLUT1. In this study we set out to determine the location of the GLUT1 nucleotide binding domain.

To answer this question we applied peptide mapping and N-terminal sequencing techniques to GLUT1 photolabeled with azidoATP, a photoactivatable, $^{32}P$ labeled, ATP analog. Our findings suggest that GLUT1 residues 301-364 form an integral part of the ATP binding domain.

## *Results*

### *Peptide Mapping*

Previous studies from this laboratory have demonstrated that GLUT1 and azidoATP associate reversibly to form a binary complex and that azidoATP binding to GLUT1 is inhibited competitively by ATP, ADP and AMP (Carruthers and Helgerson, 1989). *UV* irradiation of the azidoATP/GLUT1 complex activates the ATP azido group resulting in covalent attachment of bound nucleotide to GLUT1. In the absence of irradiation, no measurable 8-azido[$\gamma$-$^{32}$P]ATP is incorporated into the carrier protein (Carruthers and Helgerson, 1989). Limited proteolysis studies suggest that the point of photoattachment of azidoATP to GLUT1 falls within the carboxyl-terminal half of the protein (residues 270-456; see (Carruthers and Helgerson, 1989). We have now undertaken a detailed peptide mapping analysis to more precisely define the site of covalent attachment of nucleotide to GLUT1.

Peptide mapping studies of integral membrane proteins such as GLUT1 are complicated by the typically hydrophobic nature of

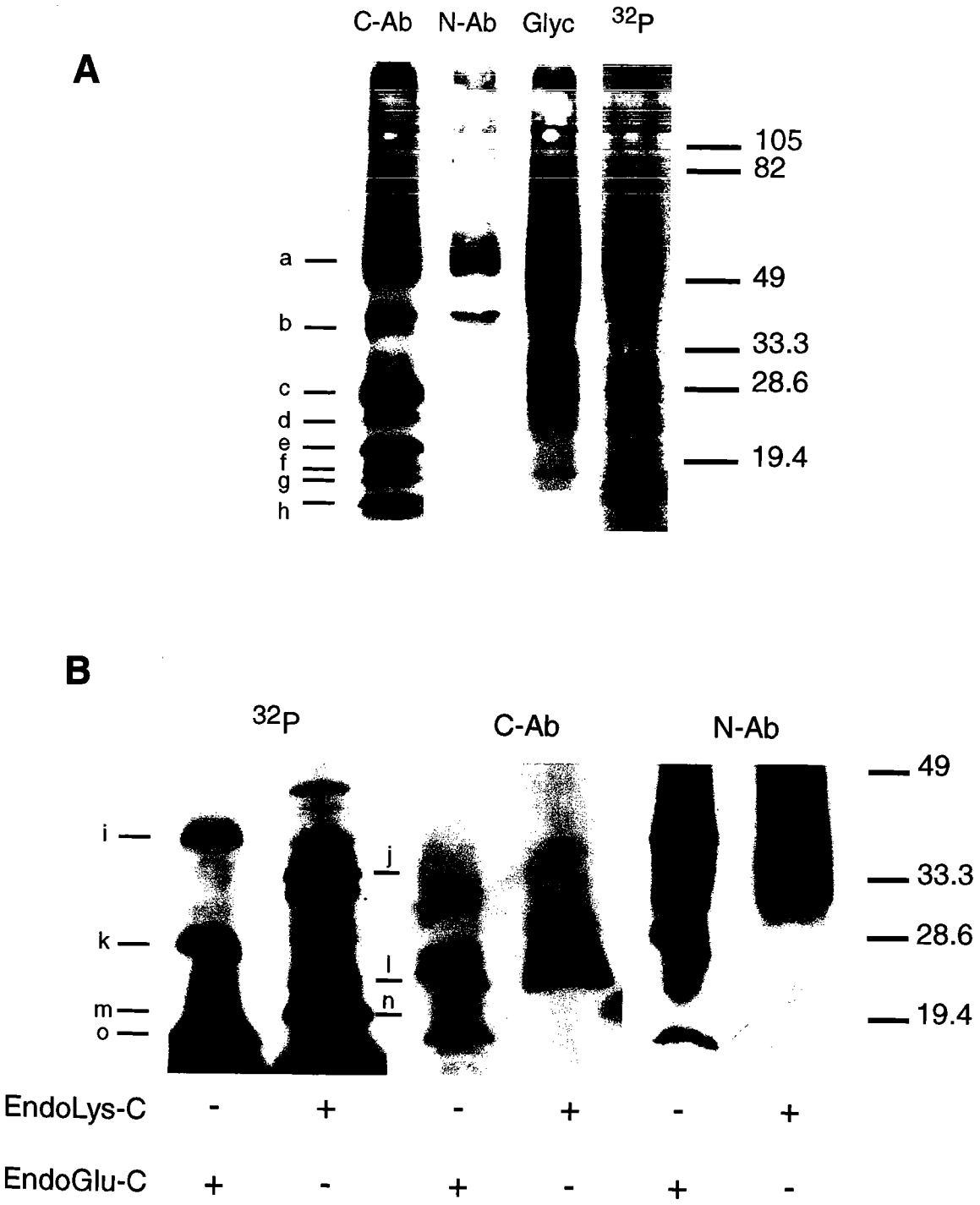
the resulting peptides and by the presence of N-linked oligosaccharide. For example, human erythrocyte band 7 proteins include the multi-spanning integral membrane glycoproteins aquaporin (28 kDa) and RhD (Mr = 45 kDa) (Smith and Agre, 1991) but are resolved as a 32 kDa protein band upon 10% SDS PAGE (Zottola et al., 1995). Intact GLUT1 (Mr = 55 kDa) is resolved as a 50 kDa band (Fig 6A). We therefore decided to use GLUT1 internal markers to assist in peptide identification. Peptides containing N-linked oligosaccharide ($asn^{45}$) were identified by blot analysis (using streptavidin-conjugated HRP) of GLUT1 fragments obtained from glucose transporter biotinylated on carbohydrate residues prior to proteolysis. Peptides containing GLUT1 residues 88-95 (N-residues) and/or 480-492 (C-residues) were identified by use of rabbit antisera (N-Ab and C-Ab respectively) raised against synthetic peptides corresponding to these GLUT1 domains. Each experiment (see Figure 6A) thus involves biotinylation of carbohydrate exposed by unsealed GLUT1 proteoliposomes, followed by GLUT1 photolabeling using azido[$\gamma$-$^{32}$P]ATP. The labeled proteoliposomes

***Figure 6.*** **Peptide mapping of photolabeled GLUT1. A.** Purified GLUT1 (40 $\mu$g biotinylated on carbohydrate residues) was photolabeled using 10 $\mu$M azido-[$\gamma$-$^{32}$P]ATP then incubated in sample buffer containing 5 $\mu$g chymotrypsin (30 minutes at 37 ˚C). Peptides were separated on a 10-20% SDS PAGE gradient gel, transferred to nitrocellulose. Azido[$\gamma$-$^{32}$P]ATP-containing peptides were detected by autoradiography ($^{32}$P). C-Ab and N-Ab reactive peptides were detected using immunoblot analysis. Biotinylated carbohydrate (Glyc) was detected by streptavidin blot analysis. C-Ab, N-Ab, $^{32}$P and Glyc analyses were performed on a single common lane of electrophoretically separated peptides. Major peptides are assigned a label (a through h) and are indicated by the leftmost solid bars. The mobility and $M_r$ of molecular weight standards are indicated by the solid bars to the right of the figure.

**B.** EndoLys-C digestion and sequencing of azido-[$\gamma$-$^{32}$P]ATP-photolabeled GLUT1. Labeled, purified GLUT1 (100 $\mu$g) was incubated in 0.1% SDS, 50 mM Tris-HCl medium, pH 7.4 containing 5 $\mu$g endoproteinase Lys-C (EndoLysC) or 5 $\mu$g endoproteinase Glu-C (EndoGlu-C; 18-24 hours at 4 ˚C). Peptides were separated on a 10-20% SDS PAGE gradient gel, which had been pre-equilibrated with 1 mM thioglycolate and subsequently transferred to Immobilon-PVDF for staining with Coomassie blue. Bands containing [$\gamma$-$^{32}$P]ATP were identified by autoradiography ($^{32}$P). C-Ab and N-Ab reactive peptides were detected using chemiluminescence immunoblot analysis. Major peptides are

assigned a label (i through o) and are indicated by the leftmost solid bars. The mobility and $M_r$ of molecular weight standards are indicated by the solid bars to the right of the figure. An intensely labeled 19 kDa band (peptide n) was excised from the membrane, washed 10 times with distilled water and subjected to sequence analysis by the UMMC Microsequencing Facility. A single sequence was obtained as AGVQQPVYAT corresponding to GLUT1 residues 301-310.

Figure 6


A
C-Ab
N-Ab
Glyc
32P
105
82
49
33.3
28.6
19.4
a
b
c
d
e
f
g
h
B
32P
C-Ab
N-Ab
49
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i
j
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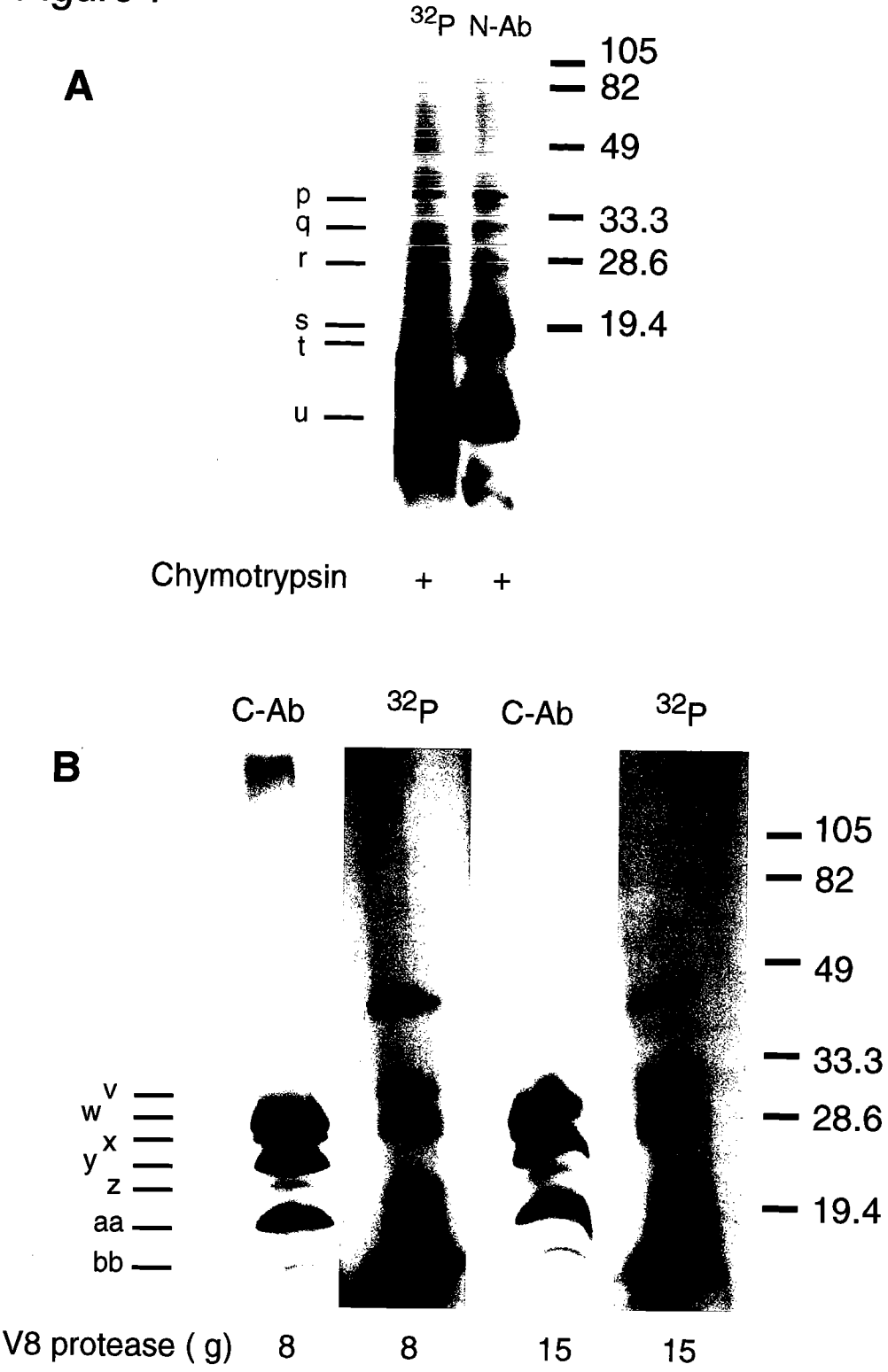
m
n
o
EndoLys-C - + - + - +
EndoGlu-C + - + - + -

were subjected to limited proteolysis, peptides are resolved by SDS-PAGE, transferred to immobilon or nitrocellulose, and biotinylated and/or $^{32}$P-labelled peptides are detected by autoradiography. The same membrane filters are then probed for the presence of peptides containing N- and C-residues by chemiluminescent immunoblot analysis.

Figure 6A indicates that not all N- or C-Ab-reactive peptides contain $^{32}$P or biotinylated carbohydrate. Similarly, not all $^{32}$P-containing peptides include N- or C-Ab-reactive sequence. Figure 6B shows EndoLys-C and EndoGlu-C (V8) digests of 8-azido[$\gamma$-$^{32}$P]ATP labeled GLUT1 which were subsequently probed for $^{32}$P and C- and N-residues. One of the $^{32}$P-labeled peptides (figure 6B, peptide n) was subjected to N-terminal sequence analysis (see below). Figure 7 summarizes additional experiments in which azidoATP-labeled, intact GLUT1 was subjected to digestion by chymotrypsin (Figure 7A) or by V8 protease (Figure 7B). Major peptides of Figures 6 and 7 have an assigned label and are aligned in Figure 8 according to size, sequence content (identified by internal markers) and proteolytic specificity. This analysis permits approximate localization of the

***Figure 7.*** **Peptide mapping of photolabeled GLUT1. A**. Purified GLUT1 (40 $\mu$g) was photolabeled using 10 $\mu$M azido-[$\gamma$-$^{32}$P]ATP then incubated in sample buffer containing 5 $\mu$g chymotrypsin (30 minutes at 37 °C). Peptides were separated on a 10-20% SDS PAGE gradient gel, transferred to nitrocellulose. [$\gamma$-$^{32}$P]ATP-containing peptides were detected by autoradiography ($^{32}$P). N-Ab reactive peptides were detected using immunoblot analysis. Major peptides are assigned a label (p through u) and are indicated by the leftmost solid bars. The mobility and $M_r$ of molecular weight standards are indicated by the solid bars to the right of the figure. **B**. V8-protease digestion of azido-[$\gamma$-$^{32}$P]ATP-photolabeled GLUT1. Labeled, purified GLUT1 (100 $\mu$g) was incubated in 0.1% SDS, 50 mM Tris-HCl medium, pH 7.4 containing 8 or 15 $\mu$g endoproteinase Glu-C (EndoGluC; 18-24 hours at 4 °C). Peptides were separated on a 10-20% SDS PAGE gradient gel and subsequently transferred to Immobilon-PVDF for staining with Coomassie blue. Bands containing [$\gamma$-$^{32}$P]ATP were identified by autoradiography ($^{32}$P). C-Ab reactive peptides were detected using chemiluminescence immunoblot analysis. Major peptides are assigned a label (v through bb) and are indicated by the leftmost solid bars. The mobility and $M_r$ of molecular weight standards are indicated by the solid bars to the right of the figure.

Figure 7


A
32P N-Ab
105
82
49
33.3
28.6
19.4
p
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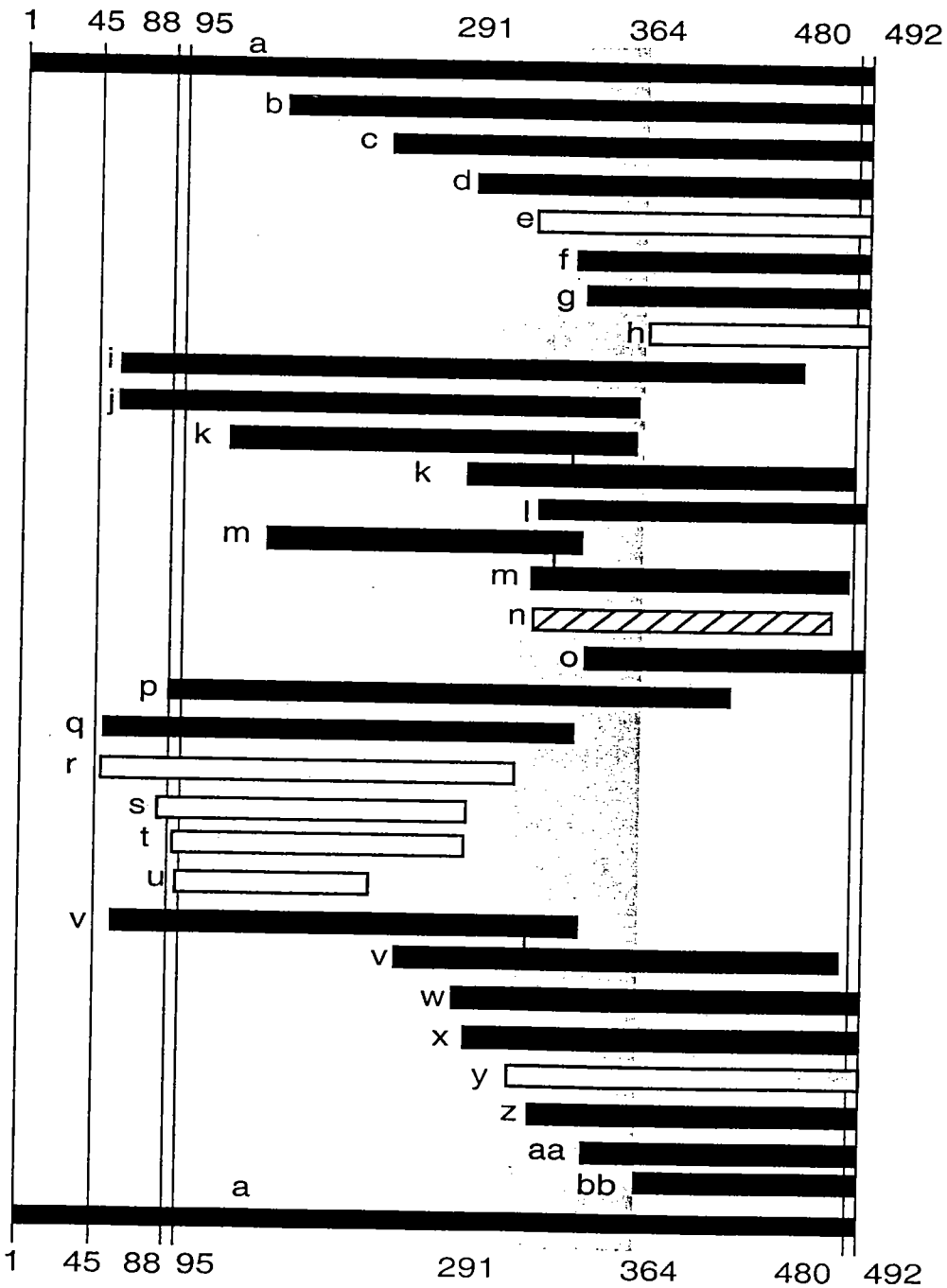
s
t
u
Chymotrypsin + +
B
C-Ab
32P
C-Ab
32P
105
82
49
33.3
28.6
19.4
v
w
x
y
z
aa
bb
V8 protease ( g) 8 8 15 15

***Figure 8.*** **Summary of peptide mapping analysis of [γ–$^{32}$P]ATP-labeled GLUT1 (Figures 6 and 7).** The horizontal axis represents GLUT1 residues 1 to 492. $^{32}$P-labeled and unlabeled peptides are indicated by filled and open bars respectively. Data are from Figures 6 and 7 and are cross-referenced by peptide labels (a - bb). Peptides containing C-Ab reactive sequence overlap with GLUT1 residues 480 – 492. Peptides containing N-Ab reactive sequence overlap with GLUT1 residues 88 – 95. Peptides containing biotinylated carbohydrate also contain GLUT1 residue asn$^{45}$. When two peptides are linked by a single vertical bar, this indicates that localization is ambiguous and the range of possibilities is shown for the linked peptides. The length of each peptide was computed by assuming that electrophoretic mobility accurately represents peptide molecular weight. The shaded vertical box spanning GLUT1 residues 291–364 represents that region of GLUT1 that most likely contains covalently attached [γ–$^{32}$P]ATP. The striped bar represents a 19 kDa, [γ-$^{32}$P]ATP-labeled, endoLysC fragment (peptide n) of GLUT1 whose N-terminal residue (A$^{301}$) was confirmed by sequencing. Peptides e and y should (but do not) contain [γ–$^{32}$P]ATP and are shaded differently for comparison. Presumed specificities of proteolysis are: Endo-LysC, K; V8 protease, D, E; chymotrypsin, F,W,Y.

Figure 8


1
45
88
95
291
364
480
492
a
b
c
d
e
f
g
h
i
j
k
k
l
m
m
n
o
p
q
r
s
t
u
v
v
w
x
y
z
aa
bb
a
1
45
88
95
291
364
480
492

site of covalent attachment of [γ-$^{32}$P]ATP to residues 291 through 364. Recognizing that the masses of hydrophobic and glycosylated proteins obtained by SDS-PAGE analysis are approximate, this region of nucleotide attachment should be viewed with some caution. Two peptides have properties inconsistent with this conclusion. Peptide e (Figure 6A) and peptide y (Figure 7B) cross react with C-Ab, are characterized by $Mr_{app}$ 19.9 and 22.4 kDa respectively but contain no detectable $^{32}$P. Three observations suggest that these peptides are dysfunctional, GLUT1 fragments present in GLUT1 proteoliposomes prior to labeling and digestion. 1) These C-Ab-reactive peptides are observed occasionally in GLUT1 preparations that are untreated by proteolytic enzymes (Hebert and Carruthers, 1992); Cloherty and Carruthers, unpublished observations). 2) Peptide y does not correspond to any possible V8-specific GLUT1 cleavage product. 3) Peptide n overlaps with peptides e and y, contains $^{32}$P and is identified definitively by N-terminal sequencing (see below).

*N-Terminal Microsequencing*

To further refine definition of the site of nucleotide attachment, azido[$\gamma$-$^{32}$P]ATP labeled, GLUT1 was subjected to endoproteinase Lys-C digestion. Endoproteinase Lys-C specifically hydrolyzes amide, ester, and peptide bonds at the carboxylic side of lysine (Jekel et al., 1983). Assuming the conditions of proteolysis reveal each cleavage site and also favor EndoLys-C specificity, this enzyme is expected to produce a 16 kDa GLUT1 fragment corresponding to residues 301 - 456. Purified, azido-ATP labeled GLUT1 was exposed to endoLys-C for 18-24 hours at 4 °C and the resulting peptides were resolved on 10-20% gradient SDS-PAGE gels. Following transfer to Immobilon-P membranes, the blots were exposed to film for autoradiography and stained with Coomassie blue. Bands of interest were excised from the membrane, and subjected to N-terminal microsequence analysis. Figure 6B indicates that a 19 kDa band (peptide n) is heavily labeled by [$\gamma$-$^{32}$P]ATP but lacks recognition sites for both N- and C-Ab. N-terminal sequence analysis of this peptide yielded 10 pmol of a single sequence of AGVQQPVYAT corresponding to GLUT1 residues 301-310.

## *Discussion*

The results of our peptide mapping studies point to GLUT1 residues 291-364 as the smallest GLUT1 cassette that contains covalently attached azido[$\gamma^{32}$P]-ATP. This result is consistent with previous indirect studies suggesting that GLUT1 residues 270-380 may be involved in nucleotide binding (Carruthers and Helgerson, 1989). The present study offers significant improvement over our earlier analyses because we have employed internal markers to indicate the approximate origin of proteolytic fragments previously assigned on the basis of peptide mass and assumed proteolytic enzyme specificity alone. These markers are: GLUT1 residues 84-96 (N-residues), GLUT1 residues 480-492 (C-residues) and asparagine-linked carbohydrate ($asn^{45}$). Even using these markers, however, interpretation of GLUT1 peptide mapping studies is complicated by the frequently anomalous mobility of GLUT1 fragments upon SDS PAGE. For example, an azido[$\gamma$–$^{32}$P]-ATP labeled endoLysC GLUT1 fragment with intact carboxyl-terminus is resolved as a 23 kDa fragment. EndoLys-C specifically hydrolyzes amide, ester, and

peptide bonds at the carboxylic side of lysine. Assuming specificity in proteolysis (an assumption supported by sequence analysis of a smaller endoLysC GLUT1 fragment), this peptide could derive from cleavage at lysine 300 or lysine 256. The predicted masses of these peptides (21 kDa and 25.8 kDa respectively) differ significantly from the observed mass on SDS PAGE.

The smaller azido[$\gamma$-$^{32}$P]-ATP labeled endoLysC GLUT1 fragment (19.1 kDa) was subjected to N-terminal sequence analysis. This confirmed the origin of the azido[$\gamma^{32}$P]-ATP labeled GLUT1 fragment as $ala^{301}$ with hydrolysis occurring at $lys^{300}$. Assuming specificity and precise molecular weight determination, this result places the site of azido[$\gamma^{32}$P]-ATP incorporation within a GLUT1 cassette comprised of residues 301-477 (theoretical molecular weight = 19223 D). Our peptide mapping studies suggest that azido[$\gamma^{32}$P]-ATP incorporation occurs between GLUT1 residues 291 and 364. This permits further refinement of the azidoATP-incorporation domain to residues 301 -364. This GLUT1 domain contains primary sequence that shares 50% identity with a region of adenylate kinase previously shown to form a hydrophobic strand of

a parallel ß-pleated sheet flanking the adenine-ribose and triphosphate chain of MgATP (Fry et al., 1986).

This sequence

| | |
|---|---|
| adenylate kinase$_{110\text{-}121}$ | GQPTLLLYVDAG |
| | \| \|\| \| \|\| |
| GLUT1$_{332\text{-}343}$ | GRRTLHLIGLAG |

is also found in the adenine nucleotide binding proteins glycogen phosphorylase (Newgard et al., 1988), RAD3 (Reynolds et al., 1985), the ATP/ADP translocase (Plano and Winkler, 1991), the human brown fat uncoupling protein (Casteilla et al., 1989; Cassard et al., 1990), phosphofructokinase (Valdez et al., 1989) and the α- & β-subunits of $F_1$-ATPase (Ohta and Kagawa, 1986). Assignment of this GLUT1 domain to the glucose transport nucleotide binding pocket, while strengthened by our peptide mapping studies, remains inferential in the absence of a detailed GLUT1 structure. It is interesting to note that GLUT1 also contains the consensus Walker motif GXXXXGKS/T, which is found in a number of ATP and GTP binding proteins (Fry et al., 1986). In GLUT1, this sequence (residues 111-118) may be exposed to the exterior of the cell

(Hresko et al., 1994) suggesting that it assumes no role in intracellular nucleotide binding.

# Chapter V

## Alanine mutagenesis of the GLUT1 consensus ATP binding sequences

Peptide mapping and n-terminal sequencing studies in this laboratory have suggested that residues 301-364 comprise part of the GLUT1 ATP binding domain. This region of GLUT1 contains primary sequence that is 50% identical with a region of adenylate kinase previously shown to form a hydrophobic strand of a parallel β-pleated sheet flanking the adenine ribose and triphosphate chain of Mg.ATP (Fry et al., 1986). Two additional consensus ATP binding sequences have been identified in GLUT1 (Fry et al., 1986). In order to delineate further the location of the ATP binding site on GLUT1 we have mutated all of the residues in these three proposed regions to alanine. Our findings suggest that residues 332-335 contribute to the efficiency of adenine nucleotide binding to GLUT1. In addition, the data also show that residues 338-340 are necessary for normal transporter function.

## *Results*

### *Purification of Human GLUT1 HA $His_6$*

GLUT1 is a glycosylated membrane protein that contains a single n-linked oligosaccharide at $asn^{45}$. The presence of this carbohydrate makes it difficult to synthesize GLUT1 in a bacterial system. We felt that either a yeast system or a transient mammalian system would fulfill our needs. Our data suggest that we were fairly successful in expressing hGLUT1 in a strain of Saccharomyces Cerevisiae (RE700A) in which seven (hxt 1-7) of 20 putative glucose transporter genes are disrupted or deleted. Unmodified, this strain of yeast is unable to survive on standard glucose media (Table 3, Figure 9B). Following transfection with human GLUT1, selected clones are able to grow on and transport glucose as a sole carbon source (Figure 9A & 9B). In addition, separation of whole cell extracts for western blotting reveals a 55 Kd protein that reacts with GLUT1 C-Ab (Figure 10). The data suggest that we have successfully, heterologously, expressed human GLUT1 in yeast. Unfortunately, this mutant yeast grows too slowly and produces insufficient GLUT1 for our purposes.

**Table 3: Characteristics of S. Cerevisiae strain RE700A transfected with GLUT1**

| Yeast | Carbon Source | D-Glucose Uptake | Growth |
|---|---|---|---|
| Wild Type[1] | Glucose | + | ++ |
| Wild Type | Galactose | + | ++ |
| Wild Type | Maltose | + | ++ |
| RE700A[2] | Glucose | - | - |
| RE700A | Galactose | + | ++ |
| RE700A | Maltose | - | ++ |
| RE700A + GLUT1[3] | Glucose | + | + |
| RE700A + GLUT1 | Galactose | + | ++ |
| RE700A + GLUT1 | Maltose | + | ++ |

[1]S. Cerevisiae
[2] RE700A is a URA 52 mutant with 7 of its glucose transporters (HXT 1-7) deleted or disrupted.
[3]RE700A transfected with HA $His_6$ epitope tagged human erythrocyte glucose transporter (GLUT1).

***Figure 9.*** **GLUT1 HA $His_6$ expression in Saccharomyces Cerevisiae. A.** [$^3$H]D-glucose uptake measured in RE700A +/- tagged GLUT1. Yeast were grown to an $OD_{600}$ = 0.6 for uptake. 1-2 mL of cells were pelleted and washed in PBS prior to addition of uptake media. All solutions except stop were preheated to 37°C. Uptake of 100 μM D-glucose was terminated at 5, 30, and 60 seconds by washing with ice cold PBS pH 7.4. Following lysis, duplicate samples of supernatant were counted in a Beckman scintillation counter. **B.** RE700A +/- tagged GLUT1 grown on YP-maltose or minimal media + glucose.

# Figure 9

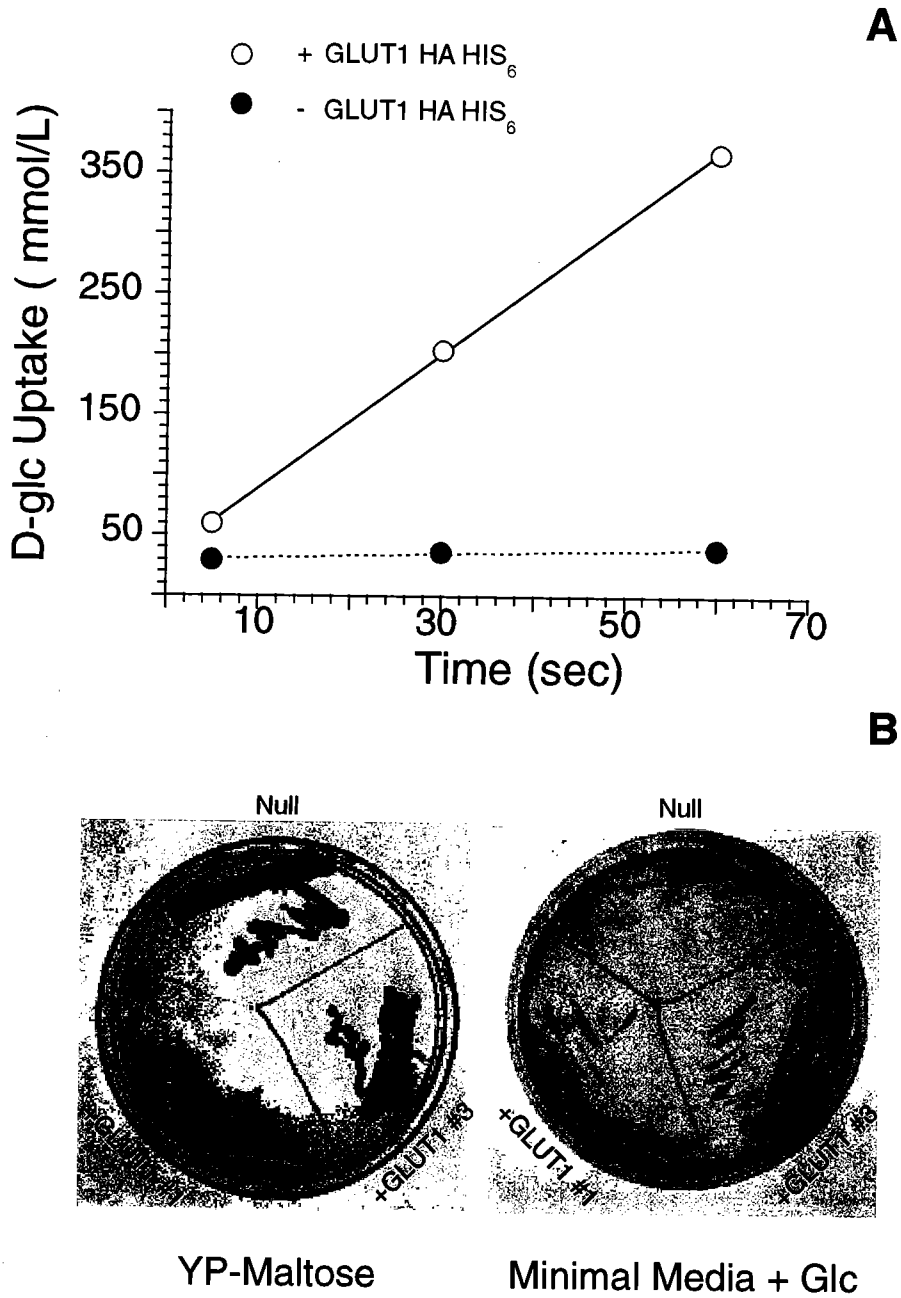
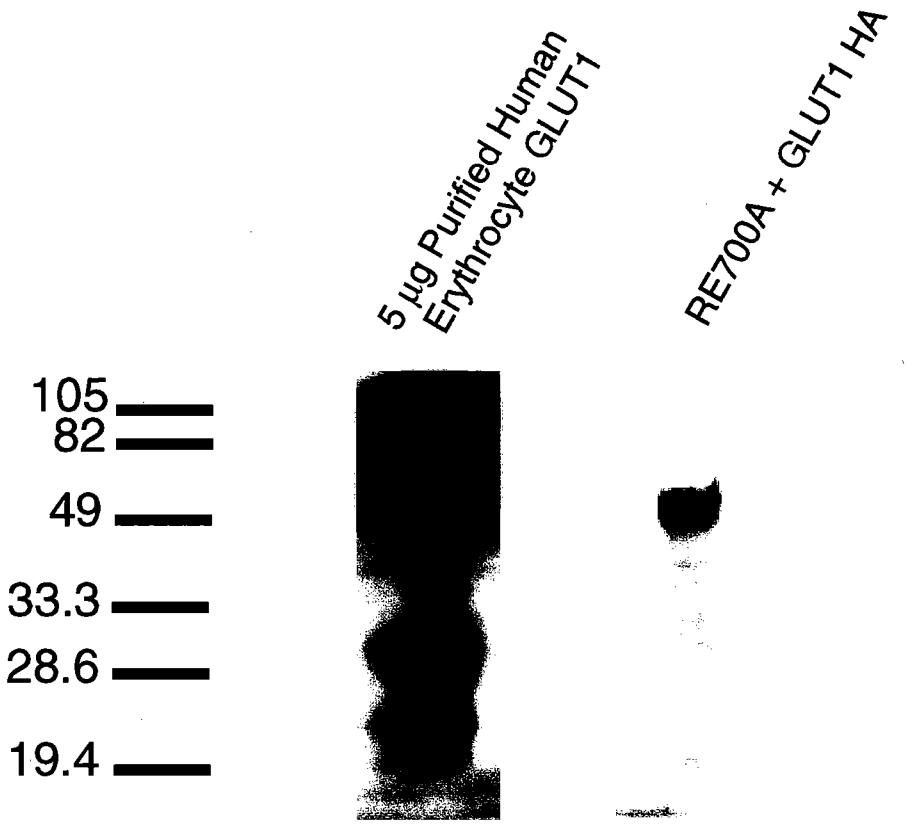

A
+ GLUT1 HA HIS6
- GLUT1 HA HIS6
D-glc Uptake ( mmol/L)
350
250
150
50
10
30
50
70
Time (sec)
B
Null
Null
+GLUT1 #3
+GLUT1 #1
+GLUT1 #3
YP-Maltose
Minimal Media + Glc

***Figure 10.*** **C-terminal GLUT1 antibody western blot of RE700A +/- GLUT1 HA $His_6$** Cell extracts were prepared from RE700A +/- tagged GLUT1 grown to an $OD_{600}$ of 0.6. Prior to extraction, the yeast were washed twice with ice cold 50mM Tris-HCl pH 7.5, 10 mM $NaN_3$. Cells were combined with 0.2 mM glass beads and 100 µl of protein sample buffer containing 1 mM PMSF. The mixture was vortexed briefly and 50 µl of each sample was resolved on 10% SDS PAGE gels that were transferred to nitrocellulose for western blotting with GLUT1 C-terminal antibody.

Figure 10


5 µg Purified Human Erythrocyte GLUT1
RE700A + GLUT1 HA
105
82
49
33.3
28.6
19.4

We therefore decided to develop a mammalian transient expression system for GLUT1. Cos-7 cells are a simian kidney cell line that is capable of correctly processing GLUT1. (Mueckler et al., 1985; Schurmann et al., 1997). We introduced a hemagglutinin (HA), 6 histidine ($His_6$) tag to facilitate the purification of the expressed human GLUT1 (GLUT1 HA $His_6$). To accomplish this, a unique Mfe1 restriction site was introduced at residue 1685 in a 1.7 Kbp fragment of GLUT1 cDNA previously subcloned into the *Bam*H1 site of pGEM3Z (pGEM3Z-GT1) (Zottola et al., 1995). Following the insertion of an HA $His_6$ epitope tag at this site, the GLUT1 was further subcloned into the *Bam*H1 site of a pcDNA3.1+ mammalian expression vector. The benefits of attaching this tag are twofold; 1) the $His_6$ region binds Nickel-NTA allowing affinity purification of recombinant protein and, 2) HA can be detected with standard western blotting techniques, allowing us to differentiate between parental Cos-7 GLUT1 and tagged human GLUT1 (GLUT1 HA $His_6$).

To purify hGLUT1, Cos-7 cells were transfected with pcDNA3.1 GLUT1 via the Fugene 6 transfection reagent system. Cells were harvested and homogenized 72 hours post transfection

and membranes were isolated by high-speed centrifugation in a Beckman TI50.2 rotor at 100,000 x g (Loo and Clarke, 1994). Due to the hydrophobic nature of hGLUT1, it is necessary to solubilize the membranes before they are applied to the Ni-NTA. A non-ionic detergent, n-dodecyl-β D maltoside, was used to prepare samples for purification because ionic detergents may interfere with the ability of the protein to bind to the column (Loo and Clarke, 1995). The solubilized protein was allowed to bind to the nickel matrix for one hour with gentle agitation. The Ni-NTA column was washed with increasing concentrations of imidazole (10-25 mM), and samples were eluted with a solution of 500 mM NaCl and 300 mM imidazole. Fractions were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose membranes for immunoblotting.

Standard western blotting techniques were used to identify the GLUT1 present on these membranes. Gels stained with Pro-blue suggest that GLUT1 HA $His_6$ containing eluate is approximately 50% pure. Rabbit antisera raised against a synthetic peptide corresponding to hGLUT1 residues 480-492 (C-Ab) was used to locate and identify intact GLUT1 in various purification fractions.

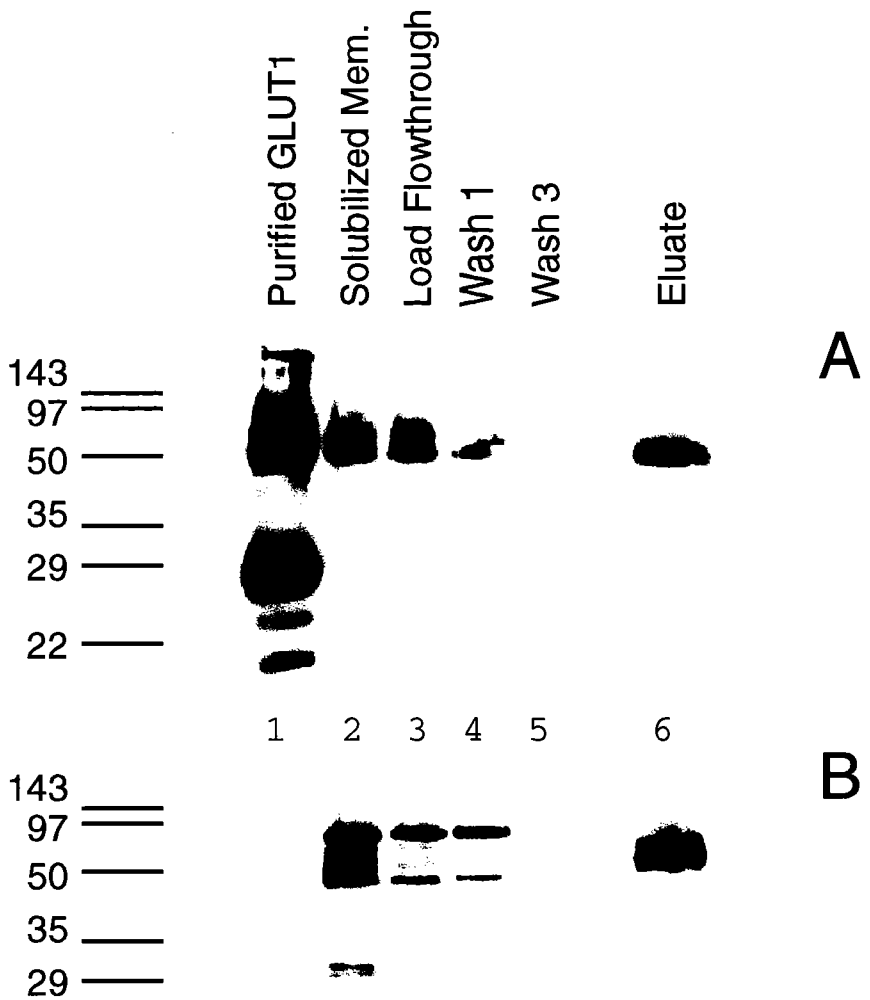
Human GLUT1 and Cos-7 GLUT1 show significant homology, thus C-terminal Ab binding is indicative of total GLUT1 concentration. To quantitate hGLUT1, we used anti-HA antisera which binds selectively to epitope tagged hGLUT1. Figures 11A & B show both the resulting C-terminal and HA western blots from a standard GLUT1 HA $His_6$ purification protocol using Cos-7 cell membranes. A single large band with an approximate molecular weight of 55 KDa is visible in the final elution fraction (eluate (E) on both blots. The presence of both an intact C-terminus and an HA tag suggests that we have successfully affinity-purified His tagged hGLUT1 protein.

*Alanine Scanning Mutagenesis of ATP Consensus Binding Sequences*

Fry et al (Fry et al., 1986) showed that GLUT1 contains three potential consensus ATP binding sequences (Figure 1). Current GLUT1 topology would place consensus site I (G-X-X-X-X-G-K-S/T residues 111-117), within transmembrane domain 3. Consensus site II (K-X-V-X-K amino acids 225-229) lies within the large cytoplasmic loop between domains 6 & 7. Finally site III (G-X-X-X-L-X-L (Y, I) X-X-

***Figure 11.*** **Purification of GLUT1 HA $His_6$ from Cos-7 cells.** HA-Ab (A) and C-Ab (B) immunoblot analysis of samples of GLUT1 HA $His_6$ -transfected cell membranes obtained during GLUT1 affinity purification on Ni-NTA. Isolated Cos-7 cell membranes were solubilized in n-dodecyl-β-D-maltoside (lane 2) and were applied to a Ni-NTA column. Following flowthrough of non bound GLUT1 (lane 3), columns were washed with 300 mM NaCl plus 10 mM imidazole (lane 4), 300 mM NaCl plus 30 mM imidazole (lane 5) and 500 mM NaCl plus 300 mM imidazole (lane 6). Fractions were collected and proteins were separated on 10% SDS-PAGE gels and transferred to nitrocellulose for immunoblot analysis. **A.** The presence of GLUT1 in each of the samples was detected with C-Ab. Purified human erythrocyte GLUT1 was placed in the far left lane as a control. GLUT1 runs as a diffuse band with an approximate molecular weight of 55 kDa. Lower molecular weight bands are proteolytic fragments of GLUT1. **B**. GLUT1 HA $His_6$ was identified with a mouse anti-HA antibody. Fractions correspond to and are aligned with those in panel A. The mobility of molecular weight standards are indicated by the solid bars to the left of the panels A and B.

Figure 11


Purified GLUT1
Solubilized Mem.
Load Flowthrough
Wash 1
Wash 3
Eluate
A
143
97
50
35
29
22
1 2 3 4 5 6
B
143
97
50
35
29

A-G residues 332-343) occupies both a portion of the cytoplasmic loop between helices 8 & 9 and transmembrane domain 9. Peptide mapping studies from this laboratory (Levine et al., 1998) suggest that the point of covalent ATP attachment to GLUT1 is between $Arg^{301}$ – $Met^{364}$. This proposed region of binding overlaps with sequence III. Once a procedure for producing and purifying GLUT1 was in place, site-directed mutagenesis could be undertaken. To test the involvement of the three domains in GLUT1 ATP binding, we changed the residues in each of the three proposed sites to alanine (Table 4) and determined whether transporter function or azidoATP binding was altered.

*D-glucose Uptake into Cos-7 Cells*

To assess whether wild type and mutant hGLUT1 are functional, we measured 2-deoxy-glucose (2DOG) uptake into Cos-7 cells transfected with each of our pcDNA3.1+ GLUT1 constructs. 2DOG is used in this instance because it undergoes phosphorylation and remains trapped in the intracellular space. Cells expressing epitope tagged hGLUT1 show a two-fold increase in cytochalasin B (CCB)

## *Table 4 - GLUT1 Alanine Mutants*

| *Mutant Name* | *Original Sequence* | *Mutation* |
|---|---|---|
| 111A | $^{111}$GFSK$^{114}$ | $^{111}$AAAA$^{114}$ |
| 225A | $^{225}$KSVLK$^{229}$ | $^{225}$AAAAA$^{229}$ |
| 332A | $^{332}$GRR$^{334}$ | $^{332}$AAA$^{334}$ |
| 335A | $^{335}$TLH$^{337}$ | $^{335}$AAA$^{337}$ |
| 338A | $^{338}$LIG$^{340}$ | $^{338}$AAA$^{340}$ |
| 341A | $^{341}$LAG$^{343}$ | $^{341}$AAA$^{343}$ |

inhibitable sugar transport above basal levels (i.e. transport measured in untransfected cos-7 cells, or in cells transfected with pcDNA3.1+ lacking GLUT1 HA $His_6$ cDNA) (Figure 12). Transfection with alanine mutants: 111A, 225A, 332A, 335A, and 341A also results in a 2-fold stimulation of transport (Figure 12). We hypothesized that this increase was a result of greater GLUT1 presence at the plasma membrane. We tested this possibility by measuring δ-antibody binding to Cos-7 cells +/- hGLUT1. Because this antibody recognizes an exofacial epitope of tetrameric hGLUT1, its binding is an approximate measurement of transporter expression at the membrane. Cos-7 cells transfected with tagged GLUT1 exhibit a 2-fold increase in δ-antibody binding above Cos-7 cell levels (Table 5). This confirms our original assumption that transport stimulation is a direct result of enhanced GLUT1 presence at the plasma membrane.

In contrast, mutant 338A shows only basal transport activity and is considered non-functional. This mutant, as we will discuss later, retains its ability to bind azidoATP. This result implies that

***Figure 12.*** **2-deoxyglucose uptake in Cos-7 cells transfected with GLUT1 HA $His_6$.** Comparison of 2DOG (0.1 mM) uptake at 37 ˚C by Cos7 cells, Cos-7 cells transfected with vector (pcDNA3.1) lacking GLUT1 sequence and by Cos7 cells expressing GLUT1 HA $His_6$ and GLUT1 HA $His_6$ alanine mutants. Ordinate: CCB-inhibitable 2DOG accumulation (mol per min per $\mu$g total cell protein). Abscissa: cells in which transport was measured. Results are shown as mean ±SEM of triplicate measurements.

Figure 12

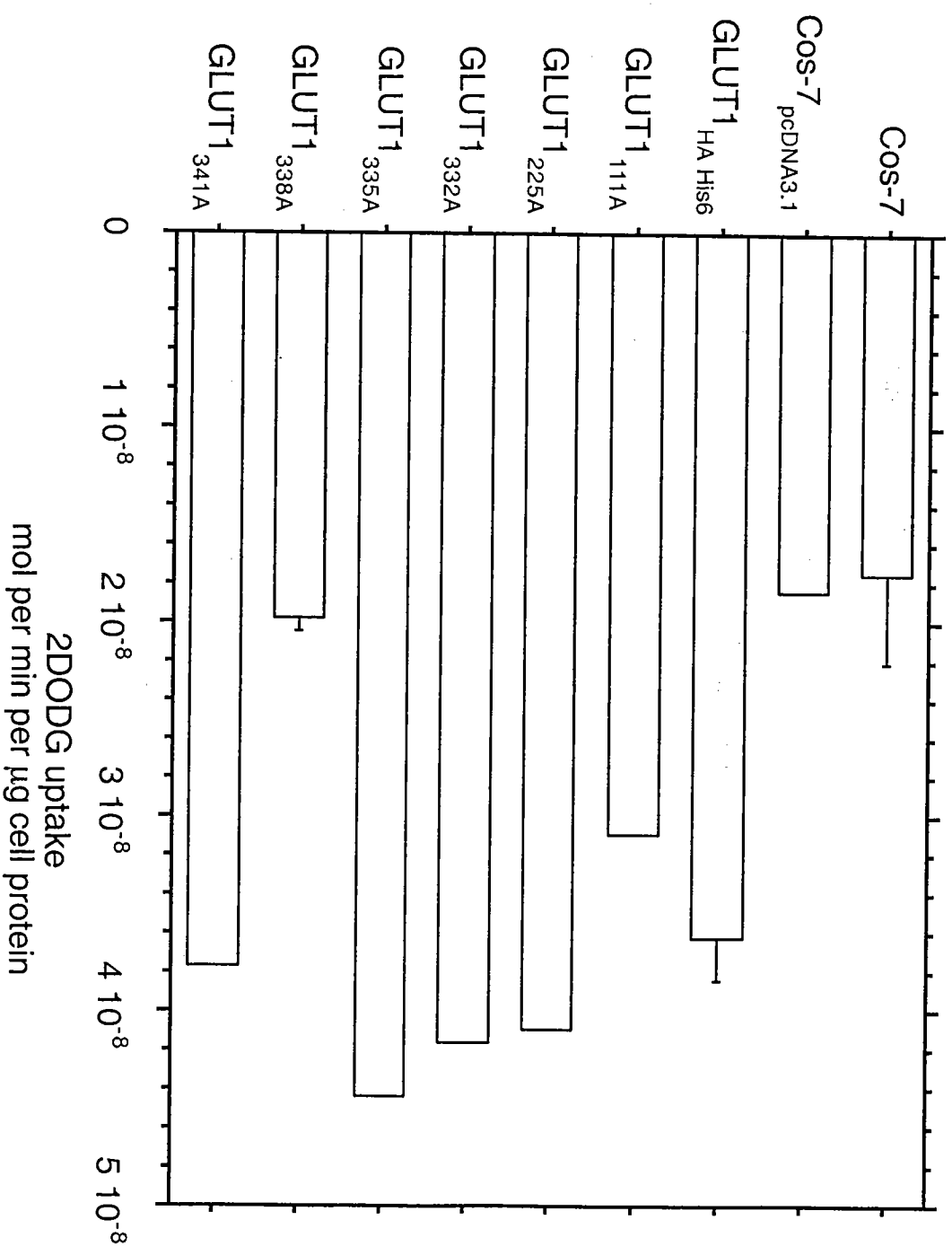

Cos-7
Cos-7 pcDNA3.1
GLUT1 HA His6
GLUT1 111A
GLUT1 225A
GLUT1 332A
GLUT1 335A
GLUT1 338A
GLUT1 341A
0
1 10-8
2 10-8
3 10-8
4 10-8
5 10-8
2DODG uptake
mol per min per µg cell protein

**Table 5: δ-Antibody binding to Cos-7 Cells transfected with GLUT1**

| | δ-Antibody Binding cpm $^{125}$I-protein G per μg total protein | SEM[1] |
|---|---|---|
| Cos-7 Cells | 2658 | 704 |
| Cos-7 Cells + GLUT1 HA $His_6$ | 5595 | 427 |
| Cos-7 Cells + 338A | 2199 | 51 |

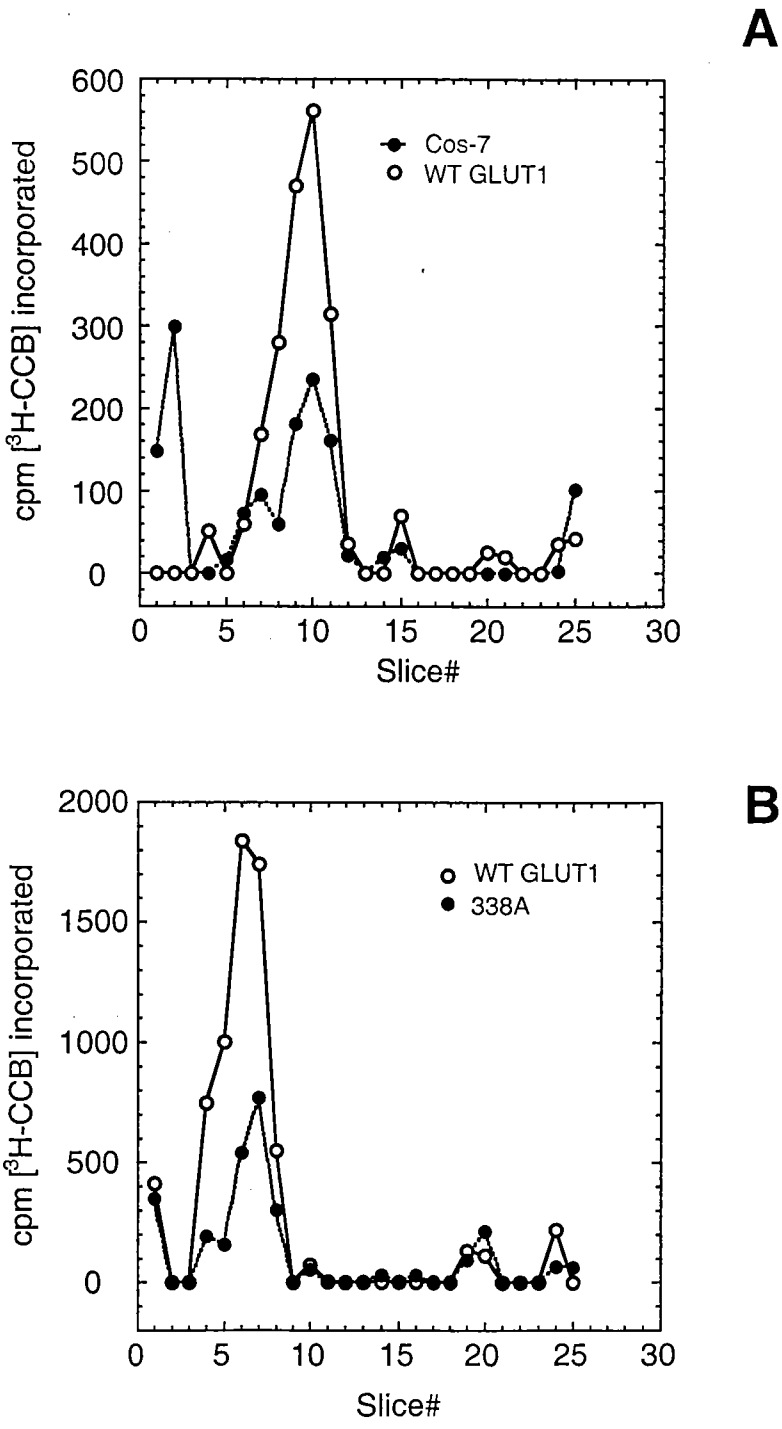
[1] *N= 2 experiments carried out in duplicate*

alanine substitution at residues 338-340 does not grossly affect transporter quaternary structure (i.e. GLUT1 is folded correctly with respect to ATP binding). To test this hypothesis, CCB (cytochalasin B) photoincorporation into GLUT1 was measured in membranes isolated from unaltered Cos-7 cells, as well as cells that had been transfected with GLUT1 HA $His_6$ and 338A. Membranes containing tagged wild type GLUT1 bound 2-fold more CCB than Cos-7 cells alone (Figure 13A). In contrast, CCB binding in 338A membranes was equivalent to that found in basal Cos-7 membranes (Figure 13B). The absence of CCB binding and the retention of azidoATP binding imply that some aspect of the mutant transporter structure is disrupted.

It is also possible that GLUT1 trafficking to the plasma membrane is disrupted. To better understand these results, δ-antibody binding studies as previously described were applied to Cos-7 cells transfected with mutant 338A. Interestingly, 338A exhibited the same level of antibody binding as untransfected Cos-7 cells, and 2-fold less binding than cells containing wild type hGLUT1 (Table 5).

***Figure 13.*** **Photolabeling of parental GLUT1 and transfected wild type and mutant human GLUT1 in Cos-7 cells by the sugar export site ligand [$^3$H]-CCB.** Cos-7 cell membranes were photolabeled in the presence and absence of 100 μM phloretin. Membranes were washed to remove unincorporated [$^3$H]-CCB and membrane proteins were separated by SDS-PAGE. Following Coomassie staining, each gel lane was sliced into 2 mm sections, dissolved and counted for associated [$^3$H]-CCB. Ordinate: phloretin inhibitable CCB incorporation (cpm). Abscissa: relative mobility (gel slice number). A. CCB incorporation in Cos-7 cells, and cells transfected with GLUT1 HA $His_6$. B. CCB incorporation in Cos-7 cells transfected with GLUT1 HA $His_6$ and alanine mutant 338A.

Figure 13


A
600
500
400
300
200
100
0
cpm [3H-CCB] incorporated
Cos-7
WT GLUT1
0
5
10
15
20
25
30
Slice#
B
2000
1500
1000
500
0
cpm [3H-CCB] incorporated
WT GLUT1
338A
0
5
10
15
20
25
30
Slice#

*AzidoATP Labeling of Wild Type & Mutant hGLUT1*

To test whether alanine substitution affects ATP binding to the transporter, we measured azidoATP incorporation in GLUT1 HA $His_6$ (mutant & wild type) purified from Cos-7 cells. Wild type and mutant hGLUT1 were affinity-purified from Cos-7 cells as described previously. Proteins eluted from the Ni-NTA column were concentrated using an Amicon centricon-10 spun at 5000 x g for 1 hour in a Beckman JA-17 rotor. Samples labeled with azidoATP were equilibrated for 30 minutes in 10-15 μM azidoATP then UV irradiated for 90 seconds at 280 nM in a Rayonet Photochemical reactor. Fractions were simultaneously resolved on three 10% SDS-PAGE gels and transferred to nitrocellulose. Two of the membranes were used for C-Ab and HA-Ab western blotting. The third blot, which contained photolabeled hGLUT1, was exposed to autoradiographic film for 5-24 hours. Densitometry was used to quantitate epitope tagged GLUT1 and azidoATP incorporation. Total protein concentration for each of the constructs was measured with a Pierce BCA kit, and a uniform quantity of each mutant protein was applied to all three gels.

Western blots and azidoATP autoradiographs for each alanine mutant are shown in Figure 14. Lane 3 (4 mM ATP) shows purified epitope tagged GLUT1 (from lane 4) labeled with azidoATP in the presence of 4 mM cold ATP. The absence of radiolabel incorporation suggests that ATP and 8-azidoATP bind to the same site on GLUT1 HA $His_6$. This same effect has been seen previously, using purified human erythrocyte GLUT1 (Carruthers and Helgerson, 1989; Levine et al., 1998).

AzidoATP binding in each alanine mutant was compared to incorporation in wild type GLUT1 HA $His_6$. The ratio of photolabel (azidoATP) to HA antibody was used as a measure of ATP attachment to tagged transporter. Mutants 111A, 335A, 338A, and 341A bound quantities of azidoATP that were equivalent to that found in GLUT1 HA $His_6$ (Figure 15). In contrast, mutants 225A and 332A exhibited a 1.5 to 2.3-fold increase in photolabel incorporation (Figure 15). Three possible explanations for this increased ATP incorporation are: 1) The small sidechain of alanine reduces steric interactions that normally limit ATP access to the transporter-binding site. 2) By exchanging polar residues with

***Figure 14.*** **Azido[$^{32}$P]ATP labeling of GLUT1 HA His$_6$ and its corresponding alanine mutants. A.** Transporter was purified by Ni-NTA affinity chromatography of membranes isolated from Cos-7 cells transfected with wild-type or mutant GLUT1 HA His$_6$ (see Figure 11). Purified GLUT1 was photolabeled using 10 $\mu$M azido[$^{32}$P]ATP, resolved on three separate 10% SDS PAGE gels (25 $\mu$g total protein per lane per gel) and transferred to nitrocellulose for western blotting and autoradiography. For each group of mutants an internal control of GLUT1HA His$_6$ was also processed. Control protein is visible at the far left of each group of blots. GLUT1 constructs were identified by C-Ab and HA-Ab immunoblot analysis. Incorporated affinity label (azidoATP) was detected by autoradiography. Antibody and ATP binding were quantitated by densitometry using NIH Image. The ratio of azidoATP labeling to HA-Ab reactivity is shown below the blots. Lane 3 (4 mM ATP) shows that Ni-NTA purified GLUT1 HA His$_6$ photolabeling by azidoATP (lane 4) is inhibited in the presence of 4 mM unlabeled ATP.

Figure 14

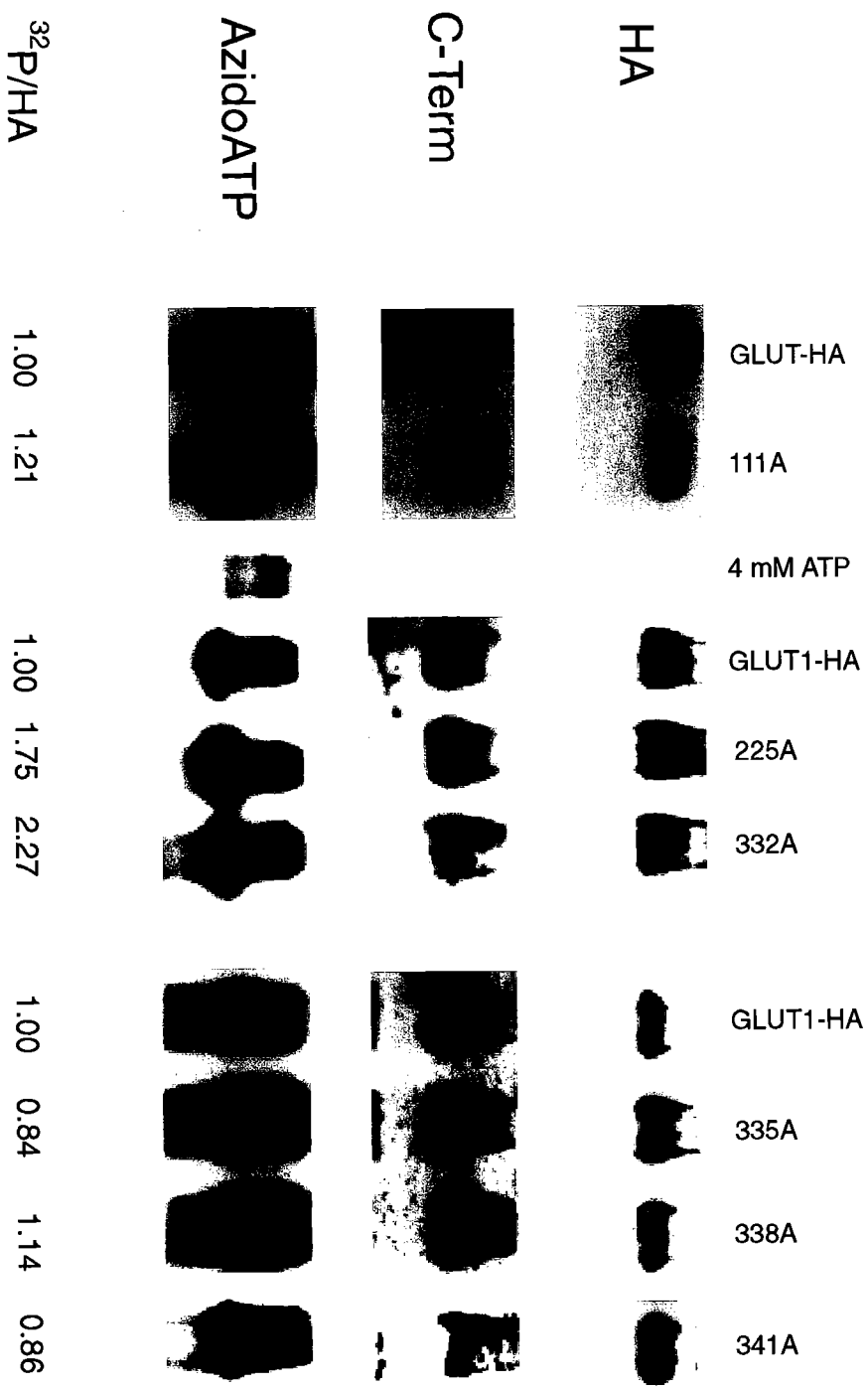
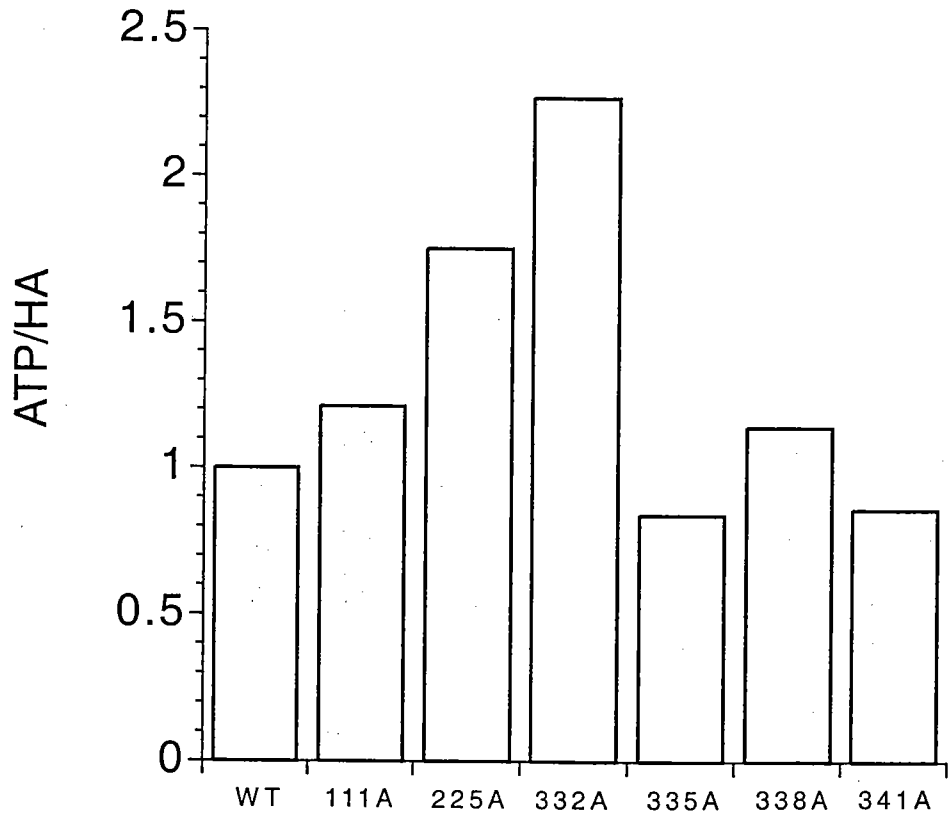

$^{32}$P/HA
AzidoATP
C-Term
HA
1.00
1.21
GLUT-HA
111A
4 mM ATP
1.00
1.75
2.27
GLUT1-HA
225A
332A
1.00
0.84
1.14
0.86
GLUT1-HA
335A
338A
341A

***Figure 15.*** **Corrected AzidoATP labeling of GLUT1 HA $His_6$ (wild type and mutant).** AzidoATP labeling of tagged GLUT1 (wild type & mutant) is presented as a ratio of ATP/ HA antibody binding. This distinguishes azidoATP labeling of recombinant human GLUT1 HA $His_6$ from parental GLUT1.

Figure 15

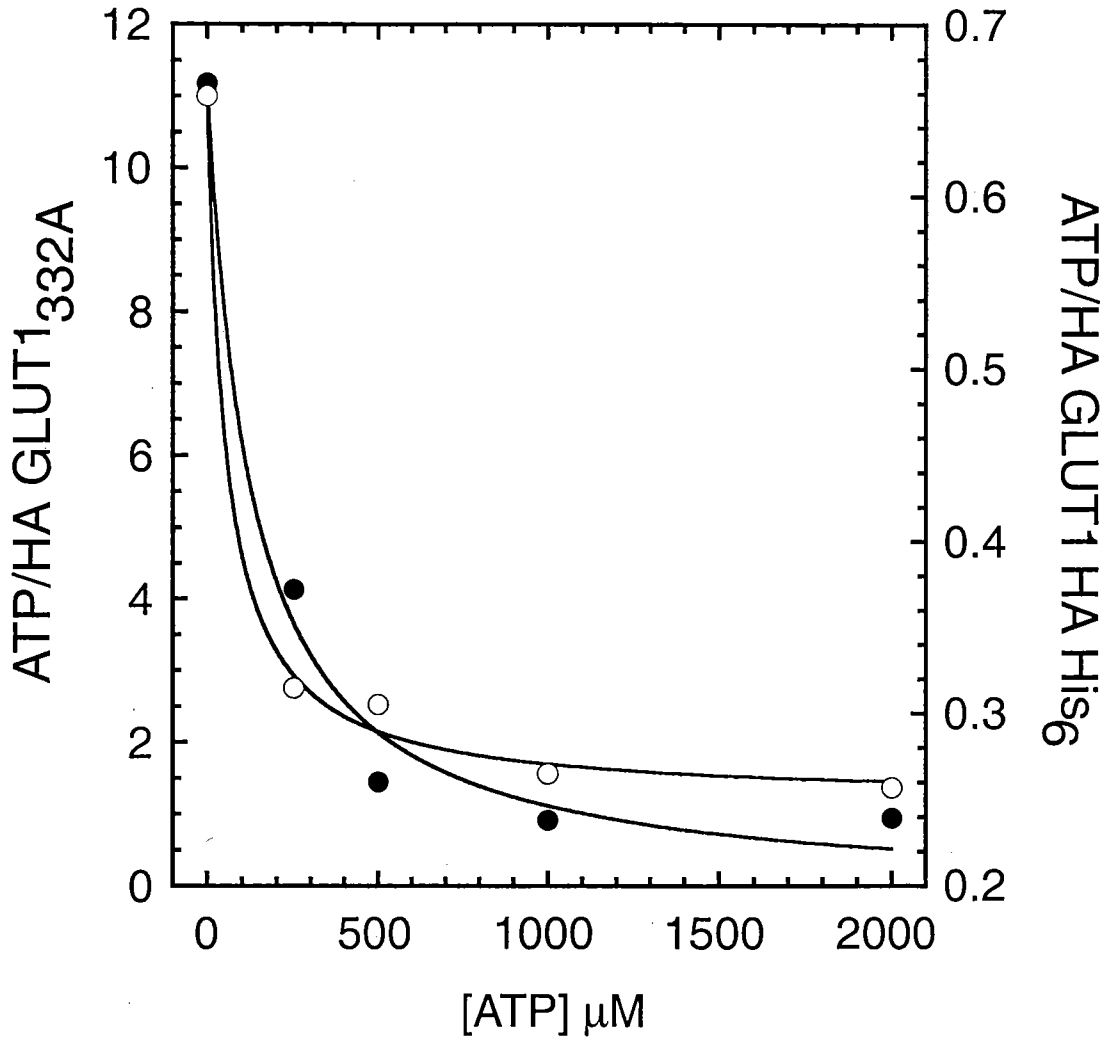


uncharged alanines, sequence hydrophobicity and subsequently ATP binding are increased. 3) Charge alterations loosen the structure of the binding pocket.

GLUT1 HA $His_6$ and 332A were also subjected to competition analysis of ATP inhibition of azidoATP photoincorporation. Affinity-purified GLUT1 HA $His_6$ and 332A were photolabeled with azidoATP in the presence of increasing concentrations of unlabeled ATP. Membranes were then subjected to SDS-PAGE, transferred to nitrocellulose and quantitated for azidoATP incorporation by autoradiography and densitometry. As with azidoATP photolabeling of isolated purified human erythrocyte glucose transport protein (Levine et al., 1998), photoincorporation of azidoATP into affinity purified GLUT1 HA $His_6$ and 332A is inhibited half-maximally by 50-150 $\mu$M ATP (Figure 16). With GLUT1 332A, however, photoincorporation of azidoATP in the absence of ATP is some 10-fold greater. This suggests that increased photoincorporation of azidoATP into mutant 332A results from increased efficiency of mutant GLUT1 labeling. HA-Ab immunoblot efficiency is unchanged by azidoATP photolabeling of GLUT1.

***Figure 16.*** **ATP-inhibition of azidoATP photolabeling of affinity purified GLUT1 HA His6** (○) **and GLUT1$_{332}$** (●). Purified, epitope-tagged GLUT1 was photolabeled as in Figure 14 in the presence of increasing unlabeled [ATP]. Proteins were resolved by SDS-PAGE and label incorporation and epitope-tagged GLUT1 were quantitated by densitometry of $^{32}$P-autoradiograms and Ha-Ab immunoblots. Ordinate: the ratio [$^{32}$P] incorporation: HA-Ab reactivity. Abscissa: [ATP] present during photolabeling in $\mu$M. Curves drawn through the points were computed by nonlinear regression assuming that inhibition of photolabeling shows simple saturation kinetics and is characterized by the expression: $\text{label}_{\text{incorporation}} = N - \frac{P[ATP]}{K_{i(app)} + [ATP]}$ where N is label incorporation in the absence of unlabeled ATP, P is the amount by which saturating unlabeled ATP inhibits label incorporation and $K_{i(app)}$ is that concentration of unlabeled ATP which reduces labeling by P/2. The following constants were obtained: GLUT1 HA His$_6$, N= 0.66 ± 0.01; P=0.41 ± 0.04, $K_{i(app)}$ = 54 ± 17 $\mu$M ATP; GLUT1$_{332\text{-}3A}$, N= 11.2 ± 0.7; P=11.3 ± 1.0, $K_{i(app)}$= 127 ± 49 $\mu$M ATP.

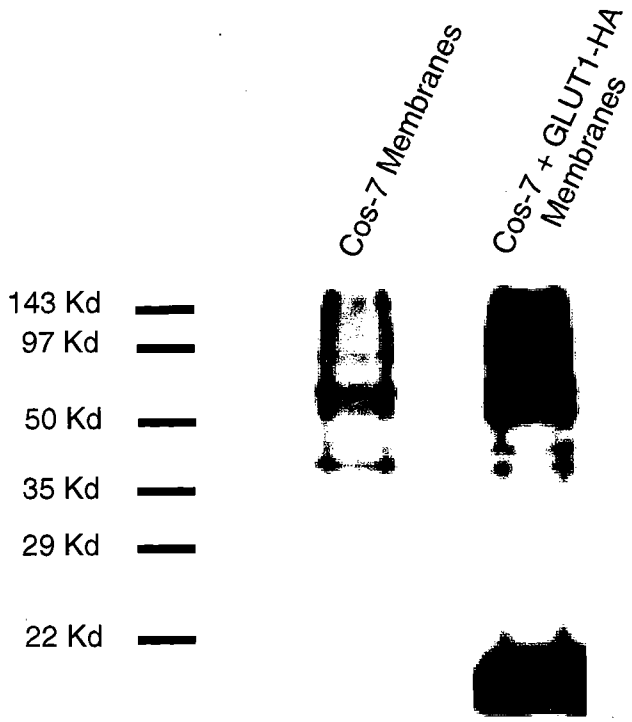
Figure 16


12
10
8
6
4
2
0
0.7
0.6
0.5
0.4
0.3
0.2
0
500
1000
1500
2000
[ATP] μM
ATP/HA GLUT1332A
ATP/HA GLUT1 HA His6

Parental Cos-7 GLUT1 may be able to form heterotetramers with GLUT1 HA $His_6$. This could make it difficult to visualize the effects of GLUT1 mutagenesis on azidoATP binding. For example, if 3 units of parental GLUT1 and 1 unit of tagged GLUT1 form a tetramer, alterations in labeling caused by alanine substitution in the single subunit may be partially camouflaged. To examine this possibility, Cos-7 membranes +/- GLUT1 HA $His_6$ were stripped of peripheral membrane proteins with an alkaline wash and labeled with azidoATP. Samples were resolved on 10% SDS-PAGE gels and photolabel incorporation was measured. The same quantity of total protein was found to bind 3-fold more azidoATP from cells transfected with GLUT1 HA $His_6$ than non-transfected cells (Figure 17). This suggests that parental Cos-7 GLUT1, present in preparations of nickel purified, epitope tagged hGLUT, should not obscure the affects of alanine mutagenesis on azidoATP binding.

***Figure 17.*** **AzidoATP labeling of Cos-7 cell membranes stripped of peripheral proteins.** Isolated Cos-7 cell membranes from control cells or from cells transfected with GLUT1 HA $His_6$ were labeled with azidoATP following removal of peripheral proteins by alkaline wash. Samples were resolved on 10% SDS PAGE gels and transferred to nitrocellulose. Protein contents were determined using a Pierce BCA assay kit, and 35 μg of total protein were loaded in each lane. Label incorporation was detected by exposure of membranes to autoradiographic film. AzidoATP binding was quantitated by densitometry using NIH Image. Membranes from GLUT1 HA $His_6$ transfected cells contain 3 fold more azidoATP label than membranes isolated from nontransfected cells.

Figure 17


Cos-7 Membranes
Cos-7 + GLUT1-HA Membranes
143 Kd
97 Kd
50 Kd
35 Kd
29 Kd
22 Kd

## *Discussion*

In this study we ask the question; Does alanine substitution at the three proposed GLUT1 consensus ATP binding sequences affect sugar transport and azidoATP binding? As a result of our alanine mutagenesis studies, we have expressed HA $His_6$ tagged human erythrocyte GLUT1 in Cos-7 cells. Intact Cos-7 cells expressing GLUT1 HA $His_6$ exhibit normal sugar transport under basal conditions. In addition, purification of the protein on a Ni-NTA column provides a transporter that is normal with respect to ATP binding capacity.

We compared 2-deoxyglucose (2DOG) uptake in Cos-7 cells +/- GLUT1 HA $His_6$ as a measure of epitope tagged GLUT1 function and/or cell surface expression. Transfection with GLUT1 HA $His_6$ (wild type) and 5 of the six mutant constructs (111A, 225A, 332A, 335A, and 341A) resulted in a two-fold stimulation of transport above basal Cos-7 cell levels, and levels observed in cells transfected with empty pcDNA3.1+ vector. This increase in transport is most probably the result of a 2-fold increase in cell surface transporter

expression. We tested this hypothesis by measuring δ-antibody binding to Cos-7 cells +/- GLUT1 HA $His_6$. We observe that cells expressing GLUT1 HA $His_6$ present 2-fold more δ-antibody reactive cell surface sites than untransfected Cos-7 cells. This result confirms our initial hypothesis that transport stimulation in transfected cells is a direct result of greater transporter presence at the plasma membrane.

In comparison, cells transfected with mutant 338A display unchanged sugar transport rates. This construct, as we will discuss later, retains its ability to bind azidoATP. This suggests that the oligomeric structure of 338A is not affected by alanine substitution, at least with respect to ATP binding. This hypothesis was examined by measuring [$^3$H]-CCB binding to membranes isolated from Cos-7 cells transfected with GLUT1 HA $His_6$, and 338A. Tagged GLUT1 (wild type) binds 2-fold more CCB than 338A or Cos-7 cells. These results suggest that some structural component of mutant 338A is altered (although ATP is still able to bind). Most probably, it is the region of GLUT1 that contains the closely related sugar efflux and CCB binding sites (Sultzman and Carruthers, 1999). Alterations in

the sugar-binding site might also explain the inability of 338A to transport sugar.

Two other possibilities could explain the inability of 338A to transport sugar. 1) Transporter trafficking to the plasma membrane may be disrupted. 2) The affinity of 338A for sugar may be altered. δ-antibody binding studies were used to test whether 338A is expressed at the plasma membrane. The data show that δ-antibody binding in 338A transfected cells is the same as that observed in untransfected Cos-7 cells. The lack of δ-antibody binding may be due to the absence of 338A at the plasma membrane or, because δ-antibody recognizes an exofacial epitope of tetrameric GLUT1, alanine substitution at 338-340 may disrupt GLUT1 oligomeric structure. The use of a configuration insensitive GLUT1 exofacial-Ab in this protocol may resolve this question.

Schurmann et al. observed that transport was lost in GLUT4 when conserved residues 333R/334R were converted to alanine (Schurmann et al., 1997). These results contrast with our work that shows normal transport activity in GLUT1 mutant 332A. This difference may be explained by their reconstitution of GLUT4 into

vesicles to measure uptake. In comparison, our experiments were carried out in intact Cos-7 cells. Substitution of alanine at this site results in a loss of charged residues. These amino acids might be responsible for retaining transporter topography in the membrane. In their absence, 333R/334R may lose topographical stability when exposed to detergent during reconstitution.

We assumed that the strategy of alanine mutagenesis would decrease, or ablate, azidoATP binding to GLUT1. However this was not the case; in fact two constructs (225A, and 332A) incorporate 1.5 to 10-fold more azidoATP than GLUT1 HA $His_6$ or the other mutants. This increase in labeling was somewhat unexpected and does not reflect altered affinity for ATP but rather, altered efficiency of photolabeling.

The overall increase in azidoATP labeling observed in 225A and 332A might be a result of alanine substitution reducing the steric hindrance that normally limits ATP access to the GLUT1 ATP binding pocket. Alternatively, the presence of additional alanines makes consensus sequences II, and III more hydrophobic. Increasing their similarity to the proposed consensus sequences may allow

more azidoATP to bind to the transporter. Finally, alterations in transporter charge may result in the loss of stabilizing cation-$\pi$ interactions (Gallivan and Dougherty, 1999), which could affect ATP binding. Assuming that increased ATP incorporation does not reflect a secondary action in which mutations introduced at a distal site nonspecifically alter ligand binding within the nucleotide-binding pocket, this effect might be consistent with a direct perturbation of the nucleotide-binding domain.

A large range appears to exist in the maximum amount of azidoATP that can be incorporated into mutant 332A. Densitometry of photolabeled 332A shows a 1.5-2.3 fold increase in azidoATP labeling (Figure15), as opposed to competition analysis (ATP inhibition of azidoATP incorporation) which suggests 10-fold higher azidoATP labeling (Figure16). This discrepancy may be a result of autoradiogram overexposure in the photolabeling experiments, which could alter the $^{32}P$ detection sensitivity of the technique.

The effects of mutating residues 332-335 on GLUT1 ATP binding are consistent with N-terminal sequence analysis of peptides obtained upon proteolysis of azido ATP-photolabeled GLUT1. These

studies indicate that the GLUT1 ATP binding domain lies between GLUT1 residues 301 and 364 (Levine et al., 1998). Since GLUT1 sequences 307 to 327 and 338 to 358 are strongly hydrophobic and thus putative membrane spanning α-helices (Mueckler et al., 1985), they are unlikely to be accessible to cytosolic ATP. In contrast, GLUT1 residues 332-335 and the remainder of the Walker B domain fall in the intervening (cytosolic) sequence between these membrane spanning regions and may be well-positioned for interaction with ATP and modulation of the sugar translocation pathway.

AzidoATP and ATP binding to purified human erythrocyte GLUT1 are mutually exclusive, suggesting that they bind at the same site on the protein (Carruthers and Helgerson, 1989). To determine that this is also the case in epitope tagged GLUT1, we labeled purified GLUT1 HA $His_6$ with azidoATP in the presence of 4 mM ATP. The absence of $^{32}P$ incorporation confirms the stereospecificity of azidoATP binding to tagged GLUT1.

It is probable that parental GLUT1 and tagged GLUT1 oligomerize to form tetrameric GLUT1. However, Cos-7 cell membranes transfected with GLUT1 HA $His_6$ bind 3-fold more

azidoATP than a comparable amount of protein from untransfected membranes. This suggests that the presence of parental GLUT1 in samples of affinity purified epitope tagged GLUT1 does not obscure changes in azidoATP binding that are a direct result of alanine mutagenesis.

# Chapter VI

# Significance & Future Directions

This thesis examines several hypotheses. First, we tested the capacity of various physiologic factors to influence ATP modulation of GLUT1 mediated sugar transport. Specifically, we looked at the effects of glycolytic intermediates, intracellular pH, and transporter oligomeric structure. Next we asked, is ATP interaction with GLUT1 sufficient for sugar transport modulation? Third, we investigated the location of the nucleotide binding domain on the human erythrocyte glucose transporter. Finally, having narrowed the possible location of the ATP binding site on GLUT1, we analyzed whether alanine mutagenesis altered sugar transport and ATP binding.

We have concluded that only $AMP_i$, and possibly $ADP_i$, inhibit $ATP_i$ regulation of GLUT1. Previous work in this laboratory has shown that $AMP_i$, and $ADP_i$, though unable to mimic the effects of $ATP_i$ compete with $ATP_i$ for access to the transport system (Carruthers and Helgerson, 1989). This suggests that the adenosine

moiety is responsible for binding, while phosphate chain length dictates the ability of a compound to modulate GLUT1 activity. The present study shows that adenosine alone is insufficient for modulation of human GLUT1 function.

An increase in glycolysis can lead to an elevation in cellular lactate levels and subsequent acidosis (Cairns et al., 1993). This encouraged us to examine how alterations in intracellular pH affect ATP regulation of GLUT1. From these studies, we observed that as pH decreases from 8.0 to 5.5, ATP modulation of GLUT1 is inhibited. We propose that this loss of ATP regulation results from altered binding kinetics (affinity or capacity of GLUT1 for ATP) or from the pH dependent binding of a regulatory molecule to the ATP-GLUT1 complex.

The data indicate that pH affects ATP-GLUT1 binding kinetics. Surprisingly, we found that low pH increases [$^{32}$P]ATP binding while the $K_{i(app)}$ for ATP-inhibition of photolabeling is unchanged. Merino et al. (Merino et al., 1997) has shown that pH also modulates the structure of the nucleotide-binding domain on $Ca^{2+}$-ATPase. Specifically, low pH was found to increase access to the ATP binding

site. A similar mechanism could explain the increase in azidoATP binding that we have observed in GLUT1 as pH is lowered from 8.0 to 5.5.

To better understand how low pH inhibits ATP regulation of GLUT1, we examined the ATP dependence of 3OMG uptake into red cell ghosts at pH 5.5 and 8.0. Uptake was found to increase in a saturable manner at pH 8.0. In contrast, 3OMG uptake at pH 5.5 was maximally stimulated at 200 μM ATP and inhibited at 2 mM ATP. Degradation of ATP at low pH was ruled out as a cause of this unexpected result.

We have concluded that ATP acts in an inhibitory fashion on sugar transport at low pH. In view of the increase seen in photolabel incorporation at pH 5.5, transport inhibition was unexpected. This result alluded to the possibility that low pH unmasks a unique transport-antagonistic ATP binding site on GLUT1. The exposure of such a site would account for both the increase in ATP binding and the inhibition of transport seen under acidic conditions. Transport inhibition could also be the result of GLUT1 interacting with a pH dependent inhibitory protein.

We have shown that GLUT1 reconstituted into proteoliposomes containing 2 mM ATP exhibits transport stimulation at subsaturating 30MG. We have therefore concluded that GLUT1, the lipid bilayer, and ATP are the only components required for ATP modulation of GLUT1 function. This same system could be used to rule out or confirm the presence of a pH inducible regulatory protein. If transport inhibition is seen in this system at pH 5.5, we can say with some certainty that a regulatory protein is not the agent arresting transport at low pH.

Wheeler (1989) was unable to recreate the ATP effect in a reconstituted system. His experimental design used the reduced or dimeric form of the transporter. In contrast, our studies employ the physiologic or tetrameric form of the transporter (Hebert and Carruthers, 1992). This laboratory has observed that exposure to reductant (dithiothreitol-DTT) inhibits ATP modulation of 30MG uptake. Specifically, conversion of tetrameric GLUT1 to its dimeric form results in loss of azidoATP labeling. This explains the disparity between the two data sets. We conclude that tetrameric GLUT1 is regulated by ATP.

Although GLUT1 functions as an ATP binding protein, the location of its nucleotide binding domain is currently unknown. Past experiments have suggested that covalent incorporation of azidoATP to GLUT1 falls within the range of C-terminal residues (270-456) (Carruthers and Helgerson, 1989). The peptide mapping studies carried out in this thesis identify residues 291-364 as a likely component of the GLUT1 azidoATP-attachment site. The strong hydrophobicity of GLUT1 presents a problem for estimating the molecular weight of GLUT1 fragments by gel mobility. To balance this uncertainty, we used internal markers (C-terminus, N-terminus, carbohydrate, and azidoATP), and proteolytic enzyme specificity to identify peptide fragments more definitively. More recent work in our laboratory has shown that ATP increases GLUT1 resistance to proteolytic digestion (Cloherty and Carruthers, unpublished observations). This may offer an explanation for the difficulty we encountered obtaining complete proteolytic digests of azidoATP labeled GLUT1.

Digestion of purified erythrocyte GLUT1 with endoproteinase

Lys-C produces a peptide fragment whose first ten amino acids were identified by N-terminal sequencing as GLUT1 residues 301-310. This sequence and the peptide mapping data allow us to further refine the proposed azidoATP-attachment site on GLUT1 to a cassette that contains residues 301-364. Importantly, this range of residues overlaps with a proposed Walker B motif in GLUT1.

We have shown that ATP competitively inhibits azidoATP binding to purified GLUT1 (Carruthers and Helgerson, 1989). This implies that azidoATP and ATP bind to the same site on the transporter. The proposed pocket structure of a nucleotide-binding domain (Fry et al., 1986) presents certain problems when using 8-azidoATP for peptide mapping. Specifically the azido moiety that sits on carbon 8 of adenine may crosslink to a peptide adjacent to the actual binding site. For this reason, GLUT1 peptide mapping experiments using 2-azidoATP should also be performed in an effort to identify other GLUT1 segments contributing to the nucleotide binding pocket. If 8-azidoATP & 2-azidoATP crosslink to adjacent peptides, digestion of 2-azido labeled GLUT1 might produce a significantly different pattern of labeled peptides.

To further delineate the location of the ATP binding site, we constructed alanine mutants for each of the three proposed consensus sequences on GLUT1 and measured azidoATP labeling of, and sugar transport by, each mutant GLUT1. This required the development of a GLUT1 expression system. Unfortunately, the yeast strain we created (RE700A + GLUT1 HA $His_6$) grew too slowly and produced insufficient GLUT1 for our purposes. Currently, more clones are being screened to select one with a high rate of growth and human GLUT1 expression.

This problem led us to develop a transient mammalian expression system for human GLUT1 using Cos-7 cells. Purification of HA $His_6$ tagged GLUT1 on a nickel column produced large quantities of a protein which run as a diffuse band with an approximate molecular weight of 55 kDa. Western blotting with GLUT1 C-Ab, and HA-Ab, identified this as epitope tagged human erythrocyte glucose transporter. In addition, in-vivo transporter was found to be normal with respect to sugar transport, and the isolated transporter was normal with respect to azidoATP binding.

Transfection of Cos-7 cells with epitope tagged wild type GLUT1, and five of the six alanine mutants, resulted in a 2-fold increase in 2-DOG uptake above basal Cos-7 cell levels. δ-Antibody binding studies have shown that cells transfected with GLUT1 HA $His_6$ present a 2-fold excess of GLUT1 at the plasma membrane relative to untransfected Cos-7 cells. This suggests that the transport stimulation we observed in Cos-7 cells expressing epitope tagged GLUT1, is the result of increased transporter expression at the cell membrane. As a cautionary note, one cannot rule out the possibility that these newly expressed proteins stimulate transport, in part, by having a higher rate of catalytic turnover than parental GLUT1. However, increased transport parallels quantitatively the change in cell surface binding of a polyclonal antiserum (δ-Ab) directed against exofacial epitopes of GLUT1. $K_{m(app)}$ for GLUT1-mediated 2DODG transport in human red cells is 15 mM (Stein, 1986). The measurements reported in the present study were made at 0.1 mM 2DOG. Transport is, therefore, given by

$$uptake = \frac{V_{max}}{K_{m(app)}}[2DODG]$$

where $V_{max}$ (the cellular 2DOG transport capacity) = $k_{cat}[GLUT1]_{cell\ surface}$ where $k_{cat}$ is the intrinsic catalytic turnover of GLUT1. Since both transport and $[GLUT1]_{cell\ surface}$ are doubled by Cos-7 cell transfection with GLUT1 HA $His_6$ this suggests that the $k_{cat}/K_{m(app)}$ ratio for expressed GLUT1 HA $His_6$ in Cos-7 cells is not significantly different from that of Cos-7 cell endogenous GLUT1.

338A was the only mutant whose catalytic activity may have been affected by alanine substitution. 30MG uptake by this construct was not significantly different from uptake in untransfected Cos-7 cells. This was suprising because these residues (338-340) are located in a proposed transmembrane domain of GLUT1 and are, like alanine, uncharged. The fact that this mutant retains its ability to bind azidoATP suggests that its oligomeric structure is unchanged with respect to the nucleotide binding site. In contrast, phloretin inhibitable photoincorporation of CCB into a 55 kDa protein in membranes expressing mutant 338A is not significantly different from that observed with membranes isolated from non-transfected cells. The absence of CCB binding implies that a portion of mutant 338A structure is disrupted. Because this

mutant retains azidoATP binding, the CCB labeling data suggest that the disrupted region in question encompasses the proposed GLUT1 CCB binding and sugar efflux sites, which are closely related (Sultzman and Carruthers, 1999).

The lack of 2-deoxyglucose uptake by the 338 mutant could result from disabled sugar transport, a result consistent with the loss of CCB binding. Alternatively, the transporter may not be expressed correctly at the plasma membrane. Incorrectly trafficked GLUT1 that is structurally intact, could still bind azidoATP in these studies because total solubilized Cos-7 cell membranes are applied to the nickel column for epitope tagged GLUT1 purification.

To test this possibility, cell surface δ-antibody binding was measured in Cos-7 cells transfected with mutant 338A. The results show that mutant 338A expressing cells bind the same amount of antibody as Cos-7 cells alone, but 2-fold fewer IgGs than cells expressing wild type epitope tagged GLUT1. The lack of δ-antibody binding may be due to the absence of 338A at the plasma membrane or alternatively, alanine substitution at 338-340 may convert tetrameric GLUT1 to dimeric GLUT1. In this instance δ-

antibody, which recognizes an exofacial epitope of tetrameric GLUT1, would be unable to recognize the protein. If this is the case, the affinity of mutant 338A for 2DOG or its ability to translocate 2DOG may be altered. Immunohistochemistry using a configuration insensitive exofacial GLUT1 antibody may resolve this question.

We therefore conclude that the lack of sugar transport in mutant 338A is a result of one or more of the following: 1) dysfunctional GLUT1 trafficking to the plasma membrane, 2) abnormal oligomeric structure in a small region of the transporter, and/or 3) an altered quaternary structure.

Originally, we hypothesized that conversion of an essential amino acid to alanine would knock out, or reduce, azidoATP binding to the transporter. Contrary to this expectation, 225A and 332A were found to bind 1.5 to 2.3-fold more azidoATP than GLUT1 HA $His_6$ or the other alanine mutants. It is possible that the charge alterations in these mutants loosen the structure of the transporter, resulting in increased access of the $N_3$ group of azidoATP to the ATP binding pocket. Another explanation is based on the small side chain found on alanine. In both 225A and 332A, alanine replaces

polar amino acids that contain large side chains. Removal of these barriers could reduce steric hindrances controlling access to the nucleotide binding pocket. Another possibility is related to the hydrophobic nature of Sequence III. This stretch of amino acids in GLUT1 was found to contain 50% homology to a Walker B box which is highly hydrophobic (Fry et al., 1986). Substitution of uncharged alanines for polar residues in GLUT1 (i.e. 332-334 GRR), increases the hydrophobic nature of this region. By making GLUT1 sequence III more like a true Walker B motif, we may have enhanced azidoATP binding to the transporter. In the absence of a change in the $k_i$ for ATP inhibition of azidoATP labeling in GLUT1 HA $His_6$ and mutant 332A, it is doubtful that the increased label incorporation that we have observed in mutants 225A, and 332A is due to altered transporter affinity for ATP. The reactive $N_3$ of azidoATP may simply be in closer proximity to (i.e. have increased probability of reacting with) side chains in the pocket.

Another obstacle that concerned us was the potential for parental GLUT1 oligomerization with tagged GLUT1 (both mutant and wild type). GLUT1 HA $His_6$ containing Cos-7 cell membranes

bound 3-fold more azidoATP than membranes from cells lacking tagged GLUT1. We propose that the presence of parental GLUT1 in these preparations should not obscure the affects of alanine mutagenesis.

We were particularly interested to observe that azidoATP incorporation into purified human erythrocyte GLUT1 at low pH increases in a manner similar to that found with epitope tagged GLUT1 mutant 332A purified from Cos-7 cells. $K_{i(app)}$ for ATP-inhibition of azidoATP GLUT1 HA $His_6$ labeling is not reduced, indicating that transporter affinity for ATP is not increased by mutation of these residues. AzidoATP labeling of red cell GLUT1 is increased 5 to 10-fold by lowering pH from 8.5 to 5.5 yet $K_{i(app)}$ for ATP-inhibition of labeling is unchanged (Levine et al., 1998). Histidine is the best candidate amino acid for side-chain protonation as pH is lowered from 8.5 to 5.5. GLUT1 contains 5 histidine residues of which four (H 160, 239, 337 and 484) are located within cytoplasmic domains. One of these, $H_{337}$, is found in the putative GLUT1 Walker B domain although direct involvement in pH-dependent modulation of azidoATP binding efficiency will require

confirmation by substitution mutagenesis. Why would $His_{337}$ protonation mimic $(alanine)_3$ substitution for $_{332}GRR$? One clue may be obtained from the hydrophobic GLUT1 sequence preceding the putative Walker B motif. This sequence contains a phenylalanine that could pair with $Arg_{333}$ or $Arg_{334}$ by cation-$\pi$ interactions (Gallivan and Dougherty, 1999). These interactions, which attain $\Delta G^{\circ}$ approaching –4 kcal/mol, can significantly stabilize protein structure (Gallivan and Dougherty, 1999). If the neighboring $His_{337}$ were protonated at reduced pH, it might disrupt Phe-Arg cation-$\pi$ interactions by charge repulsion of $Arg^{+}$. Removal of $Arg_{333}$ or $Arg_{334}$ by mutagenesis or protonation of $His_{337}$ could relax local GLUT1 structure permitting closer interaction of the *uv* activated azidoATP nitrene with GLUT1 side chains or backbone. This structural relaxation could increase the efficiency of label photoincorporation without changing the affinity of the transporter for nucleotide. While speculative, this hypothesis warrants experimental scrutiny.

Fluorescence resonance energy transfer (FRET) is another technique that can be used to further delineate the location of the GLUT1 nucleotide binding domain. We have collaborated with a

visiting organic synthetic chemist (Dr. Boris Yagen, Hebrew University, Jerusalem) to synthesize a fluorescent ATP derivative, anthraniloyl-ATP (AN-ATP). AN-ATP has an absorbance maximum at 343 nm and an emission maximum at approximately 450 nm. AN-ATP binding to 2 $\mu$M GLUT1 is half maximal at 7 $\mu$M AN-ATP with $k_{on}$ and $k_{off}$ constants of 0.3 per $\mu$M per min and 2.1 per min respectively (Cloherty, Yagen and Carruthers, unpublished). AN-ATP also mimics the ability of ATP to modulate GLUT1-mediated transport in red cells.

Using the intrinsic tryptophan fluorescence of GLUT1 (excitation at 298 nm, emission max 332 nm) to excite AN-ATP bound to GLUT1, we have observed fluorescence resonance energy transfer (FRET) between GLUT1 tryptophan residues and bound AN-ATP. AN-ATP fluorescence requires GLUT1 tryptophan fluorescence because in the absence of this energy source, AN-ATP shows extremely low fluorescence. Using this technique, we can map the location of the GLUT1 nucleotide-binding domain as it relates to tryptophan residues within the transporter. By utilizing Ni-NTA column purification of epitope tagged human GLUT1, we can

synthesize and isolate GLUT1 mutants in which tryptophan has been converted to glycine or leucine, thereby identifying tryptophan residues in close proximity to bound AN-ATP

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