We synthesized a transportable diazirine derivative of D-glucose, 3-deoxy-3-azi-D-glucopyranose (3-DAG), and studied its interaction with purified human erythrocyte facilitative glucose transporter, GLUT1. 3-DAG was rapidly transported into human erythrocytes and their resealed ghosts in the dark via a mercuric chloride-inhibitable mechanism and with a speed comparable with that of 3-O-methyl-D-glucose (3-OMG). The rate of 3-DAG transport in resealed ghosts was a saturable function of 3-DAG concentration with an apparent \( K_m \) of 3.2 mM and the \( V_{\text{max}} \) of 3.2 \( \mu \text{mol}/\text{ml} \cdot \text{d} \). Glucose inhibited the 3-DAG flux competitively with an apparent \( K_i \) of 2.4 \( \times \) 10\(^{-7} \) M. Cytochalasin B inhibited this 3-DAG flux in a dose-dependent manner with an estimated \( K_i \) of 2.4 \( \times \) 10\(^{-7} \) M. Cytochalasin E had no effect. These findings clearly establish that 3-DAG is a good substrate of GLUT1. UV irradiation of purified GLUT1 in liposomes in the presence of 3-DAG produced a significant covalent incorporation of 3-DAG into GLUT1, and 200 mM D-glucose abolished this 3-DAG incorporation. Analyses of trypsin and endoproteinase Lys-C digestion of 3-DAG-photolabeled GLUT1 revealed that the cleavage products corresponding to the residues 115-183, 256-300, and 301-451 of the GLUT1 sequence were labeled by 3-DAG, demonstrating that not only the C-terminal half but also the N-terminal half of the transmembrane domain participate in the putative substrate channel formation. 3-DAG may be useful in further identification of the amino acid residues that form the substrate channel of this and other members of the facilitative glucose transporter family.

A family of structurally related intrinsic membrane proteins known as facilitative glucose transporters catalyzes the movement of glucose and other selected sugars across the plasma membrane diffusion barrier in mammalian cells (1). Six isoforms have been identified in this family (2); these include GLUT1 or erythrocyte type (3), GLUT2 or liver/pancreatic beta cell type (4), and GLUT4 or muscle/adipose cell type (5). Homology analyses of cDNA-deduced amino acid sequences together with the biochemical information obtained from purified GLUT1 (1–3) suggest that this protein family shares the common transmembrane topology composed of a highly conserved, large (amounting to approximately 50% of protein mass) transmembrane domain, with less conserved, grossly asymmetric, cytoplasmic and exoplasmic (nonmembrane) domains. Evidence (1, 3, 6–8) further suggests that the transmembrane domain is made of 12 transmembrane \( \alpha \)-helices (TMHs, \( \alpha \)-helices (TMHs, \( \alpha \)-helical segments. For the putative glucose channel structure, Mueckler et al. (3) have first noted the possible significance of the presence of amphipathic TMHs, which may line an aqueous channel for glucose movement and provide hydroxyl and amide hydrogens for hydrogen bond formation with glucose. Conspicuously lacking in the current literature is an effort to identify such a channel residue or residues that interacts with glucose or other substrates (substrate binding sites) in this protein.

The identification of GLUT1 inhibitor binding sites, in contrast, has been quite successful. Photo labeling with ATB-BMPA (2-N-[4-(1-azido-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(o-mannos-4-yloxy)-2-propylamine) (12), cytochalasin B (13), and

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* This work was supported in part by National Institutes of Health Grant DK17736 and Buffalo Veterans Administration Medical Center Medical Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: TMH, transmembrane helix; 3-DAG, 3-deoxy-3-azi-D-glucopyranose; 3-OMG, 3-O-methyl-D-glucose; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; BSS, balanced salt solution.
forskolin (14) revealed that all of these inhibitors label exclusively at residues within the C-terminal half of the protein. Based on these findings, Holman and his co-workers (15) have proposed a model for GLUT1 structure suggesting that the N- and C-terminal halves are two separate domains and that only the C-terminal half is directly involved in substrate binding and translocation. This model implies that the C-terminal half alone forms a putative glucose channel.

The aim of the present study is to identify experimentally the channel-forming transmembrane $\alpha$-helices in GLUT1 by affinity labeling using a photoactive substrate analog. We synthesized a diazirine derivative of $\alpha$-glucose, 3-DAG, and demonstrated that this photoreactive glucose analog is a good substrate of GLUT1. The analog was rapidly transported by intact human erythrocytes and their ghosts, which was inhibited by cytochalasin B but not by cytochalasin E. 3-DAG upon UV irradiation was covalently incorporated into purified GLUT1 in liposomes. Analyses of the trypsin and endoproteinase Lys-C cleavage products of 3-DAG-labeled GLUT1 clearly revealed that both the N- and C-terminal halves were affinity-labeled by this substrate analog. Based on these findings, we propose that the putative glucose channel is formed between the N- and C-terminal halves of the transporter rather than exclusively within the C-terminal half of the transmembrane domain.

**EXPERIMENTAL PROCEDURES**

Materials—Cytochalasins B and E were from Sigma. 3-O-methyl-$^3$H$\alpha$-glucose was purchased from Amersham Corp. $[^3]$HNaBH$_4$ was obtained from DuPont NEN. Trypsin and Lys-C were from Wako Chemicals (Richmond, VA). N-Glycosidase F was purchased from Boehringer Mannheim. Diacetoxy glucose, hydroxyamine O-sulfonic acid, molecular sieve, and 2-methoxyethyl ether were from Aldrich.

Synthesis of 3-DAG—3-DAG, a novel diazirine derivative of $\alpha$-glucose, was synthesized in our laboratory: diacetoxy glucose was oxidized according to Baker et al. (16). The ketone was stirred with liquid ammonia and molecular sieve first at dry ice temperature and then at room temperature. Solids were removed, and the filtrate was reacted with hydroxyamine O-sulfonic acid in 2-methoxyethyl ether (17). Oxidation of the reaction product with iodoacetethylamine in methanol gave, after extensive purification, 3-deoxy-3-azido-1,2,5,6-di-O-isopropylidene-110, 160, 313 mm, $\varepsilon = 90$; $K_r = 0.7$ (10% ethyl acetate in hexane). Deprotection was carried out by treatment with trifluoroacetic acid/water (9:1). After chromatography on C-18 Sep-Pak cartridge (Waters), 3-DAG $I_{max} 313 \text{nm}$. A $^{1}R_{f}$ for glucose was 0.45 was obtained as an amorphous white solid.

Assay for 3-DAG and 3-DAG Transport Measurement—Human erythrocytes were isolated free of white cells and cell debris from freshly drawn blood, and hemoglobin-free, resealed erythrocyte ghosts were prepared as described elsewhere (18). The rates of 3-DAG transport into resealed erythrocytes and their resealed ghosts were measured by following the time course of 2 mM mercuric chloride-inhibitable net influx and efflux of 3-DAG, in the absence and in the presence of additives as specified in each figure legend. For the determination of the amount of 3-DAG uptake, ghosts or erythrocytes (200 $\mu$l) were vigorously vortexed with chloroform (200 $\mu$l) and water (200 $\mu$l). The aqueous layer was separated, and the organic layer was extracted with water (200 $\mu$l) once more. The combined aqueous extracts were made up to 1 ml. The glucose analog was quantitated in this solution according to Park and Johnson (19) by assaying glucose through oxidation at C-1, with a slight modification (the time of the incubation at 70 °C was increased to 30 min). In experiments measuring 3-DAG transport in the presence of glucose, 3-DAG was measured after derivatization with 1-phenyl-3-methyl-5-pyrazolone, followed by capillary zone electrophoretic separation in the presence of 100 mM borate buffer, pH 9.5, detected at 245 nm (e = 24500). Prior to digestion with Lys-C, lipids and unbound ligands were removed by size exclusion chromatography on a 1% SDS on a TSK 3000 column (21). Peptides produced by digestion were separated electrophoretically using Tricine/SDS-polyacrylamide gel according to Schagger and Von Jagow (25) and after sonication for 30 s followed by a 30-min incubation at 37 °C with shaking. This digestion is known to digest all the nonmembrane portion of the protein (26).

Other Analytical Methods—Protein was determined by the method of Lowry et al. (27). Separation of proteins and peptides by electrophoresis was carried out using SDS-polyacrylamide gel as described elsewhere (28). Radioactivities of $[^3]$H3-OMG and $[^3]$HNaBH$_4$ were measured using a liquid scintillation counter (LKB Rackbeta, Pharmacia Biotech Inc.). Partial amino acid and N-terminal sequence analysis of peptide fragments was performed by Dr. Audree V. Fowler (UCLA School of Medicine).

**RESULTS AND DISCUSSION**

3-DAG Is Transported by GLUT1 in Human Erythrocytes—A 60-s time course of the mercuric chloride-sensitive net uptake of 3-DAG by human erythrocytes was followed in the dark at 20 °C by measuring time-dependent uptake of 3-DAG by cells (Fig. 1). The uptake of this glucose analog in the dark by erythrocytes was very fast, being complete within 1 min. This mercuric chloride-sensitive 3-DAG uptake was completely inhibited in the presence of 10 $\mu$M cytochalasin B (Fig. 1). The uptake was not affected at all in the presence of 10 $\mu$M cytochalasin E (not illustrated).

Resealed human erythrocyte ghosts were suspended in 1:10 BSS (18) at a cytocrct of approximately 65% and incubated in the presence of 0.2 mM 3-DAG in the dark. The concentration of 3-DAG inside the ghosts measured after 10 min of this incubation was essentially identical to that in the medium (not illustrated). The time course of 3-DAG net exit from these prequillibrated ghosts suspended (10% cytocrct) in 1:10 BSS buffer was then followed in the dark at 20 °C (Fig. 2). The 3-DAG exit from ghosts was also fast, being virtually completed within 10 s. This 3-DAG exit was sensitive to the presence of cytochalasin B; the exit rate was reduced approximately 50% by 0.2 $\mu$M cytochalasin B and more than 80% by 1 $\mu$M cytochalasin B but not at all significantly by 10 $\mu$M cytochalasin E (Fig. 2). More
The time course of 3-DAG net uptake by human erythrocytes. Erythrocytes were suspended (20% hematocrit) in a balanced salt solution (18) of 50 mM Tris-HCl buffer, pH 7.4, containing no cytochalasin B (control) or 10⁻⁵ M cytochalasin B in a final volume of 1 ml. A small aliquot of 3-DAG from a 10 mM stock solution was quickly introduced to give a final concentration of 0.2 mM and incubated under gentle magnetic stirring. After each specified incubation time, 5 ml of prechilled (ice temperature) BSS buffer containing 2 mM HgCl₂ was quickly added to the cell suspension. Cells were separated from suspension medium by centrifugation at 7°C, and the 3-DAG content in cell lysates was assayed as described under “Experimental Procedures.” The amounts of 3-DAG in cells (in μg per 0.1 ml of packed cells) are plotted as a function of incubation time, for control (solid circles) and in the presence of cytochalasin B (solid squares). Each data point represents an average of duplicate measurements.

Kinetic analysis of the rate of 3-DAG uptake by human erythrocytes as a function of 3-DAG concentrations. Cells were suspended in BSS containing specified concentrations of 3-DAG at t = 0 as in the experiments of Fig. 1. The initial velocities for 3-DAG uptake were measured by quantitating the first 3-s uptake of 3-DAG by cells by arresting the flux with HgCl₂ and separating cells free of medium by centrifugation. The results were plotted as kS/v versus S according to the relationship, (S/v)Kₘ/Vₘₐₓ = Kₘ/Vₘₐₓ + (S)/Vₘₐₓ, where v and S are initial velocity (in μmol/s) and 3-DAG concentration in ghosts at the start (t = 0) of the flux measurement, respectively, Kₘ and Vₘₐₓ are the Michaelis-Menten constant and maximal velocity, respectively, and k is a composite constant and equal to 1.81 × Vₘₐₓ. Each data point represents an average of triplicate measurements whose S.D. were less than 10%.

Photolytic Incorporation of 3-DAG into GLUT1 Transmembrane Domain—UV irradiation of purified GLUT1 proteoliposomes in the presence of 3-DAG resulted in a significant incorporation of 3-DAG into GLUT1 protein. A 30-min photolysis with 1 mM 3-DAG in 10 sessions (see “Experimental Proce-
GLUT1 (or 0.027 μmol of 3-DAG incorporation/mol of GLUT1 protein, n = 3). The presence of 200 mM D-glucose during photolysis abolished this 3-DAG incorporation by more than 80% (Fig. 5).

An extensive pepsin digestion (see “Experimental Procedures”) of 3-DAG-photolyzed GLUT1 did not cause any significant reduction in the 3-DAG incorporation of GLUT1 protein (not illustrated). Since this pepsin digestion cuts all individual TMHs free of nonmembrane segments (26), the finding indicates that 3-DAG was incorporated into TMH residues with little incorporation into nontransmembrane domain including loops.

A mild trypsin digestion of GLUT1 after photolysis with 3-DAG followed by SDS-PAGE and protein staining (Fig. 6a) revealed the two well-known trypic fragments (23), namely a broad faintly stained band around 30 kDa of the glycosylated N-terminal half and a sharp 19-kDa band of the C-terminal half of the protein, respectively. A broad band at around 55 kDa was rather evident (Fig. 6a), which was identified in immunoblot (not illustrated) as an undigested GLUT1 monomer. Quantitation of 3-DAG labeling after blank subtraction (Fig. 6b) revealed a significant 3-DAG incorporation to each of these protein-staining bands. For mild trypsin-treated sample, consistent with incomplete digestion revealed by protein staining (Fig. 6a), the 30- and 19-kDa trypic fragments accounted for approximately 50% of total protein label. Although the labeling of the 19-kDa fragment was sharp and noticeable, the overall level associated over the broad 30-kDa fragment was significant; it amounted to 64 ± 9% (n = 3) of the label associated with the 19-kDa fragment in each of three independent estimations. Treatment with N-glycosidase F sharpened the broad 30-kDa band to a 21-kDa band for protein staining and 3-DAG labeling (Fig. 6, a and b). This finding clearly demonstrates that both the N- and C-terminal halves of GLUT1 are labeled by 3-DAG, although labeling is significantly greater at the C-terminal half than at the N-terminal half. It is also important to note that 3-DAG labels the protein at more than one site. This would indicate that the transport process involves...
physical proximity of glucose to the channel residues at multiple sites. This is entirely possible as the length of the channel approximated by the thickness of the lipid bilayer is more than 3 times that of α-glucose.

Digestion of purified GLUT1 with endoproteinase Lys-C produced six deavage fragments (fragments A–F in Table I), all of which except fragment B were separately identifiable on SDS-PAGE (Fig. 7a). These fragments were individually eluted from the gel, and their relationship to individual TMHs was established based on partial N-terminal amino acid sequence determination (Table I). Similar experiments using 3-DAG-incorporated GLUT1 (Fig. 7b) revealed at least three major 3-DAG-labeled peaks. Electrophoretic mobilities unequivocally identified the first two peaks as the Lys-C fragments A (residues 301–451) and C (residues 118–183), respectively. Assignment of the third labeled peak to Lys-C fragments was equivocal. The mobility of this labeled peak failed to match with either of the fragments D and E, although it was significantly closer to the fragment D, indicating that the label is largely at fragment D (residues 256–300), with a slight if any at label fragment E (residues 184–225). Although exact quantitation was not possible, relative intensities of 3-DAG label among fragments A, C, and E/D, respectively. The 3-DAG incorporation to the fragments A and D, which includes TMH8–12 and TMH7, respectively, demonstrates that the C-terminal half of the protein participates in glucose channel formation. Of particular interest is the significant 3-DAG incorporation to the fragments D and E, which includes TMH7 and TMH6, respectively. This further supports the conclusion based on the results of tryptic digestion discussed above and demonstrates that the N-terminal half of the GLUT1 transmembrane domain, more specifically TMH4 and TMH5, is also directly involved in glucose channel formation. The intense labeling found over the fragment D, indicating that the label is largely at fragment D (residues 256–300), with a slight if any at label fragment E (residues 184–225).

Table I

| Peptide fragments produced by Lys-C digestion of purified GLUT1 |
|-------------------------|-----------------|-----------------|-----------------|
| **Fragments** | **Molecular mass (Da)** | **Bands** | **N-terminal sequences** | **TMH(s)** |
| A | 15,993 (Ala<sup>301</sup>–Lys<sup>321</sup>) | 14.0 | AGVQQPVYATIGSG1V | B–12 |
| B | 8,389 (Val<sup>32</sup>–Lys<sup>114</sup>) | Not visible | | 2, 3 |
| C | 6,910 (Ser<sup>115</sup>–Lys<sup>183</sup>) | 6.6 | SFMLLGYFIIYVAGNTTFV | 4, 6 |
| D | 5,070 (Lys<sup>184</sup>–Lys<sup>225</sup>) | 4.4 | KVNLGSLXPSYXOP | 7 |
| E | 3,648 (Asp<sup>184</sup>–Lys<sup>225</sup>) | 4.2 | DLWLLLSSIPQALLQICIVLP | 6 |
| F | 3,171 (Leu<sup>8</sup>–Lys<sup>38</sup>) | 3.2 | RLGLMLAVGAVLGLQFG | 1 |

*Calculated from amino acid composition between expected points of cleavage.

**Molecular mass (in kDa) as separated by Tricine/SDS-polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining.

**Partial N-terminal amino acid sequences were carried out after purification of digestion fragments by SDS-PAGE separation followed by high pressure liquid chromatographic fractionation.

**Deduced from the transmembrane topology of TMHs proposed by Mueckler et al. (3).

Fig. 7. SDS-PAGE separation of endoproteinase Lys-C digestion products of photolyzed GLUT1 with 3-DAG. Photolysis of purified GLUT1 with 3-DAG was identical to that in Fig. 6, except that 500 μg of GLUT1 protein was applied in streak. Protein molecular weight markers (Promega, low range) are indicated (from the top): carbonic anhydrase, soybean trypsin inhibitor doublet, horse heart myoglobin, lysozyme, and myoglobin F1, -2, and -3, respectively. Lys-C digestion was carried out as described under "Experimental Procedures." SDS-PAGE, Coomassie Blue staining, gel slicing, and radioactivity counting were as in Fig. 6. The protein staining pattern (panel a) and the 3-DAG incorporation pattern (panel b) are shown. The results were reproduced in two other independent sets of experiments.

Definitive assignment of the 3-DAG labeling to individual TMHs and the amino acid residues was not possible in the present study. The findings discussed above nevertheless strongly indicate that the N-terminal half of the transmembrane domain is also directly involved in the channel formation in GLUT1 and argue against the model that only the C-terminal half of the protein is directly involved in the putative glucose transport pathway (15). The findings, on the other hand, are consistent with an alternative model of GLUT1 structure, where we (32) proposed that both the N- and C-terminal halves of GLUT1 transmembrane domain form a glucose channel at their interface. This alternative model emphasizes required physical dimension and solvent accessibility of the channel in GLUT1 as well as the α-domain (28) structural motifs known in other proteins. Five amphiphilic TMHs of either 3, 4, 7, 8, and 11 or 2, 5, 7, 8, and 11 are thought to form the glucose channel in this model.

Mapping of Glucose Channel Structure by 3-DAG—In search
of covalently reactive, transportable substrate analogs for the facilitated glucose transport in human erythrocytes, Midgley et al. (29) have studied two diazirine derivatives of α-glucose, namely 4-deoxy-3,3-azi-D-glucopyranose (4-DAG) and 6-deoxy-3,3-azi-D-glucopyranose (6-DAG) and shown that both of these analogs induce counterflow (or transient accumulations) of D-galactose in human erythrocytes, suggesting that they are substrates of GLUT1. We have previously synthesized 4DAG and found that, although this compound is transportable by GLUT1 in human erythrocytes, its low synthetic yield limits the usefulness of this analog as a molecular probe for glucose channel structure mapping by affinity labeling. Synthesis of 3-DAG, however, gave a much higher yield. Our data in the present study clearly demonstrate that 3-DAG is a good substrate of GLUT1, allowing one to affinity photolabel the putative glucose binding site or glucose channel in GLUT1.

Evidence indicates that all of the isoforms of the facilitative glucose transporter family have a common transmembrane domain structure, including that of the putative glucose channel. GLUT1 of human erythrocytes is the only glucose transporter isoform currently available as a pure and functional protein with which the transmembrane domain structure can be studied. The detailed description of this protein structure would provide insight into the basic molecular and subcellular mechanisms underlying the intrinsic transport activity and its regulation of not only this isoform but probably also of other isoforms.

Detailed protein structure may be best studied by x-ray crystallography. To obtain high quality crystals of the intrinsic membrane protein such as glucose transporters, however, is extremely difficult and not likely to be forthcoming in the near future. The biochemical approach to the structural determination of GLUT1 described here, on the other hand, is quite promising although the protocol for the separation of individual transmembrane segments requires further optimization. Preliminary results obtained in our laboratory already indicate that labeled residues can be individually identified by biochemical and biophysical methods. Improved methodology on protein chemistry together with the availability of covalently reactive GLUT1 substrates such as 3-DAG, 4-DAG, and 6-DAG would allow one to map the transmembrane glucose channel structure of not only facilitative glucose transporters but also many other hexose transporters including sodium-glucose co-transporter (30) and bacterial phoshatransferase-linked hexose transporters (31).

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GLUT1 Transmembrane Glucose Pathway: AFFINITY LABELING WITH A TRANSPORTABLE D-GLUCOSE DIAZIRINE
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J. Biol. Chem. 1996, 271:5225-5230.
doi: 10.1074/jbc.271.9.5225

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