Structural Domains That Contribute to Substrate Specificity in Facilitated Glucose Transporters Are Distinct from Those Involved in Kinetic Function: Studies with GLUT-1/GLUT-2 Chimeras†

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ABSTRACT: GLUT-2 differs from other members of the facilitated glucose transporter family because it transports a wider range of substrates and exhibits a higher K_m for transport of glucose analogs such as 2-deoxyglucose (2-DOG). In order to investigate the structural determinants of the unique substrate specificity and kinetic function of GLUT-2, recombinant adenoviruses were used to express native, mutant, and chimeric glucose transporters in the kidney cell line CV-1, yielding the following key observations. (1) A chimera consisting of GLUT-1 with the C-terminal tail of GLUT-2 had a K_m for 2-DOG of 9.9 ± 1.5 that was intermediate between that of native GLUT-1 (3.7 ± 0.4) and native GLUT-2 (26.3 ± 3.3). In contrast to the effect of the GLUT-2 C terminus on K_m for 2-DOG, this substitution did not confer enhanced uptake of three alternative substrates (fructose, arabinose, or streptozotocin) which are transported efficiently by native GLUT-2 but not by GLUT-1. (2) A chimera consisting of GLUT-2 with the N-terminal 87 amino acids of GLUT-1 exhibited no change in K_m for 2-DOG relative to native GLUT-2 but exhibited a significant reduction in capacity for transport of the three alternative substrates. (3) Mutation of asparagine 62 in GLUT-2 to glutamine produced a transporter lacking its N-linked oligosaccharide that exhibited a 2.5-fold increase in K_m for 2-DOG but equally efficient transport of the three alternative substrates relative to native GLUT-2. These data provide insight into structural domains that affect substrate specificity in facilitated glucose transporters and demonstrate that they are distinct from elements involved in glucose transport kinetics.

Glucose is transported into mammalian cells by members of a family of facilitated transporters known as GLUT-1−5, numbered in the order of their discovery (Pilch, 1990). The GLUT transporters share at least 40% nucleic acid identity (Bell et al., 1990) and appear to adopt a 12-transmembrane domain topology with intracellular N and C termini, as originally proposed from the hydropathy plot of GLUT-1 (Mueckler et al., 1985; Walmsley, 1988; Baldwin, 1992). The transmembrane domains are connected by loops of various lengths, and one asparagine is glycosylated in the first extracellular loop of GLUT-1, -3, and -4 (Brant et al., 1992). The first extracellular loop of GLUT-2 also contains the consensus sequence for N-linked glycosylation, but it has been suggested that the site is not modified as in other facilitated transporters (Brant et al., 1992).

Each transporter has a characteristic tissue distribution and kinetic profile. For example, GLUT-1 is widely distributed in most tissues and cell lines and has a relatively low K_m for transport of the nonmetabolizable glucose analogs 2-deoxyglucose (2-DOG)[1] and 3-*O*-methylglucose (3-O-MG) (Gould & Holman, 1993). GLUT-2 is found only in glucose-responsive tissues such as liver and β-cells of the islets of Langerhans and has the highest K_m for glucose, 2-DOG, or 3-O-MG among the GLUTs (Baldwin, 1990; Bell et al., 1990; Johnson et al., 1990; Thomas et al., 1992; Mueckler, 1994). The high K_m of GLUT-2 means that its rate of transport is linearly dependent upon the blood glucose concentration within the normal range (Johnson et al., 1990; Thorens et al., 1990). The different transporters also display differences in substrate specificity. Thus, while galactose is transported by GLUT-1−3, GLUT-2 has the lowest affinity and the highest transport rate for this sugar (Gould et al., 1991). GLUT-2 is also differentiated from GLUT-1, -3, and -4 by its ability to transport fructose (Okuno & Gliemann, 1986; Gould et al., 1991; Colville et al., 1993a,b). The substrate binding pocket of GLUT-2 may therefore be less sterically restrictive (or more flexible) than that of the other GLUTs. Consistent with this concept, we have recently shown that the glucose analog streptozotocin [2-deoxy-2-(3-methyl-ϵ-nitrosourido)-D-glucopyranose] is transported by

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[1] Abbreviations: 2-DOG, 2-deoxy-D-glucose; 3-O-MG, 3-*O*-methyl-D-glucose; ATB-BMPA, 2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis(D-mannos-4-yloxy)-2-propylamine; βGal, β-galactosidase; BSA, bovine serum albumin; DMEM, Dulbecco's Modified Eagle's Medium; DTT, dithiothreitol; ER, endoplasmic reticulum; FCS, Fetal Calf Serum; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HRP, horseradish peroxidase; MOI, multiplicity of infection, viral pfu (plaque-forming units) divided by the number of cells infected; MTT, *C*,*N*-diphenyl-*N*′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SD, standard deviation; SDS, sodium laurel sulfate; SEM, standard error of the mean; SOE, gene splicing by overlap extension; streptozotocin, 2-deoxy-2-(3-methyl-ϵ-nitrosourido)-D-glucopyranose; Tris, tris(hydroxymethyl)aminomethane.

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GLUT-2 but not by GLUT-1 (Schnedl et al., 1994).

Site-directed mutagenesis has identified arginines 333 and 334 of GLUT-4 (Wandel et al., 1995) and numerous residues of GLUT-1 [W412 (Katagiri et al., 1991), N415 (Ishihara et al., 1991), W388 (Garcia et al., 1992), Q161 (Mueckler et al., 1994b), Y293 (Mori et al., 1994), C-terminal cysteines C347, C421, and C429 (Wellner et al., 1995), and P196 (Wellner et al., 1991)] that are required for 2-DOG transport. A V197I mutation in GLUT-2 from a patient with non-insulin-dependent diabetes mellitus (NIDDM) also results in loss of 2-DOG transport (Mueckler et al., 1994a). These residues as well as others required for the binding of competitive inhibitors are all located between the fifth transmembrane domain and the C terminus of the transporter. The catalytic residues believed to form the substrate pore are therefore clustered in the C-terminal half of the GLUTs. The major functions proposed for the N-terminal half of GLUT-1 are to serve as a scaffold upon which the active C-terminal half folds (Cope et al., 1994) and to promote oligomerization of GLUT-1 to a tetramer (Zottola et al., 1995).

Mutant and chimeric transporters have also been used to identify structural domains of GLUT-2 that contribute to its high K_m for glucose uptake. A glucose transporter consisting of GLUT-1 with a GLUT-2 C-terminal tail transported glucose with lower affinity (higher K_m) than GLUT-1, although comparison with native GLUT-2 was complicated by a low expression efficiency of this transporter (Katagiri et al., 1992). Similar studies on GLUT-4/GLUT-2 chimeras confirmed the effect of the GLUT-2 C terminus but also identified other segments of the C-terminal half of GLUT-2 as important for the transporter's high K_m for glucose (Buchs et al., 1995).

While these studies have provided some insights, a number of outstanding issues remain. For example, the structural domains that determine the broader substrate profile of GLUT-2 have not been identified. Further, the relationship between regions of GLUT-2 that govern its substrate specificity and those that confer its high K_m have not been explored. Finally, evidence for N-linked glycosylation of GLUT-2 has not been presented, and if it occurs, the role of such a modification in determining the kinetic function or substrate preference of GLUT-2 is unknown. In the current study, we have addressed these and other issues by using recombinant adenoviruses to express native, mutant, and GLUT-1/GLUT-2 chimeric glucose transporters in mammalian cells.

MATERIALS AND METHODS

Materials. L-Glucose was purchased from Fluka, and all other nonradioactive chemicals were purchased from Sigma. ^{3}H-labeled L-glucose, 2-DOG, 3-O-MG, D-galactose, and ^{14}C-labeled D-fructose were from DuPont. ^{14}C-labeled L-arabinose and ^{3}H-labeled D-xylose were from American Radiolabeled Chemicals. Rabbit anti-rat GLUT-1 and GLUT-2 antibodies were purchased from East Acres Biologicals and were raised against the respective C-terminal peptides. The secondary antibody used for Western blotting was HRP-conjugated goat anti-rabbit IgG and was purchased from Amersham.

Construction of Mutant and Chimeric GLUTs. All of the native, mutant, and chimeric GLUT constructs described in this section were validated by complete sequence analysis prior to functional studies. To mutate asparagine 62 of GLUT-2 to glutamine (N62Q) and thereby create a glycosylation-deficient GLUT-2, a fragment called G25′ consisting of the 5′ half of the rat GLUT-2 cDNA (Johnson et al., 1990; Hughes et al., 1992) encoding the N-terminal half of the GLUT-2 protein (amino acids 1−280) was treated with *Sal*I and subcloned into similarly treated M13mp19. The N62Q mutation was engineered with the double-primer method (Zoller & Smith, 1984) using the oligonucleotide N62Q (AACTATGACATC**C**A**G**GG**T**AC**C**GACACCCCACTC, base changes from the native cDNA in bold). The 5′ GLUT-2-N62Q fragment was excised from M13mp19 with restriction endonucleases *Cla*I and *Sal*I and was then ligated with the remainder of the GLUT-2 cDNA (G23′, cut with *Sal*I and *Hind*III) into the pACCMV.pLpA adenovirus vector (Gomez-Foix et al., 1992). This plasmid was designated pacCMV-GLUT-2-N62Q and encodes the protein GLUT-2-N62Q in Figure 1.

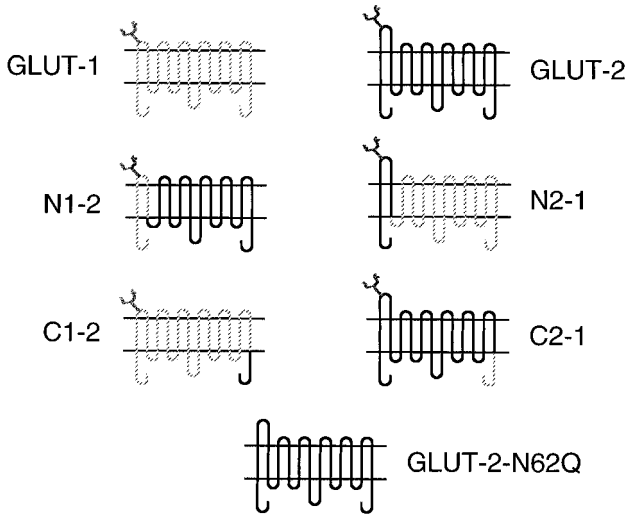

FIGURE 1: Schematic representation of expressed native, mutant, and chimeric glucose transporters. Regions of glucose transporters comprised of the GLUT-2 sequence are shown in black, and regions comprised of GLUT-1 are shown in gray.

Chimeric GLUTs were created with rat GLUT-2 and human GLUT-1 sequences. This approach was valid because human GLUT-1 is approximately 98% identical to rat GLUT-1 (Bell et al., 1990), including exact identity in the C-terminal tail. To create a GLUT-2 protein with a GLUT-1 N terminus, polymerase chain reaction (PCR) splicing by overlap extension (SOEing) (Horton et al., 1990) was employed. An amplified fragment called N12AB was generated from the human GLUT-1 cDNA (graciously provided by G. I. Bell, University of Chicago) with the forward oligonucleotide SpIgIpcr (ATCGATTTGCATGCGGGGTCGGAGTCAGAGTCGCAGTGGG) that hybridizes to nucleotides 50−68 and the reverse oligonucleotide N12B (CCGAGTTTGTTAACGAAAGGCCC) that hybridizes to nucleotides 426−442. A second PCR fragment called N12CD was generated by amplification of the G25′ template (see above) with a forward oligonucleotide called N12C (TTTCGTTAACAAACTCGGAA) that hybridizes at nucleotides 460−471 and a reverse oligonucleotide called L12F (CTCTAGAGTCGACGCCTC) that hybridizes to sequences in the cloning cassette of the M13mp19 vector. The N12AB and N12CD fragments were united by PCR SOEing to create

the chimeric fragment N12ABCD, which was subcloned into pBLUESCRIPT (Stratagene, La Jolla, CA). To complete the cDNA for this chimera, the N12ABCD fragment was excised from pBLUESCRIPT with *Bam*HI and *Sal*I and ligated with G23′ into pACCMV.pLpA. The resultant vector was designated paCCMV-N1-2 and encodes the N1-2 protein in Figure 1.

The inverse of the N1-2 transporter was created with a forward PCR primer called G2F (TTCA**GA**TC**T**GCTTGCTCCTCCTCCTACAATG) that hybridizes to the cloning cassette of M13mp19, and nucleotides 1−27 of G25′, introducing a *Bgl*II site, and a reverse primer called H1G2pcr (CCTTCCGAGTTTG**TTA**ACAAGCCACCCACCAAAG) that hybridizes to nucleotides 441−457 of GLUT-2, introducing a *Hpa*I restriction site, were used with the G25′ template to make fragment N21AB, which was subcloned into pBLUESCRIPT and excised with restriction endonucleases *Bgl*II and *Hpa*I. The GLUT-1 cDNA was treated with *Hpa*I and *Hind*III to generate a fragment of 1290 bp that encodes amino acids 88−492 and includes 80 nucleotides of the 3′ untranslated region. These two fragments, N12AB and GLUT-1 88−492, were ligated into *Bam*HI- and *Hind*III-treated pACCMV.pLpA to construct pacCMV-N2-1, which encodes the N2-1 protein in Figure 1.

Two C-terminal chimeras were also constructed. The first, a GLUT-2 protein with the GLUT-1 C-terminal intracellular domain, was constructed from a GLUT-2 fragment called C21AB which was generated with the forward oligonucleotide C21A (GAGATGAGGAAAGAAAAGGAAG) that hybridizes to nucleotides 925−946 and the reverse oligonucleotide C21B (CTCAGGAACTTTAAAAAATGTGAACAG) that hybridizes to nucleotides 1537 −1566 of GLUT-2 and to nucleotides 1530−1543 of GLUT-1. The second fragment, C21CD, was created by PCR amplification of the GLUT-1 cDNA with the forward oligonucleotide C21C (TTCACATTTTTTAAAGTTCCTGAGACT) that hybridizes to nucleotides 1530−1545 of GLUT-1 and to nucleotides 1537−1552 of GLUT-2 and the reverse oligonucleotide LEC53 (GTCAGGTTTGGAAG**CT**TCATCCAGC) that hybridizes to nucleotides 1724−1748 in the 3′ untranslated region of GLUT-1 and introduces a *Hind*III restriction site. The two fragments were united by SOEing, and the resulting fragment, C21ABCD, was then cloned into *Cla*I- and *Hind*III-treated paCCMV. pLpA with the *Cla*I- and *Sal*I-treated G23′ fragment (*Cla*I-*Sal*I-*Hind*III) to create the construct paCCMV-C2-1, which encodes the C2-1 protein.

Finally, the inverse of the C2-1 protein was made by PCR after engineering of silent restriction sites into the GLUT-1 and GLUT-2 cDNAs. Oligonucleotides FClaG1 (TACTCCAC**ATCG**ATCTTCGAGAAG), which hybridizes to nucleotides 1056−1079 of GLUT-1, and C12C (TTCATCTTCACCTACTTCAAAGTTCCAGAAACCAAAGGAAAG), which hybridizes to nucleotides 1509-1529 of GLUT-1 and 1549−1567 of GLUT-2, served as primers to amplify fragment C21AB. The second fragment was amplified from the GLUT-2 cDNA with forward oligonucleotide C12B (GGAACTTTGAAGTAGGTGAAGATGAA), which hybridizes to nucleotides 1549−1567 of GLUT-2 and 1056−1079 of GLUT-1, and the reverse oligonucleotide LEC53 (see above). The two fragments were joined by PCR SOEing to form C12ABCD and ligated to the remainder of GLUT-1 in pACCMV.pLpA to create paCCMV-C1-2, which encodes the C1-2 protein.

Cell Culture. All cell lines were propagated in 37 °C humidified incubators with 5% CO_2. Human embryonic kidney 293 and monkey kidney CV-1 cells were grown in DMEM (Gibco) containing 25 mM glucose supplemented with 10% FCS, 100 units/mL penicillin, and 100 mg/mL streptomycin. RIN 1046-38 cells (Clark et al., 1990) were grown in medium 199 (Gibco) supplemented with 5% FCS, 100 units/mL penicillin, and 100 mg/mL streptomycin.

Preparation and Use of Recombinant Adenoviruses. Recombinant adenoviruses containing the various glucose transporter cDNAs were prepared by previously described methods (Becker et al., 1994b). Briefly, pACCMV.pLpA vector constructs containing the cDNAs encoding the native or mutant glucose transporters were cotransfected with the pJM17 plasmid (McGrory et al., 1988) into 293 cells, and cell lysates containing the recombinant virions were collected and stored as described (Becker et al., 1994b). The viruses were named with the prefix AdCMV- and a suffix corresponding to the specific native or mutant glucose transporter cDNA as listed in Figure 1.

CV-1 cells were treated with serial dilutions of each viral stock, and expression of the transgenic protein was monitored by Western blot. A 1 h treatment of 70−90% confluent CV-1 cells with a range of multiplicities of infection was run 2 days prior to an experiment. Cells were then rinsed with PBS, returned to their regular medium, and replaced in the incubator. RIN cells at 30−50% confluence received the appropriate MOI of adenovirus 1 day before the experiment, and treatment was continuous for 24 h until the start of each experiment. Control experiments were conducted with a virus containing the gene for bacterial β-galactosidase (AdCMV-βGal; Herz & Gerard, 1993). Control transgene expression was confirmed with X-Gal staining.

Trypsin and N-Glycanase Treatment. Intact CV-1 cells expressing various transporters were subjected to extracellular digestion with 20 μg/mL TPCK-treated trypsin as described (Thorens et al., 1993). In other experiments, CV-1 cells were treated with 1.2 units of *N*-glycanase (genzyme) in 80 μL of 0.5% NP-40, 0.2% SDS, 2 TIU/mL aprotinin, 10 mM AEBSF (Calbiochem), and 1 mg/mL antipain in 0.1 M Tris at pH 7.6 and 37 °C for 2 h.

In Vitro Transcription and Translation. Transporter cDNAs in the psp73 vector (Promega) were transcribed from the Sp6 promoter and translated *in vitro* in the presence of [^{35}S]-methionine and [^{35}S]-cysteine with the Sp6 TNT kit (Promega).

Western Blots. Cells were scraped from tissue culture plates, washed and resuspended in 0.05−0.50 mL of PBS, and lysed by freezing and thawing. Samples containing a measured amount of total protein (Bradford Assay, BioRad, Hercules, CA) were digested for 10 min with 20 units/mL RQ1 RNase and DNase ONE (Promega), followed by addition of loading buffer containing DTT and SDS. Samples were run on Tris-Glycine 10% Bio-Rad Ready Gels, transferred to Immobilon P membrane (Millipore), and probed with anti-GLUT-1 or anti-GLUT-2 antibodies. Blots were developed with ECL reagents (Amersham).

Northern Blot. Total RNA was isolated with Triazol (Gibco) from CV-1 cells. RNA was electrophoresed on a 1.2% agarose gel containing formaldehyde and 2.2 M formalin, transferred to nitrocellulose by capillary elution with 20× SSC solution (Sambrook et al., 1989) and

hybridized with a randomly primed, ^{32}P cDNA probe that included the entire N2-1 cDNA.

Sugar Transport Assays. The assay for sugar uptake was a modification of the method of Hsu and Molday (1991). Following treatment with adenovirus, $9-20 \times 10^6$ CV-1 cells were detached from tissue culture plates with 100 mM EDTA in PBS, suspended in 15 mL of 37 °C assay buffer (114 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.16 mM $MgSO_4$, 20 mM HEPES, 2.5 mM $CaCl_2$, and 0.2% BSA at pH 7.2), and centrifuged gently to pellet cells. The pellet was resuspended in 10 mL of assay buffer and incubated in a 37 °C water bath under a light stream (<1 psi) of 90% O_2/10% CO_2 for 15 min. Following a second centrifugation and resuspension in 2−3 mL of assay buffer, cells were returned to the 37 °C water bath, where they remained for the duration of the experiment (1−2.5 h).

To initiate sugar uptake, 40 μL of the cell suspension was pipetted into a 1.5 mL microcentrifuge tube prewarmed to 37 °C and containing 10 μL of 5× reaction mixture (5× the desired concentration of substrate plus 5−25 mCi/mmol of ^{3}H-labeled 2-DOG, 3-O-MG, L-glucose, D-xylose, or D-galactose or 1−12.5 mCi/mmol of ^{14}C-labeled D-fructose or L-arabinose tracer in assay buffer). One milliliter of stop solution (RPMI medium without glucose at room temperature, containing 25 μM cytochalasin B and 400 μM phloretin) was added to quench the reaction after an incubation period of 2 s to 5 min (measured with a metronome or stopwatch). Background uptake for the zero time point was determined by adding the stop solution before the cell suspension. The cells were then pelleted by a 6 s spin in a microcentrifuge, resuspended in 1 mL of fresh stop solution, repelleted with another 6 s spin, and dissolved in 50 μL of 0.1 N NaOH. Reaction tubes were stored at −20 °C, thawed, and then acidified with 10 μL of 17.4 M acetic acid. Radioactivity was measured in 10 mL of Biosafe scintillation fluid (Research Products International). Data were normalized to total cellular protein.

Rates of uptake were derived from least-squares fitting (by the LINEST function of Microsoft Excel 5.0) of the linear portion of the uptake measurements at various concentrations of substrate. Measurements of the L-glucose uptake rate were performed at each concentration and were subtracted from the rates obtained with 2-DOG or 3-O-MG. Note that L-glucose uptake may not be a complete control for 2-DOG or 3-O-MG "leakage" or transbilayer diffusion but should be viewed as a general control for cell integrity and GLUT stereospecificity. L-Glucose uptake rates remained below 10% of substrate uptake rates for concentrations of substrate of ≤60 mM.

Streptozotocin Killing of RIN Cells. RIN cells were uniformly seeded in 96-well tissue culture dishes and treated with adenoviruses as described above. Medium was replaced with 0−20 mM streptozotocin in 100 μL of medium 199 and placed in the incubator for 24 h. The streptozotocin solution was replaced with medium 199 containing 0.5 mg/mL *C*,*N*-diphenyl-*N*′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and the cells were returned to the incubator for 1 h. One hundred microliters of 2-propanol with 0.04 N HCl was then added to each well, and the optical density at 595 nm was measured with a spectrophotometer. Cell survival was normalized to the optical density of cells expressing the same transgene but not treated with streptozotocin.

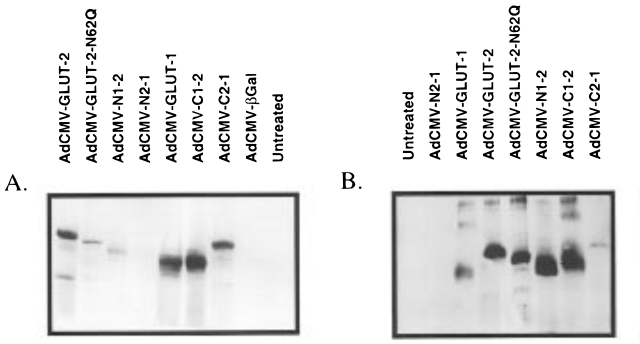

FIGURE 2: Expression of native, mutant, and chimeric glucose transporter proteins in CV-1 and RIN 1046-38 cells. CV-1 cells (A) or RIN 1046-38 cells (B) were either not treated (untreated) or treated with the indicated recombinant adenoviruses and harvested 2 days later for Western blot analysis. For both panels, 10 μg of protein was loaded per lane, and the blots were hybridized with a mixture of anti-GLUT-1 and anti-GLUT-2 antibodies, as described in Materials and Methods.

RESULTS

Expression of Glucose Transporters in CV-1 Cells. Wild-type, mutant, and chimeric glucose transporters corresponding to the structures depicted in Figure 1 were expressed in CV-1 and RIN cells by transduction with recombinant adenoviruses. Western blots of expressed proteins are shown in Figure 2. When untreated or treated with AdCMV-βGal, neither CV-1 nor RIN cells express proteins that are detected by the anti-GLUT-2 or GLUT-1 antibodies. GLUT-1 but not GLUT-2 protein could be detected in both cell lines by allowing development with the ECL reagent to occur under less stringent conditions (data not shown), consistent with previous observations (Ferber et al., 1994).

The expressed wild-type GLUT-1 has an apparent molecular mass of 45 kDa. The higher apparent molecular mass of 60 kDa for GLUT-2 is partly explained by the presence of 32 extra amino acids in the first extracellular loop of this transporter relative to human GLUT-1. Although the change in mobility is larger than predicted by the difference in molecular mass, our findings are consistent with those of other investigators. The anomalous migration patterns of glucose transporters on SDS−PAGE gels has been ascribed to variable glycosylation and hydrophobicity of the transporters (Mueckler et al., 1985; Thorens et al., 1988). C2-1, which includes the extra amino acids, has the same mobility as wild-type GLUT-2, while N1-2 and C1-2 proteins have the same mobility as GLUT-1 because they contain the shorter GLUT-1 N terminus.

The N2-1 Transcript is Translated in Vitro. Buchs et al. (1995) have reported that mRNAs encoding chimeric transporters in which the GLUT-2 N terminus was placed on the GLUT-4 protein were nonfunctional when injected into *Xenopus* oocytes, but no information about the expression levels of these transporter proteins was provided. In the current study, attempts to express the analogous GLUT-2/GLUT-1 transporter construct by treatment of CV-1 or RIN cells with AdCMV-N2-1 (Figure 2) or transient transfection with the pACCMV-N2-1 plasmid (data not shown) failed to produce detectable transporter protein. Treatment of a variety of other cell lines (A549, CHO, or INS-1) with AdCMV-N2-1, also failed to cause expression of protein reactive with the anti-GLUT-1 antibody (data not shown). Complete sequence analysis of the N2-1 cDNA revealed that

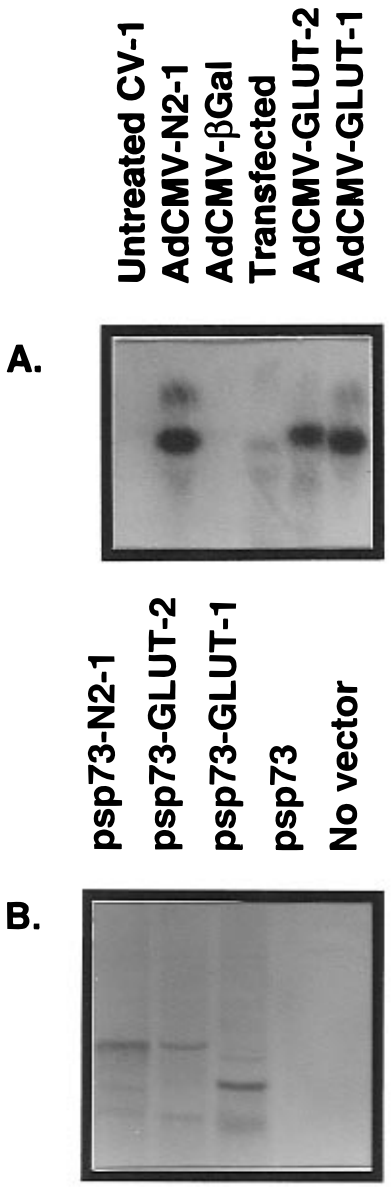

FIGURE 3: Expression of N2-1 mRNA in CV-1 cells and its *in vitro* translation. (A) Cells were either not treated (untreated), treated with the indicated recombinant adenoviruses, or transiently transfected with the pACCMV-N2-1 expression vector by Ca_2PO_4 precipitation and harvested for isolation of total RNA 48 h later (transfected). The RNA blot was then probed with randomly primed N2-1 cDNA. (B) *In vitro* transcription and translation were carried out as described in Materials and Methods, and ^{35}S-labeled proteins were resolved by SDS−PAGE and developed as an autoradiogram. Lanes: no vector, *in vitro* translation reaction performed without added template; psp73, *in vitro* translation reaction performed with vector only; psp73-GLUT-1, *in vitro* translation reaction performed with expressed native GLUT-1 transcript; psp73-GLUT-2, *in vitro* translation reaction performed with expressed native GLUT-2 transcript; psp73-N2-1, *in vitro* translation reaction performed with expressed N2-1 transcript.

the lack of expression was not attributable to a mutation in the insert. In order to more fully understand the mechanism responsible for the failure to express this construct, we attempted to detect N2-1 mRNA in AdCMV-N2-1-treated CV-1 cells. Figure 3A shows that treatment with the virus or transient transfection with the pACCMV.pLpA plasmid containing the N2-1 cDNA resulted in the appearance of an mRNA of the predicted size that hybridizes to the appropriate cDNA probe. The capacity of this mRNA to be translated was then tested by an *in vitro* assay. The autoradiogram of Figure 3B proves that, at least when transcribed and translated *in vitro*, the cDNA of N2-1 encodes a protein of the predicted size. Thus, the failure to synthesize N2-1 protein within adenovirus-treated cell lines is likely attributable either to a failure to translate its mRNA in the intracellular environment or to inherent instability of the protein, leading to its rapid degradation.

Expression of Chimeric Transporters at the Cell Surface. In order to ascertain whether the expressed proteins with GLUT-2 N termini were correctly delivered and inserted into the plasma membrane of CV-1 cells, whole cells were treated with a low dose of trypsin. This assay was chosen in lieu of treatment with photosensitive ligands such as 2-*N*-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA) that have been used to quantify cell surface GLUT-1 or GLUT-4 (Clark & Holman, 1990; Clark et al., 1991; Palfreyman et al., 1992; Vannucci et al., 1992; Yang et al., 1992; Lund et al., 1993; Mori et al., 1994; Kozka et al., 1995; Araki et al., 1996) because such photolabels fail to bind efficiently to GLUT-2 (Wandel et al., 1995; K. Timmers and S. Cushman, personal communication). Trypsin treatment has been shown to digest plasma membrane resident GLUT-2 into a lower-migrating band due to a cleavage site in its large extracellular loop, while leaving intact any protein still in transit from the ER (Thorens et al., 1996). The GLUT-1 portion of N2-1 is reported to contain an extracellular trypsin site as well (Coderre et al., 1995), but this site is not cleaved at the low concentrations of trypsin used in these experiments. Figure 4 demonstrates efficient trypsin digestion of native GLUT-2, GLUT-2-N62Q, and C2-1 proteins from whole CV-1 cells and confirms that all of these proteins are correctly delivered to the plasma membrane with their trypsin epitope situated extracellularly. In addition, the migration of the GLUT-2-N62Q protein coincides with that of *N*-glycanase-treated GLUT-2 (Figure 4), confirming that loss of glycosylation fully explains the mobility of GLUT-2-N62Q, and providing the first direct demonstration of glycosylation of GLUT-2.

Effect of Viral MOI on Kinetic Function. Recent targeting studies with expressed GLUT-1/GLUT-4 chimeras have suggested that the level of expression of the transporters can affect results, especially in the case of vast overexpression (Marsh et al., 1995). To determine if the level of expression could impact kinetic function, we measured the apparent K_m for 2-DOG uptake for a range of multiplicities of infection (MOIs) for all of the adenovirus constructs. In CV-1 cells treated with AdCMV-GLUT-1, a constant K_m of approximately 4 mM was obtained over the range of MOIs from 0.05 to 5, but above this threshold, a surprising increase in K_m was observed (Figure 5A). The K_m for 2-DOG uptake of CV-1 cells treated with AdCMV-GLUT-2-N62Q also was dependent upon viral titer. MOIs of less than 1 did not achieve sufficient levels of GLUT-2-N62Q expression to mask the kinetic contribution of endogenous GLUT-1, while very high titers caused a similar increase in K_m as observed with AdCMV-GLUT-1 (Figure 5B). Similar findings were obtained with viruses encoding all other active transporters. On the basis of these data, all subsequent kinetic studies were performed following treatment of cells with an MOI that was at the center of the range over which the K_m was constant. For all viruses, the MOI chosen caused clear expression of the desired protein on the basis of Western blot analysis. Note that, despite our ability to effectively titrate expression

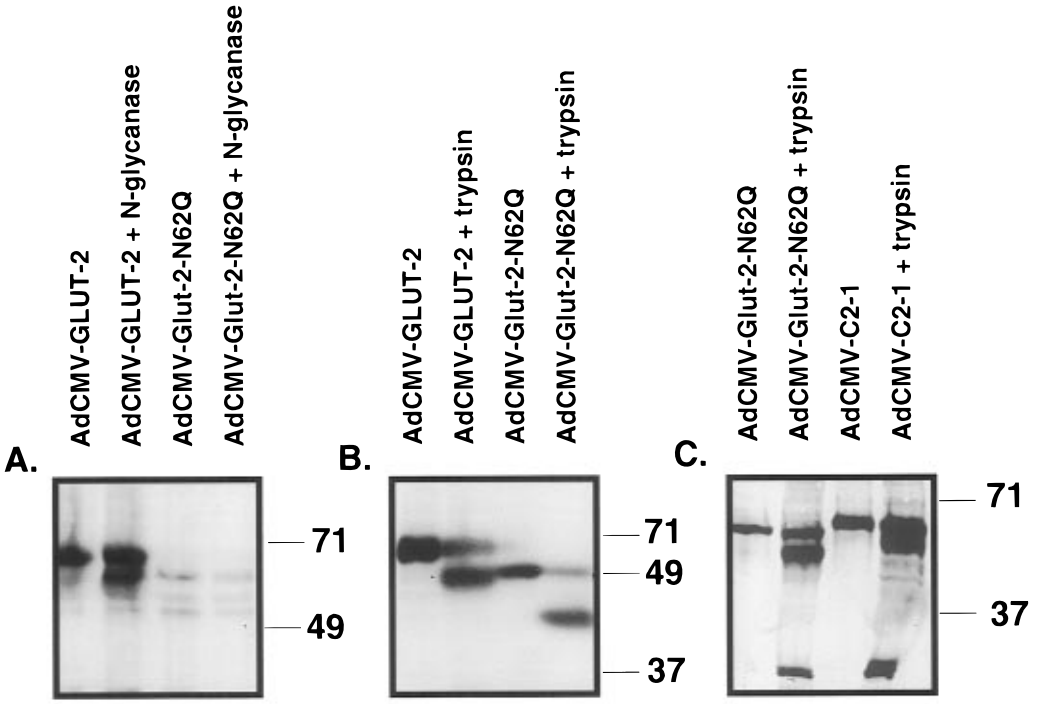

FIGURE 4: Trypsin and *N*-glycanase treatment of CV-1 cells expressing native, mutant, and chimeric glucose transporters. (A) CV-1 cells were treated with the indicated adenoviruses and, 48 h later, not treated or treated with *N*-glycanase as described in Materials and Methods. Following *N*-glycanase treatment, whole cell extracts were resolved by SDS−PAGE and blots were treated with anti-GLUT-2 antibody. Note that *N*-glycanase-treated GLUT-2 has the same mobility as untreated or *N*-glycanase-treated GLUT-2-N62Q, indicating that GLUT-2 is glycosylated and that GLUT-2-N62Q is not. (B and C) CV-1 cells were treated with the indicated adenoviruses, and 48 h later, intact cells were either not treated or treated with trypsin, as described in Materials and Methods. Following trypsin treatment, whole-cell extracts were resolved by SDS−PAGE and blots were treated with anti-GLUT-2 antibody (B) or a mixture of anti-GLUT-2 and anti-GLUT-1 antibodies (C). Note that the three glucose transporters with the GLUT-2 sequence at their N terminus (native GLUT-2, GLUT-2-N62Q, and C2-1) are all sensitive to trypsin digestion, indicating that these proteins were correctly inserted in the CV-1 cell plasma membrane with the correct display of the first extracellular loop.

of the transporters, no attempt was made to calculate V_{max} in the following experiments because the use of two different antibodies in Western blotting and the failure of GLUT-2-based transporters to bind available photolabels precluded accurate comparison of expression levels.

The reasons for the paradoxical increase in K_m at high levels of expression of the transporters are not known. One possibility might have been that very high levels of transporter expression could cause 2-DOG phosphorylation to become saturated, thereby impacting the apparent K_m. We feel that this is unlikely, given that experiments conducted with AdCMV-GLUT-1 and 3-O-MG as substrate showed the same increase in K_m as observed with 2-DOG at high MOIs (data not shown). An alternative explanation is that very high levels of expression promote oligomerization of transporters. This idea is based on the fact that the oligomeric state of GLUT-1 has been assigned a role in that transporter's kinetic function (Pessino et al., 1991; Hebert & Carruthers, 1992; Coderre et al., 1995; Zottola et al., 1995). Whether such mechanisms are operative in our system remains to be established.

Kinetics of 2-DOG Uptake. K_m values for 2-DOG or 3-O-MG were determined in multiple experiments, and an average value is reported for each transporter in Table 1. Surprisingly, AdCMV-βGal treatment slightly but significantly increased the apparent K_m of the CV-1 cells for either substrate. Previous metabolic studies with AdCMV-βGal have shown that this virus has no effect on glycolytic rate or glycogen synthesis in primary islets or hepatocytes (Becker et al., 1994a; O'Doherty et al., 1996). The mechanism by which AdCMV-βGal induces a modest increase in the K_m of endogenous glucose transporters (mainly GLUT-1) in CV-1 cells is unknown.

The K_m for 2-DOG uptake by cells treated with AdCMV-GLUT-1 was low (3.7 ± 0.4 mM), while that of cells treated with AdCMV-GLUT-2 was high (26.3 ± 3.3 mM). The clear difference in K_m indicates that the recombinant adenovirus expression system allows appropriate discrimination between these GLUT isoforms and validates the system for study of chimeric transporters. Cells expressing the glycosylation mutant GLUT-2-N62Q had a K_m for 2-DOG that was 2.5 times higher than that of cells expressing native GLUT-2 ($p < 0.005$). While these are the first kinetic data reported for nonglycosylated GLUT-2, an increase in K_m of the same magnitude has been reported when comparing native and nonglycosylated GLUT-1 (Asano et al., 1991). The K_m for 2-DOG of cells treated with AdCMV-C1-2 was intermediate between that of cells treated with AdCMV-GLUT-1 and AdCMV-GLUT-2, consistent with results obtained with a similar transporter chimera expressed in CHO cells (Katagiri et al., 1992). In that study, the comparison between the chimera and GLUT-2 was complicated by difficulty in expression of GLUT-2, while in the current study, the K_m for 2-DOG of C1-2 is statistically different ($p < 0.005$) from both GLUT-1 and -2, each of which is well expressed.

Cells treated with AdCMV-N1-2 exhibited a K_m for 2-DOG that was indistinguishable from that measured in cells treated with AdCMV-GLUT-2. These cells had a high K_m because the majority of the N1-2 amino acid sequence,

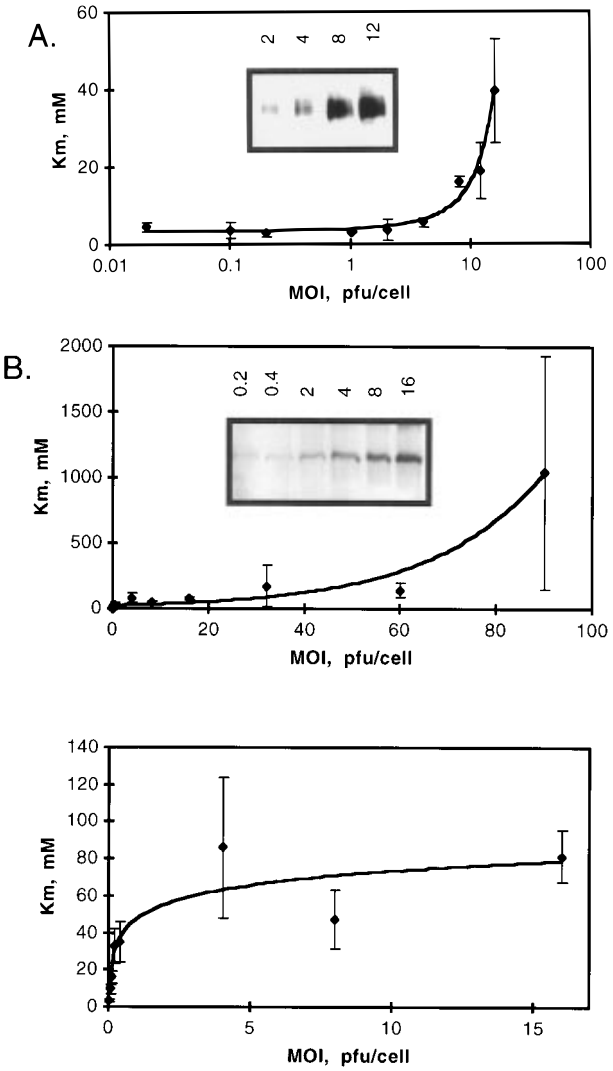

FIGURE 5: Effect of viral titer on transporter kinetic function. CV-1 cells were transduced with different multiplicities of infection (MOIs) of recombinant adenovirus, measured as plaque-forming units per cell (pfu/cell) and used for determination of the K_m for 2-DOG uptake. (A) Cells treated with AdCMV-GLUT-1. The inset shows the GLUT-1 protein level determined by Western blot as a function of MOI. (B) Cells treated with AdCMV-GLUT-2-N62Q. The inset in the upper graph shows the GLUT-2-N62Q protein level determined by Western blot as a function of MOI. The lower graph represents an enlargement of the upper graph with the changes occurring at low MOI being highlighted (note the difference in scale between the top and bottom graphs). Points represent the $K_m \pm$ standard absolute error as derived by the curve fitting of the k_{cat} computer program from six rates of uptake, each derived from five separate uptake measurements.

including its C-terminal half that is vital to transport, is derived from GLUT-2. This transporter is similar to a GLUT-4/GLUT-2 chimera examined in *Xenopus* oocytes that included the GLUT-2 sequence from the end of the sixth transmembrane domain to the C terminus (Buchs et al., 1995). Although that study measured equilibrium exchange of 3-O-MG rather than initial uptake of 2-DOG, the chimera was also found to have a K_m similar to that of GLUT-2.

A goal of this investigation was to express the inverse chimeras to N1-2 and C1-2. As detailed above, the N2-1 construct failed to direct protein expression in a variety of different cell lines, despite the capacity to translate its mRNA by *in vitro* methods. Due to this lack of expression, no information about its kinetics could be obtained. Kinetic

Table 1: Kinetic Constants for 2-DOG and 3-O-MG Uptake into CV-1 Cells Expressing Native, Mutant, and Chimeric Glucose Transporters[a]

adenovirus	K_m for 2-DOG (mM)	K_m for 3-O-MG (mM)
none	$1.7 \pm 0.6^{b,c,e,i}$ (exp = 1, n = 32)	$3.7 \pm 1.3^{c,d,h}$ (exp = 3)
AdCMV-βGal	$4.6 \pm 0.9^{b-e,i}$ (exp = 1, n = 40)	$8.5 \pm 0.5^{c,d,h}$ (exp = 3)
AdCMV-GLUT-1	$3.7 \pm 0.4^{b,d,e,h}$ (exp = 4)	$17.1 \pm 2.1^{e-g}$ (exp = 7)
AdCMV-GLUT-2	$26.3 \pm 3.3^{c,f,g}$ (exp = 5)	$21.0 \pm 3.1^{f,g}$ (exp = 2, n = 71)
AdCMV-GLUT-2-N62Q	$62.6 \pm 12.6^{b-d,h}$ (exp = 4)	$16.7 \pm 4.4^{f,i}$ (exp = 1, n = 30)
AdCMV-N2-1	no protein	no protein
AdCMV-N1-2	$19.9 \pm 3.2^{c,f,h}$ (exp = 4)	$19.1 \pm 3.8^{f,i}$ (exp = 1, n = 30)
AdCMV-C1-2	$9.9 \pm 1.5^{b-d,h}$ (exp = 3)	$25.8 \pm 0.6^{f,h}$ (exp = 3)
AdCMV-C2-1	expressed but inactive	expressed but inactive

[a] CV-1 cells were treated with the indicated recombinant adenoviruses (see Figure 1 for summary of proteins encoded by these viruses), and transport of 2-DOG or 3-O-MG was measured as described in Materials and Methods. The K_m for uptake of the two glucose analogs is presented as the mean $\pm$ SD or SEM for the number of experiments indicated in parentheses. Superscript letters refer to statistical analysis. [b] Significantly different ($p < 0.005$) from K_m of the same transporter for 3-O-MG. [c] Significantly different ($p < 0.005$) from K_m of GLUT-1 for the same substrate. [d] Significantly different ($p < 0.005$) from K_m of GLUT-2 for the same substrate. [e] Significantly different ($p < 0.005$) from K_m of untreated cells for the same substrate. [f] Statistically the same ($p < 0.005$) by ANOVA. [g] Results expressed as mean $\pm$ SD of two determinations. [h] Results expressed as mean $\pm$ SEM of three or more determinations. [i] Results expressed as $K_m \pm$ constant absolute error as determined by the k_{cat} program.

investigation of CV-1 cells transduced with AdCMV-C2-1, which directs expression of the inverse of the C1-2 chimera, yielded K_m values of 3.9 ± 0.4 and 7.3 ± 1.5 mM for 2-DOG and 3-O-MG, respectively, very similar to those obtained for AdCMV-βGal-treated CV-1 cells. As with AdCMV-βGal-treated cells, treatment with AdCMV-C2-1 did not enhance the rate of 5 mM 2-DOG uptake relative to that of untreated cells (data not shown). The slight increase in K_m therefore appears to occur by a similar but as yet undefined mechanism in AdCMV-βGal- and AdCMV-C2-1-treated CV-1 cells.

Kinetics of 3-O-MG Uptake. In contrast to the 7-fold difference in K_m obtained in GLUT-1- and GLUT-2-expressing cells with 2-DOG, the K_m values for 3-O-MG uptake in cells treated with AdCMV-GLUT-1 or AdCMV-GLUT-2 were indistinguishable (Table 1). One possible explanation for these results might have been that the adenovirus treatment itself raised the K_m for 3-O-MG of CV-1 cells to about 20 mM. This possibility is eliminated by the finding that the K_m values of AdCMV-βGal- and AdCMV-C2-1-treated cells, while higher than those of untreated cells, are still well below those in cells treated with AdCMV-GLUT-1 or AdCMV-GLUT-2 (Table 1).

One difference between 2-DOG and 3-O-MG is that only the former is phosphorylated by hexokinases within cells. CV-1 cells contain only low-K_m hexokinase activity and do not express the high-K_m glucose-phosphorylating enzyme glucokinase (H. Berman and C. B. Newgard, unpublished observations). Thus, if low-K_m hexokinase activity was rate-limiting for 2-DOG uptake, spuriously low 2-DOG K_m values

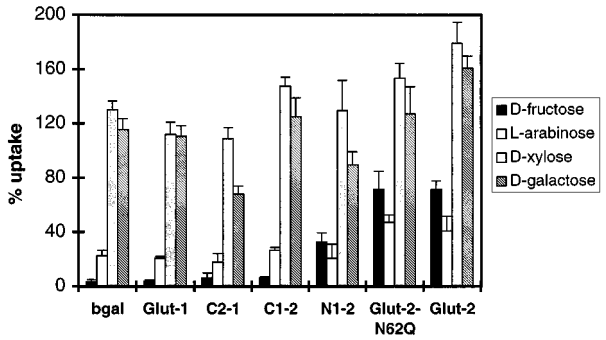

FIGURE 6: Relative efficiency of transport of different substrates by native, mutant, and chimeric glucose transporters. CV-1 cells were treated with the indicated adenoviruses and studied 48 h later. Cells were incubated with each of the radiolabeled sugars (all 40 mM) shown in the legend at the right of the figure for 15 s for measurement of their transport. Sugar uptake values determined as picomoles per milligram of protein were scaled for each group of cells to the uptake of 3-O-MG (treated as 100%) and the uptake of L-glucose (treated as 0%). Data represent the mean ± standard deviation for six determinations for each treatment condition.

may have been derived for AdCMV-GLUT-1-expressing cells. This possibility was ruled out by measuring the transport rates of 20 mM 2-DOG or 3-O-MG into AdCMV-GLUT-1-treated CV-1 cells in the presence of either 20 mM mannoheptulose (an inhibitor of hexokinase in intact cells; Malaisse et al., 1969) or 20 mM L-glucose (to control for osmolarity). Mannoheptulose did not alter the rates of uptake of either substrate (data not shown), confirming that phosphorylation is not rate-limiting in this system.

Because CV-1 cells transduced with either AdCMV-GLUT-1 or AdCMV-GLUT-2 had K_m values for 3-O-MG that were indistinguishable, comparison of the K_m values of cells expressing the other transporters was uninformative when this substrate was used. In fact, cells expressing any of the active transporters (the wild-type proteins, GLUT-2-N62Q, N1-2, or C1-2) had statistically identical K_m values for 3-O-MG uptake.

Substrate Specificity. Uptake of 40 mM 3-O-MG, L-glucose, D-fructose, L-arabinose, D-xylose, and D-galactose by CV-1 cells treated with adenoviruses encoding each glucose transporter variant or β-galactosidase was measured over 15 s at 37 °C. Results for each sugar were normalized for each adenovirus-treated cell population relative to 3-O-MG uptake (set to 100%) and L-glucose uptake (set to 0%). Normalization in this way allowed us to compare relative transport efficiencies for each sugar within a population of cells and controlled for differences in levels of transgene expression between populations.

As shown in Figure 6, cells expressing GLUT-1 or GLUT-2 had clearly distinct profiles of sugar uptake. Consistent with previous studies on fructose uptake and that sugar's ability to inhibit 2-DOG uptake (Gould et al., 1991), AdCMV-GLUT-2-treated cells exhibited intermediate fructose uptake. Such cells also had lower L-arabinose uptake and robust xylose and galactose uptake, while AdCMV-GLUT-1-treated cells displayed negligible fructose uptake, low L-arabinose uptake, and robust xylose and galactose uptake. An important confirmation of the integrity of these data emerges from comparison of the CV-1 cells treated with AdCMV-βGal with those treated with AdCMV-GLUT-1. The identical substrate profiles of these cells indicates that specificity is not affected by overexpression of the transporter, since GLUT-1 is also expressed at low levels in the AdCMV-βGal-treated cells.

The data of Figure 6 provide new insight into determinants of the substrate specificity of GLUT-2. First, a hallmark of GLUT-2 function is the transport of fructose. CV-1 cells treated with AdCMV-GLUT-2 or with viruses encoding the "GLUT-2-like" transporters GLUT-2-N62Q or N1-2 all transported fructose efficiently. An important finding, however, is that cells treated with AdCMV-C1-2 exhibited no enhancement in fructose transport relative to cells treated with AdCMV-GLUT-1. Furthermore, the presence of the GLUT-2 C terminus also failed to impart a significantly improved capacity for transport of arabinose. Thus, the C terminus of GLUT-2 appears to be a determinant of affinity for glucose analogs but does not participate in conferring the broad substrate specificity of this transporter.

In contrast to the lack of effect of the C terminus on substrate preference, the N-terminal chimera N1-2 undergoes significant functional changes. Substitution of the N terminus of GLUT-1 for that of GLUT-2 markedly reduces the efficiency of fructose and arabinose transport to roughly half of the corresponding values for cells expressing native GLUT-2 (33 vs 71% for fructose and 20 vs 41% for arabinose transport by AdCMV-N1-2- and AdCMV-GLUT-2-treated cells, respectively). These data indicate that the chimera discriminates more strongly against these sugars relative to 3-O-MG than does GLUT-2 and provide the first evidence that the N terminus of GLUT-2 participates in its substrate specificity.

CV-1 cells treated with the glycosylation mutant virus, AdCMV-GLUT-2-N62Q, behave almost exactly like cells treated with AdCMV-GLUT-2. We conclude that the oligosaccharide attached to asparagine 62 of GLUT-2 has no role in defining its relative affinities for the four sugars that were studied.

As expected, the substrate specificity pattern of AdCMV-C2-1-treated cells was similar to that of AdCMV-βGal-treated cells, since neither virus directs expression of a transporter protein that is active toward 2-DOG or 3-O-MG. Further experiments that measured uptake rates revealed no difference between the two cell populations in terms of uptake of 40 mM 3-O-MG [AdCMV-C2-1-treated cells, 1.8 ± 0.4 pmol s^{-1} (mg of protein)$^{-1}$; AdCMV-βGal-treated CV-1 cells, 2.2 ± 0.4 pmol s mg of protein)$^{-1}$] or 5 mM D-galactose [AdCMV-C2-1-treated cells, 0.50 ± 0.02 pmol s^{-1} (mg of protein)$^{-1}$; AdCMV-βGal-expressing cells, 0.64 ± 0.07 pmol s^{-1} (mg of protein)$^{-1}$], but AdCMV-C2-1-treated cells did exhibit a significantly enhanced uptake of 5 mM D-xylose compared to AdCMV-βGal-treated cells (Figure 7).

Streptozotocin Cytotoxicity. Previous work in our laboratory demonstrated streptozotocin transport into RIN 1046-38 or AtT-20_{ins} cells expressing GLUT-2 but not GLUT-1 (Schnedl et al., 1994). RIN cells are highly susceptible to streptozotocin cytotoxicity and grow more slowly than CV-1 cells. These properties allow us to evaluate streptozotocin uptake into RIN cells treated with various GLUT adenoviruses using a simple colorimetric viability assay for cell killing (Schnedl et al., 1994). As shown in Figure 8, untreated RIN cells or cells treated with AdCMV-GLUT-1, AdCMV-βGal, or AdCMV-C2-1 required approximately 12 mM streptozotocin to kill 50% of the cells. Cells treated with AdCMV-C1-2, while generally similar in streptozotocin

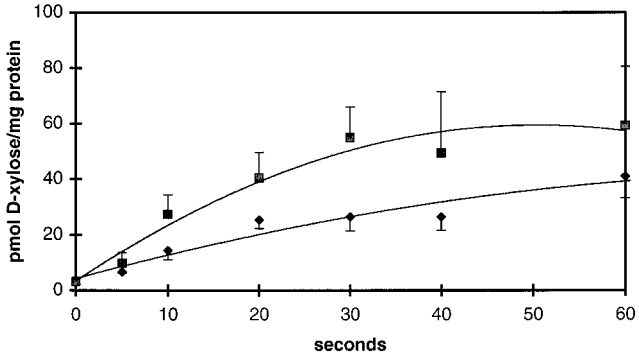

FIGURE 7: Transport of xylose by CV-1 cells treated with AdCMV-βGal or AdCMV-C1-2. CV-1 cells were treated with AdCMV-βGal (diamonds) or AdCMV-C1-2 (squares), and uptake of ^{3}H-labeled xylose was studied 48 h later. Rate values were derived as described in Materials and Methods, and each data point represents the mean $\pm$ standard deviation for three independent determinations. Calculation of the rate of xylose uptake based on these data yielded values of 1.8 $\pm$ 0.2 for cells treated with AdCMV-C2-1 and 1.1 $\pm$ 0.1 for cells treated with AdCMV-βGal ($p < 0.005$).

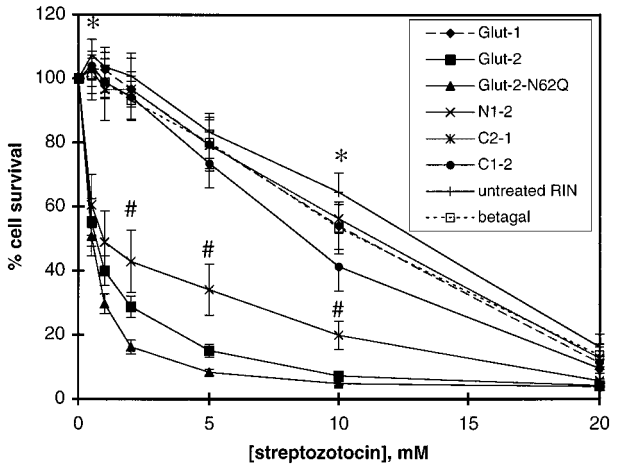

FIGURE 8: Streptozotocin cytotoxicity in RIN cells expressing native, mutant, or chimeric glucose transporters. RIN 1046-38 cells were treated with the recombinant adenoviruses indicated in the legend at the right of the figure. Cells were treated with the indicated concentrations of streptozotocin for 24 h, and cell survival was measured with the MTT assay, as described in Materials and Methods. Data are expressed as the percentage of cells surviving, normalized to cells expressing the same transgene but not treated with streptozotocin (100%). Each data point represents the mean $\pm$ standard deviation for four separate experiments, each involving three determinations. (*) Cells treated with AdCMV-C1-2 were more sensitive to streptozotocin than the other cells in the upper group ($p < 0.05$ compared to cells treated with AdCMV-GLUT-1, AdCMV-C2-1, of AdCMV-βGal or untreated). (#) Cells treated with AdCMV-N1-2 were less sensitive to streptozotocin than cells treated with AdCMV-GLUT-2 ($p < 0.05$).

sensitivity to the first three cell populations, exhibited a slightly greater streptozotocin cytotoxicity at two of the concentrations tested, 0.5 and 10 mM. In contrast, less than 1 mM streptozotocin was required for killing of 50% of the cells treated with AdCMV-N1-2, AdCMV-GLUT-2-N62Q, or AdCMV-GLUT-2. At 0.5 or 1 mM streptozotocin, RIN cells expressing any of these three transporters exhibited a similar percentage of cell survival. At 2, 5, and 10 mM streptozotocin, however, cells treated with AdCMV-N1-2 were less effectively killed than cells treated with AdCMV-GLUT-2-N62Q or AdCMV-GLUT-2 ($p < 0.05$). These data indicate that the GLUT-1 N terminus, which when attached to GLUT-2 caused reduced efficiency of fructose and arabinose uptake relative to native GLUT-2, had a similar discriminatory effect on streptozotocin uptake.

DISCUSSION

These studies provide new insights into structural determinants of kinetic attributes and substrate preference in facilitated diffusion glucose transporters. The panel of mutant and chimeric transporters used in this work allow some specific conclusions to be drawn about the contribution of the N terminus, the C terminus, and the N-linked oligosaccharide to these functions. The role of each of these elements is discussed in turn.

Role of the N Terminus of GLUT-2. Previous investigation of the role of the N terminus of GLUT-2 in determining its kinetic function is limited to one study. A chimera consisting of the GLUT-2 protein with a GLUT-4 N terminus had a K_m for 3-O-MG similar to that for wild-type GLUT-2 when the two transporters were compared by expression in *Xenopus* oocytes (Buchs et al., 1995). In the current study, the N-terminal region of GLUT-1 is shown to have a similar lack of effect on the K_m of GLUT-2 for 2-DOG or 3-O-MG transport. An important advance of the current work is that the studies were carried out in mammalian cell lines, in which highly efficient expression of the transporter constructs could be demonstrated.

In contrast to its lack of effect on the kinetics of 2-DOG or 3-O-MG uptake, the N-terminal domain is shown to have a clear effect on substrate specificity. Thus, native GLUT-2 is a permissive transporter relative to GLUT-1 because it effectively takes up fructose, arabinose, and streptozotocin (Burant & Bell, 1992; Schnedl et al., 1994; this work). Substitution of the N-terminal domain of GLUT-1 for that of GLUT-2 in the N1-2 protein results in a decrease in the efficiency of transport of these three compounds. These experiments define the N-terminal sixth of GLUT-2 as a partial determinant of substrate specificity, although it is also clear that other elements from the central region of the sequence must participate in order to obtain the full phenotype. Further confirmation of such a role for the N terminus of GLUT-2 might have been achieved with the inverse chimera, N2-1, but this transporter could not be expressed in RIN or CV-1 cells, despite efficient expression of the N2-1 transcript. The failure to produce an active transporter when the GLUT-2 N terminus is placed on GLUT-1 is consistent with the fact that GLUT-4 proteins with the GLUT-2 N terminus are also inactive when expressed in the *Xenopus* system (Buchs et al., 1995), although no experiments designed to determine the reason for the lack of activity were provided in the latter study. On the basis of our studies with N2-1, it now seems clear that molecules containing an N terminal domain of GLUT-2 fused to GLUT-1 either are never synthesized or are highly unstable in the intracellular environment.

The N-terminal domain of the facilitated glucose transporters is thought to serve as a scaffold upon which the C terminal substrate transport domain folds (Cope et al., 1994). We propose that the N terminal domain of GLUT-2 is required for the folding of its C-terminal domain into a conformation that allows transport of a variety of substrates. Apparently, the GLUT-2 N terminus is unable to direct folding of the GLUT-1 or GLUT-4 C-terminal domains in a similar fashion, resulting in inactive N2-1 or N2-4 chimeras.

In contrast, the GLUT-1 N terminus can pair with the GLUT-2 C terminus to form an active transporter, and may also direct folding of the GLUT-2 C terminus into a conformation that resembles GLUT-1 in its reduced efficiency of transport of alternate substrates.

Role of the C Terminus of GLUT-2. Our results demonstrate that the C terminus of GLUT-2 in the C1-2 chimera is sufficient to shift the K_m for 2-DOG toward that of GLUT-2, but does not enhance transport capacity for fructose, L-arabinose, or streptozotocin. Previous experiments with C-terminal deletions of GLUT-1 have shown that truncated transporters do not take up 2-DOG or bind the extracellular ligand ATB-BMPA (Oka et al., 1990; Muraoka et al., 1995). The mechanism proposed for the loss of 2-DOG transport held that an intact C terminus was required in order for GLUT-1 to switch back and forth between its two alternate conformations (Oka et al., 1990; Muraoka et al., 1995). Similarly, when a GLUT-1 protein with a C-terminal tail was expressed in CHO cells, the increase in the V_{max} for 2-DOG of the chimera compared to that of wild-type GLUT-1 was attributed to an increased interconversion of conformers (Katagiri et al., 1992). Such a model does not account for the chimera's increased K_m toward 2-DOG, which presumably reflects the binding of the sugar analog to the transport site. Our data show that the C terminus provides no enhancement in transport of alternate GLUT-2 substrates, suggesting either that the binding determinants for these substrates reside outside the C terminus and/or that the C terminus has differential effects on conformer interconversion which are dependent upon the substrate that is being transported.

Role of the Glycosylation at Asparagine 62 of GLUT-2. The elevated K_m of GLUT-2-N62Q for 2-DOG was not mirrored by a similar increase in K_m for 3-O-MG compared to that of wild-type GLUT-2. These data suggested that the 2.5-fold increase in K_m observed for glycosylation-deficient GLUT-1 (Asano et al., 1991) and glycosylation-deficient GLUT-2 (this study) was specific to the 2-DOG substrate. Further support for this idea comes from the finding that GLUT-2-N62Q behaves exactly like wild-type GLUT-2 with regard to fructose and L-arabinose transport activities. Of the sugar substrates studied, therefore, only 2-DOG distinguishes the glycosylation mutant from GLUT-2.

Xylose as a Substrate for Glucose Transporters. The C2-1 chimera does not transport either 2-DOG or 3-O-MG. Perhaps, like the C-terminal truncated GLUT-1, it has lost its extracellular ATB-BMPA binding site but retained its intracellular cytochalasin B binding site (Oka et al., 1990). This point could not be directly investigated because of the low affinity of GLUT-2 for these two photolabels. The ability of the C2-1 chimera to transport xylose argues that it is not locked in one of two dynamic conformations unless xylose transport can occur without conformer interchange. Xylose, like glucose, has no axial substituents in the *C*-1 chair conformation, which is the preferred conformation for substrates of GLUT-1 (Reinwein et al., 1957; LeFevre & Marshall, 1958). The only difference between these two sugars is the presence of a sixth carbon as an equatorial substituent of glucose, where the pentose xylose has only a hydrogen. Xylose may therefore fit more easily through the pore than any other substrate, and the ability to transport xylose could define the minimal function of a glucose transporter. It may be possible for a transporter to allow passage of xylose down its concentration gradient without the normal conformational dynamics required for passage of glucose so that even a kinetically impaired chimera transports xylose.

Summary. In sum, these studies provide the first insight into structural domains that affect substrate specificity in facilitated glucose transporters and demonstrate that elements involved in glucose transport kinetics are distinct from those involved in substrate discrimination. The model that emerges for GLUT-2 holds that the C terminus contributes to its specialized kinetic function, while the N terminus contributes to its broader substrate specificity, but that these elements must interact with as yet undefined regions from the central portion of the primary sequence to confer the complete phenotype. Further studies will focus on identification of the complementary "central" epitopes. These results also suggest an evolutionary purpose for the longer first extracellular loop of GLUT-2. Our study shows that this region of the transporter works together with other sequences in the center of the molecule to enhance uptake of substrates not recognized by GLUT-1, -3, and -4. One of the tissues in which GLUT-2 is expressed is the intestine, and its presence there may be explained by the need to absorb dietary monosaccharides other than glucose that are derived from digestion of complex dietary polysaccharides.

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NOTE ADDED IN PROOF

After submission of this paper, a paper by Arbuckle et al. (1996) appeared describing kinetic features of a panel of GLUT-2/GLUT-3 chimeric glucose transporters. In keeping with the predictions of our study, these authors have identified a centrally located epitope of GLUT-2, encompassing membrane-spanning region 7, that confers capacity for fructose uptake. This region may work in tandem with N-terminal domains described in our report to provide full substrate specificity. Also consistent with our findings, the authors describe a chimera consisting of the GLUT-2 sequence with a GLUT-3 C-terminal tail termed G2(12Ed) (analogous to our chimera C2-1) that exhibits an increase in K_m for 2-deoxyglucose uptake relative to GLUT-3. Our findings differ from those of Arbuckle et al., however, in that our chimera C2-1 exhibited no fructose transport, while their G2(12Ed) transporter appears to have some fructose transport capacity. The reason for this difference is unclear but may be related to the use of injected oocytes by Arbuckle et al., in comparison to our studies that were performed in mammalian cells.

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