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Selective Labeling of the Erythrocyte Hexose Carrier with a Maleimide Derivative of Glucosamine: Relationship of an Exofacial Sulfhydryl to Carrier Conformation and Structure†

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ABSTRACT: Sulfhydryl-reactive derivatives of glucosamine were synthesized as potentially transportable affinity labels of the human erythrocyte hexose carrier. *N*-Maleoylglycyl derivatives of either 6- or 2-amino-2-deoxy-D-glucopyranose were the most potent inhibitors of 3-*O*-methylglucose uptake, with concentrations of half-maximal irreversible inhibition of about 1 mM. Surprisingly, these derivatives were very poorly transported into erythrocytes. They reacted rather with an exofacial sulfhydryl on the carrier following a reversible binding step, the latter possibly to the exofacial substrate binding site. However, their reactivity was determined primarily by access to the exofacial sulfhydryl, which, as predicted by the one-site model of transport, required a carrier conformation with the exofacial substrate binding site exposed. Once reacted, the carrier was "locked" in a conformation unable to reorient inwardly and bind cytochalasin B. In intact erythrocytes the *N*-maleoylglycyl derivative of 2-[^{3}H]glucosamine labeled predominantly an M_r 45 000–66 000 protein on gel electrophoresis in a quantitative and cytochalasin B inhibitable fashion. By use of changes in carrier conformation induced by competitive transport inhibitors in a "double" differential labeling method, virtually complete selectivity of labeling of the carrier protein was achieved, the latter permitting localization of the reactive exofacial sulfhydryl to an M_r 18 000–20 000 tryptic fragment of the carrier.

A variety of poorly permeant (VanSteveninck et al., 1965; Krupka, 1985a) or impermeant (Batt et al., 1976; Roberts et al., 1982) sulfhydryl reagents have been shown to irreversibly inhibit hexose transport in the human erythrocyte, probably by reacting with an external or exofacial sulfhydryl group on the carrier protein (Abbott & Schachter, 1976; Roberts et al., 1982). The enhanced selectivity of such otherwise nonspecific reagents for this sulfhydryl results from their impermeant nature, from their progressive covalent chemical reactivity, and from the fact that their access to the exofacial sulfhydryl can be manipulated by modifying the conformation of the carrier with competitive transport inhibitors. With regard to

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the latter, this sulfhydryl appears to be exposed simultaneously with the outward-facing substrate binding site (Krupka & Devés, 1986; May, 1988a). Thus, cytochalasin B, which binds only to the inward-facing carrier conformation (Devés & Krupka, 1978), protects the exofacial sulfhydryl and prevents both irreversible transport inhibition and carrier labeling by impermeant maleimides (Abbott & Schachter, 1976; Batt et al., 1976; Roberts et al., 1982). However, the sulfhydryl and substrate binding site appear not to overlap with one another, since binding of the impermeant disaccharide maltose to the outward-facing carrier potentiates rather than inhibits reaction with the exofacial sulfhydryl (Krupka, 1985a; May, 1988a). Such data have prompted Krupka and Devés (1986) to describe the exofacial sulfhydryl in terms of an allosteric inhibitory site under the one-site or alternating conformation model of transport (Vidaver, 1966; Gorga & Lienhard, 1981).

As useful as the impermeant maleimides have been in defining the presence and reactivity of the exofacial sulfhydryl, they do not provide direct information regarding the relationship of this site to the outward-facing substrate binding site, nor do they have under the usual conditions the potency and selectivity of carrier labeling required for carrier quantitation and structural studies. For these reasons, maltose–maleimide, a mixture of isomeric monoethers of maltose and methylolmaleimide, was recently synthesized in this laboratory (May, 1988b). This impermeant reagent proved to have both increased affinity and selectivity for the exofacial carrier compared to nonspecific impermeant maleimides, but its use was limited by its low specific activity, its chemical impurity, and the expense of [^{14}C]maltose.

In the present work *N*-iodoacetyl and *N*-maleoylglycyl derivatives of glucosamine were synthesized with the rationale that they should interact with the exofacial substrate binding site, undergo transport by the hexose carrier, and potentially label carrier sulfhydryls or other groups in addition to the exofacial sulfhydryl. It was found that maleimide derivatives of glucosamine, which were the most potent transport inhibitors tested, were in fact poorly transported into the cell, reacting predominantly if not exclusively with the exofacial carrier sulfhydryl. However, the latter permitted study of several functional characteristics of the carrier mechanism as well as initial localization of the exofacial sulfhydryl within the primary amino acid sequence of the carrier protein.

Experimental Procedures

Materials. Radionuclides were obtained as follows: 3-*O*-[*methyl*-^{14}C]methylglucose (40 Ci/mol) from ICN; [4-^{3}H]cytochalasin B (15 Ci/mmol) and 2-amino-2-deoxy-D-[6-^{3}H(N)]glucopyranose hydrochloride (30 Ci/mmol) from New England Nuclear. U.S. Biochemical supplied the 6-amino-6-deoxy-D-glucose hydrochloride (6-GlcN).[1] All studies were performed in 12.5 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl (PBS), except as noted.

Syntheses of Sugar Derivatives. Synthesis of *N*-(iodoacetyl)-6-amino-6-deoxy-D-glucopyranose (6-GlcNIA) was accomplished by coupling *N*-succinimidyl iodoacetate with 6-GlcN. The former was synthesized according to the method used by Bernatowicz and Matsueda (1986) to prepare *N*-succinimidyl bromoacetate. In the coupling reaction, 50 mg (232 μmol) of 6-GlcN was dissolved in 2 mL of anhydrous methanol in the presence of 34 μL of triethylamine, followed by addition of 100 mg (350 μmol) of *N*-succinimidyl iodoacetate and incubation at room temperature for 30 min. The reaction mixture was purified by preparative thin-layer chromatography (TLC) on 20 × 20 × 0.2 cm silica gel G-60 plates in chloroform:methanol:water (65:25:4 v/v). The product was detected by staining a small portion of the plate sequentially for sulfhydryl reactivity (May, 1988b) and for carbohydrate (a 5% sulfuric acid spray followed by heating at 110 °C for 5 min). The product was identified as the peak containing both sulfhydryl reactivity and carbohydrate (R_f = 0.28). Staining with 0.1% fluorescamine in acetone followed by UV illumination revealed no evidence of a free amino group in the product. The remaining product was scraped from the plate and eluted with methanol, yield 15–20%. Upon heating, the product on the TLC plate showed yellow discoloration due to degradation of the iodoacetate moiety: ^{1}H NMR (D_2O) δ 3–4 (series of multiplets, 6 H), 3.6 (s, CH_2 I, 2 H), 4.5 [large doublet (β anomer)], 5.1 (small doublet, (α anomer), total 1 H]; mass spectrum ([+] ion FAB), *m/z* 348 (*M* + 1), 370 (*M* + Na^+). The half-life of 6-GlcNIA in PBS at 37 °C measured by residual sulfhydryl reactivity was 200 min (not shown). 2-GlcNIA was prepared in an analogous fashion to 6-GlcNIA (R_f = 0.33).

N-(*N*-Maleoylglycyl)-6-amino-6-deoxy-D-glucopyranose (6-GlcNMG) was synthesized by coupling the *N*-hydroxysuccinmide ester of *N*-maleoylglycine with 6-GlcN. The method of Rich et al. (1975) was used to prepare *N*-maleoylglycine, which was then esterified with *N*-hydroxysuccinimide according to the procedure of Keller and Rudinger (1975). In a typical synthesis of the glucosamine derivative, 50 mg (232 μmol) of 6-GlcN was dissolved in 2 mL of anhydrous methanol containing 34 μL of triethylamine, and this combined with 60 mg (238 μmol) of *N*-hydroxysuccinimidyl *N*-maleoylglycinate in 0.2 mL of anhydrous Me_2SO. The solution was allowed to incubate for 30 min at room temperature before removal of solvents under nitrogen. The derivative was purified by TLC as described for 6-GlcNIA and the area showing both carbohydrate and maleimide reactivity (R_f = 0.29) scraped from the plate, eluted with methanol, and dried. The yield was usually 20–25%: UV_{max} 200, 300 nm; ^{1}H NMR (D_2O) δ 3–4 (series of multiplets, 6 H), 4.2 (s, glycyl CH_2, 2 H), 4.5 [large doublet (β anomer)], 5.1 [small doublet (α anomer), total 1 H], 6.8 (s, maleoyl protons, 2 H). The spectra also showed a trace of an AB quartet (J = 12.0 Hz, υ_A = 6.5 ppm, υ_B = 6.2 ppm), which was assigned to maleamic acid protons: mass spectrum ([+] ion FAB), *m/z* 339 (*M* + Na^+). Mass spectroscopy was somewhat complicated by apparent reaction under the conditions of FAB of the maleimide with solvent (methanol) and matrices (glycerol, sulfolane, *m*-nitrobenzyl alcohol). The half-life of 6-GlcNMG in PBS at 37 °C was 154 min. *N*-(*N*-Maleoylglycyl)-2-amino-2-deoxy-D-glucopyranose (2-GlcNMG) was prepared in a fashion identical with that used for 6-GlcNMG (R_f = 0.26).

Radioactive 2-GlcNMG was synthesized in a typical experiment by drying 70 μCi of tritiated 2-GlcN under nitrogen in a glass culture tube, adding 2.5 mg of unlabeled 6-GlcN (11.6 μmol), and dissolving both in 200 mL of anhydrous methanol containing 10 μL of triethylamine. To this solution was added 2.9 mg of *N*-hydroxysuccinimidyl *N*-maleoylglycinate (11.6 μmol) in 40 μL of anhydrous Me_2SO. Following a 30-min incubation at room temperature, the reaction

[1] Abbreviations: 6-GlcN, 6-amino-6-deoxy-D-glucopyranose hydrochloride; PBS, phosphate-buffered saline; 2-GlcN, 2-amino-2-deoxy-D-glucopyranose; TLC, thin-layer chromatography; Me_2SO, dimethyl sulfoxide; 6-GlcNIA, *N*-(iodoacetyl)-6-amino-6-deoxy-D-glucopyranose; 2-GlcNIA, *N*-(iodoacetyl)-2-amino-2-deoxy-D-glucopyranose; 6-GlcNMG, *N*-(*N*-maleoylglycyl)-6-amino-6-deoxy-D-glucopyranose; 2-GlcNMG, *N*-(*N*-maleoylglycyl)-2-amino-2-deoxy-D-glucopyranose; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EC_{50}, concentration of half-maximal effect; GSH, reduced glutathione; K_i, inhibitory constant; K_D, equilibrium dissociation constant; B_0, total specific binding.

mixture was chromatographed as described above on a silica gel G-60 thin-layer plate (20 × 20 × 0.025 cm) in chloroform:methanol:water (65:25:4 v/v). The labeled product was detected by scraping a parallel lane on the plate, eluting with 0.5 mL of water, and counting the radioactivity in 5 mL of scintillation fluid. The appropriate area remaining on the plate was removed by scraping and eluted and stored in methanol at −20 °C until use within 24 h. Yields were routinely 30–35% of the initial amount of 2-[^{3}H]GlcN. The maleimide reactivity of the radiolabeled sugar derivative was confirmed by coupling to thiopropyl-Sepharose beads as previously described (May, 1988b).

The concentration of the unlabeled GlcN derivatives in methanol was determined as their maleimide reactivity, which was measured by an assay involving 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as previously described (May, 1988b). The derivatives were often quite hygroscopic and were usually stored in methanol at −20 °C for up to 3 weeks without loss of activity. Just before use, the methanol was removed under a stream of nitrogen and the derivative reconstituted in the appropriate aqueous buffer.

Erythrocyte Preparation and Assays. Erythrocytes were prepared, and uptake of 3-*O*-methylglucose (5.2 μM, 40 Ci/mol) was measured in triplicate as previously described (May, 1988b). Total glutathione content was measured by the method of Hissin and Hilf (1976). Binding of [^{3}H]cytochalasin B to intact erythrocytes was measured as previously described (May, 1988a). In experiments designed to measure the equilibrium dissociation constant (K_D) and total specific binding (B_0) in intact cells, erythrocytes in PBS at a 10% hematocrit were incubated with 62.5 nCi of [^{3}H]cytochalasin B, 10 μM cytochalasin E, and one of several concentrations of unlabeled cytochalasin B (10, 62.5, 125, 250, and 3000 nM) for 15 min at 37 °C prior to termination of the assay. The data for different concentrations of cytochalasin B were subjected to Scatchard analysis as modified by Rosenthal (1967). Correction was made for nonspecific binding measured in the presence of 3000 nM cytochalasin B. Maltose inhibition of the binding of a low concentration of cytochalasin B (10 nM) was performed exactly as previously described (May, 1988a).

Erythrocyte Lysis and Membrane Protein Electrophoresis. Hypotonic cell lysis and preparation of leaky white ghosts were performed according to the method of Fairbanks et al. (1971). The membranes were stored at −20 °C until sodium dodecyl sulfate gel electrophoresis in 9% acrylamide gels, unless noted otherwise. Electrophoresis was performed as previously described (May, 1988a). Samples were counted until at least 1000 disintegrations had accumulated in the gel slices of interest.

Data Analysis. Data are shown as mean ± standard error from the indicated number of experiments. Statistical comparisons were made by using the Student's *t*-test for paired values.

Results

Transport Inhibition by Glucosamine Derivatives. Influx of 3-*O*-methylglucose into erythrocytes was irreversibly inhibited by low millimolar concentrations of several of the glucosamine derivatives (Figure 1). The *N*-maleoylglycyl derivatives of either 2- or 6-GlcN were substantially more potent transport inhibitors than 6-GlcNIA (Figure 1) or 2-GlcNIA (not shown). A 50% decrease in transport rates occurred at 2- and 6-GlcNMG concentrations of about 1 mM, whereas for 2-GlcNIA the corresponding value was about 5 mM. About 20% of control transport rates were not inhibited by 2- and 6-GlcNMG. The lack of complete inhibition of

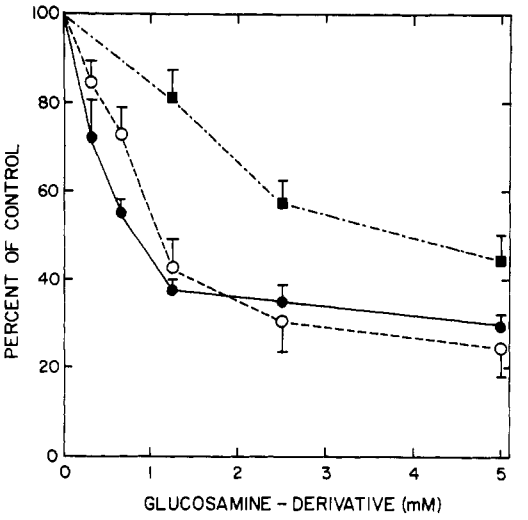

FIGURE 1: Inhibition of 3-*O*-methylglucose uptake by sulfhydryl-reactive glucosamine derivatives. Erythrocytes (0.8 mL) at a 20% hematocrit were incubated for 30 min at 37 °C with the indicted extracellular concentration of 6-GlcNMG (●), 2-GlcNMG (○), or 6-GlcNIA (■). The reagents were removed by three washes by centrifugation in 5 mL of PBS at 37 °C, the original volume was restored, and the assay of hexose transport was performed as described under Experimental Procedures. Rates of transport are expressed as a percent of rates in control cells. The data are from eight experiments with 6-GlcNMG, seven with 2-GlcNMG, and nine with 6-GlcNIA.

transport was not due to hydrolysis of the reactive groups, since the iodoacetyl and *N*-maleoylglycyl moieties were relatively stable under the conditions of incubation (see Experimental Procedures). Because of the relatively low inhibitory potencies of the iodoacetyl derivatives, only the maleimidie derivatives were studied further.

In time-course studies not shown, the extent of irreversible inhibition of transport by various concentrations of 2- and 6-GlcNMG was essentially complete by 30 min of incubation at 37 °C. If the incubation of cells with the maleimide derivatives was performed in an ice bath for a few seconds before the washing procedure with ice-cold PBS and the transport assay at the same temperature, no inhibition of transport could be detected (not shown). When the transport assay was performed at ice-bath temperature in the presence of the maleimide derivatives (i.e., without washes), no inhibition of transport could be detected at concentrations of 2-GlcNMG up to 3 mM, whereas 6-GlcNMG inhibited transport half-maximally at 2.5 ± 0.12 mM (N = 5), providing an estimate of the apparent K_i for reversible transport inhibition independent of sulfhydryl reactivity. Whereas the ultimate extent of irreversible transport inhibition by 2- and 6-GlcNMG was similar, in the absence of covalent reaction, 6-GlcNMG appeared to have a greater affinity for the carrier and presumably for the outward-facing substrate binding site.

If the sugar–maleimides are affinity labels for the hexose carrier, their rate of transport inactivation at 37 °C should be a saturable function of their concentration (Groman et al., 1977; Mullins & Langdon, 1980). In Figure 2 are plotted the logarithms of transport rates as a function of the duration of exposure to 6-GlcNMG. Rates of transport inactivation derived from these data did saturate with increasing concentrations of 6-GlcNMG, as shown in the inset to Figure 2. When analyzed as detailed by Mullins and Langdon (1980) using nonlinear least-squares analysis (Wilkinson, 1961), these data resulted in a dissociation constant for the reversible reaction with the carrier of 1.5 ± 0.1 mM and a maximal rate of inactivation of 0.16 ± 0.005 min^{-1}. The former agrees well

Table I: DTNB Protection from Transport Inhibition by 6-GlcNMG[a]

treatment	% of untreated control	
	PBS wash	cysteine wash
DTNB	55 ± 4[b]	95 ± 4
6-GlcNMG	ND	42 ± 4[b]
DTNB + 6-GlcNMG	ND	85 ± 5[c]

[a] Erythrocytes (0.8 mL) at a 20% hematocrit were incubated for 30 min at 37 °C with either 4 mM DTNB or 2 mM 6-GlcNMG (first two rows) or for 30 min at 37 °C with 4 mM DTNB, followed by 30 min at the same temperature with 2 mM 6-GlcNMG (last row). The samples were then subjected to either the usual three washes in PBS (see legend to Figure 1) or to a 10-min incubation with 5 mL of 10 mM cysteine in PBS at 37 °C, centrifugation, and removal of the supernatant. This was repeated once, followed by a final wash in PBS before the transport assay. The cysteine wash did not affect control rates of transport (not shown). Data from three experiments are expressed relative to an untreated control carried through the same wash steps. "ND" indicates not determined. [b] $p < 0.01$ vs untreated control. [c] $p < 0.01$ vs treatment with 6-GlcNMG alone.

with the K_i value determined at the same low 3-*O*-methylglucose concentration and under conditions in which irreversible reaction with the carrier was negligible.

Erythrocyte Penetrance and Reactivity of 2- and 6-GlcNMG with the Exofacial Sulfhydryl. Entry of the maleimide derivatives of glucosamine into erythrocytes was determined by measurement of intracellular GSH content in 10% erythrocytes after a 30-min incubation at 37 °C with the maleimide, followed by three 25-volume washes to remove unreacted reagent. Neither 2- nor 6-GlcNMG had any effect on intracellular GSH content at concentrations up to 4–5 mM, whereas 0.3 mM *N*-ethylmaleimide decreased GSH content by 37% (not shown). These results suggest that substantial entry of the sugar–maleimides into the cells does not occur under these conditions and that the derivatives react predominantly with an exofacial nucleophile to inhibit transport.

Evidence that the *N*-maleoylglycyl derivatives react with the exofacial sulfhydryl is provided in Table I. In these experiments, the specific sulfhydryl reagent DTNB was used to protect the exofacial sulfhydryl from reaction with 6-GlcNMG. At concentrations below 5 mM, DTNB does not lower intracellular GSH (May, 1988c) and thus may also be considered largely impermeant. Incubation of erythrocytes with 4 mM DTNB followed by several washes in PBS inhibited hexose transport by 45%, whereas incubating and washing in 10 mM cysteine reversed the inhibitory effect of DTNB (Table I). When cells reacted first with DTNB were subsequently incubated with 2 mM 6-GlcNMG and then washed and incubated with 10 mM cysteine to release the DTNB adduct, the inhibitory effect of the maleimide (58%) was largely prevented. These results, taken with the lack of penetrance of 2- or 6-GlcNMG, are compatible with the notion that DTNB and the sugar–maleimides react with the same exofacial sulfhydryl on the carrier.

Assessment of the Relationship of the Exofacial Sulfhydryl to Carrier Conformation Using the Sugar–Maleimides. If

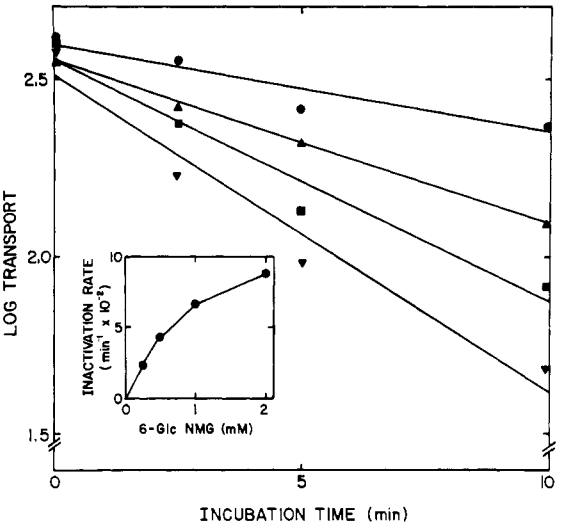

FIGURE 2: Dependence of the rate of transport inactivation on the concentration of 6-GlcNMG. Cells (20%) in 0.8 mL of PBS were incubated with one of several concentrations of 6-GlcNMG [(●), 0.25 mM, (▲) 0.5 mM, (■) 1 mM, and (▼) 2 mM] for 0, 2.5, 5, and 10 min at 37 °C, and the reaction was stopped with the addition of 5 mL of 37 °C PBS containing 10 mM cysteine. After 10 min, the cells were pelleted by centrifugation and the supernatant was discarded. The cysteine incubation and wash was repeated, followed by a wash in ice-cold PBS alone. The suspension was restored to the original volume before assay of 3-*O*-methylglucose uptake. The time dependence of transport inactivation for each 6-GlcNMG concentration is plotted, expressing transport as the logarithm of the percent of equilibrium space occupied by 3-*O*-methylglucose. (Inset) Rates of transport inactivation are calculated as the slopes of the best-fit lines through the data points (Mullins & Langdon, 1980). The results are from three experiments at each reagent concentration.

the sugar–maleimides react with the exofacial sulfhydryl, their ability to irreversibly inhibit transport should be modified by agents known to affect carrier orientation. Such agents include maltose, which pulls the carrier outward by binding to the outward-facing substrate binding site in intact cells (Krupka & Devés, 1986), and cytochalasin B, which, although penetrant, pulls carriers to an inward-facing conformation by binding only to the carrier conformation with substrate binding site facing inward (Devés & Krupka, 1978). As shown in Table II, preincubation of cells with maltose significantly enhanced the inactivation of transport caused by both 2- and 6-GlcNMG compared to the sucrose control. On the other hand, preincubation with cytochalasin B had a significant protective effect compared to preincubation with cytochalasin E. The concentration of the maleimides (1.4 mM) and times of incubation (20 min) were chosen to produce submaximal transport inactivation (see Figure 1). In studies not shown, neither 100 mM maltose nor 6.25 μM cytochalasin B alone had any residual effect on transport following the wash steps.

If carrier orientation determines access to the exofacial sulfhydryl, an important corollary is whether reaction of the latter with 2- or 6-GlcNMG permanently locks the carrier in an outward-facing mode unable to bind cytochalasin B on the

Table II: Modification of 6- and 2-GlcNMG-Induced Transport Inhibition by Competitive Transport Inhibitors[a]

derivative	% of control					
	sucrose (100 mM)	*p*	maltose (100 mM)	cyto E (6.3 μM)	*p*	cyto B (6.3 μM)
2-GlcNMG	48 ± 8	<0.01	22 ± 6	46 ± 5	<0.05	68 ± 8
6-GlcNMG	49 ± 9	<0.01	30 ± 4	43 ± 6	<0.05	63 ± 10

[a] Erythrocytes (0.8 mL) at a 20% hematocrit were incubated for 5 min at 37 °C with the indicated inhibitor (cyto E = cytochalasin E, cyto B = cytochalasin B), followed by addition of 2- or 6-GlcNMG to a final concentration of 1.4 mM for 20 min at the same temperature. The cells were washed twice in 5 mL of PBS containing 20 mg/mL bovine serum albumin (to aid in the removal of the cytochalasins) and twice more in PBS alone before readjustment to the original volume and assay of hexose transport. Data are from six experiments for 2-GlcNMG and five for 6-GlcNMG, expressed relative to an untreated control sample from the same experiment.

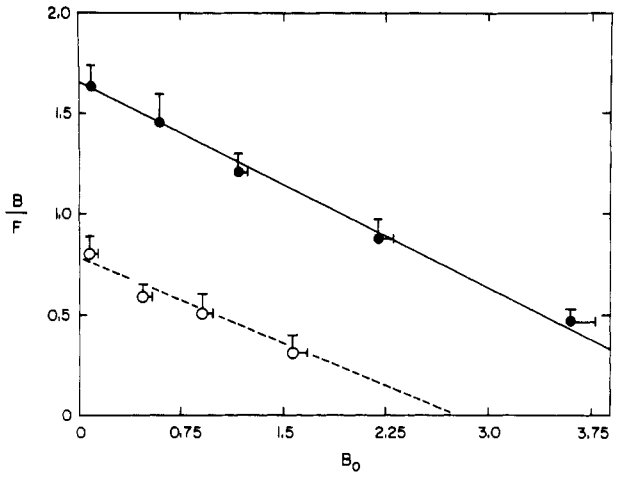

FIGURE 3: Inhibition of [^{3}H]cytochalasin B binding to cells pretreated with 6-GlcNMG. Erythrocytes were treated under the conditions of Figure 1 without (●) or with (○) 2 mM GlcNMG. Following the wash steps the binding of [^{3}H]cytochalasin B was measured and displayed according to the method of Rosenthal (1967), with the units of B_0 expressed as nanomoles per milliliter of packed cells. Data are from five experiments.

inward-facing carrier. Therefore, the ability of 6-GlcNMG to irreversibly inhibit the high-affinity binding of cytochalasin B to intact cells was measured under conditions similar to those used in the transport experiments. In preliminary studies, it was found that 3 μM cytochalasin B decreased the ratio of bound to unbound radioactivity (B/F) of 10 nM cytochalasin B by 90–95% and that 200 mM maltose decreased B/F by 85–90%, confirming the specificity of the assay (not shown). It should be noted that 25 μM cytochalasin E was included in the assay to prevent binding to sites that were not displaced by sugar. It can be seen in Figure 3 that pretreatment with 2 mM 6-GlcNMG decreased the number of specific cytochalasin B binding sites from 5.0 to 2.9 nmol/mL packed cells ($p < 0.01$), whereas the K_D was unchanged (108 vs 103 nM, for control and 6-GlcNMG, respectively). The total specific binding of cytochalasin B calculated from the control studies of Figure 3 corresponds to 300 000 sites/cell. In agreement with the lack of effect of 6-GlcNMG on the affinity of cytochalasin B for the carrier, the apparent K_i for maltose inhibition of cytochalasin B binding to remaining sites was also unaffected by treatment with 2 mM 6-GlcNMG compared to control (41 ± 2 vs 38 ± 2 mM, respectively) (not shown).

Reaction of 2-[^{3}H]GlcNMG with Intact Erythrocytes. Incubation of intact cells with 2-[^{3}H]GlcNMG under the usual conditions followed by several washes to remove unreacted reagent resulted in the incorporation of radioactivity into two major broad electrophoretic bands. About 65–75% of the label was in the band 4.5 region (M_r 45 000–66 000) extending up to but not including band 3, whereas most of the remainder on several gels was in a smaller peak with M_r 25 000–35 000 (Figure 4). Labeling of membrane proteins exposed to the inside of the cell (e.g., spectrin, actin, or glyceraldehyde-3-phosphate dehydrogenase) was not observed, although these bands were present in Coomassie Blue stained gels of leaky ghosts (not shown). However, when aliquots of the first lyse wash obtained during ghost preparation were deproteinated with trichloroacetic acid and their radioactivity was determined, it was evident that a small amount of 2-[^{3}H]GlcNMG had entered the cells, which averaged less than 0.5% of the measured extracellular concentration. Additionally, in cells pretreated with 50 μM cytochalasin B (Figure 4A) or 100 mM maltose (Figure 4B), uptake into the cell interior was suppressed by about 50% of cytochalasin E and sucrose controls, respectively. This indicates that there was a small amount of carrier-mediated uptake into erythrocytes.

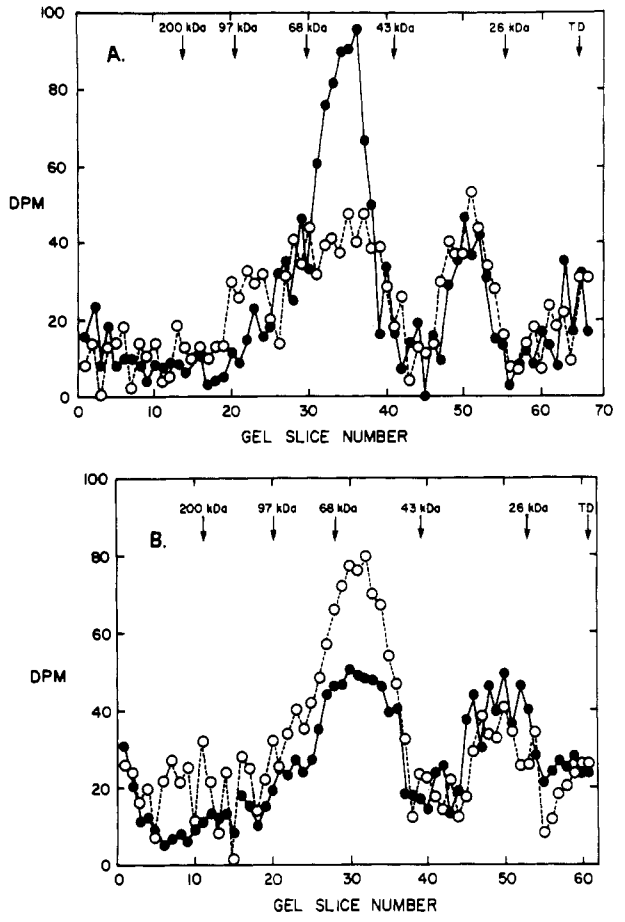

FIGURE 4: Labeling of membrane protein prepared from intact cells incubated with 2-[^{3}H]GlcNMG. (A) Erythrocytes at a 50% hematocrit were preincubated for 30 min at 37 °C with 50 μM cytochalasin E (●) or 50 μM cytochalasin B (○), followed by addition of 2-[^{3}H]GlcNMG (6 Ci/mol) to a final extracellular concentration of 2 mM for another 30 min at the same temperature. The cells were washed four times by centrifugation in 5 mL of PBS at 37 °C and subjected to lysis and membrane preparation with subsequent gel electrophoresis as described under Experimental Procedures. (B) The procedures were identical with those of (A) except that cells were preincubated with either 100 mM sucrose (●) or 100 mM maltose (○) before exposure to 1.8 mM 2-[^{3}H]GlcNMG (6 Ci/mol).

As shown in Figure 4A, the labeling of the band 4.5 electrophoretic peak by 2-[^{3}H]GlcNMG was decreased following pretreatment with 50 μM cytochalasin B compared to pretreatment with the same concentration of cytochalasin E. In two such experiments labeling of band 4.5 was suppressed 41 ± 4% by cytochalasin B compared to the cytochalasin E control. On the other hand, pretreatment with 100 mM maltose enhanced band 4.5 labeling by a less than saturating concentration of 2-[^{3}H]GlcNMG compared to that observed with 100 mM sucrose pretreatment (Figure 4B). The maltose effect was 160 ± 6% of the sucrose control in two experiments. There was no significant effect of cytochalasin B or maltose on labeling of the M_r 25 000–35 000 peak (Figure 4).

The total radioactivity incorporated into band 4.5 in several experiments was a saturable function of the 2-[^{3}H]GlcNMG concentration (Figure 5). From these data an EC_{50} of 1.7 ± 0.6 mM and a maximal number of sites of 360 000/cell was calculated by nonlinear least-squares regression (Wikinson, 1961). The EC_{50} is in the same range as that of about 1 mM derived from studies of transport inhibition by 2-GlcNMG

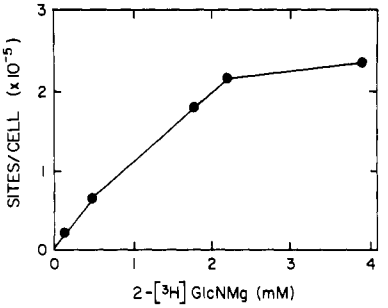

FIGURE 5: Saturation of 2-[^{3}H]GlcNMG labeling of band 4.5. Cells were labeled with the indicated extracellular concentration of 2-[^{3}H]Glc NMG as described in the legend to Figure 4 without modifying pretreatment, and the number of labeled band 4.5 sites/cell was calculated on the basis of specific activity of the agent with the assumptions of 10^{10} cells/mL of packed cells and 1.39 million cells/μg of leaky ghost membrane protein (Dodge et al., 1963). Data are shown from five experiments.

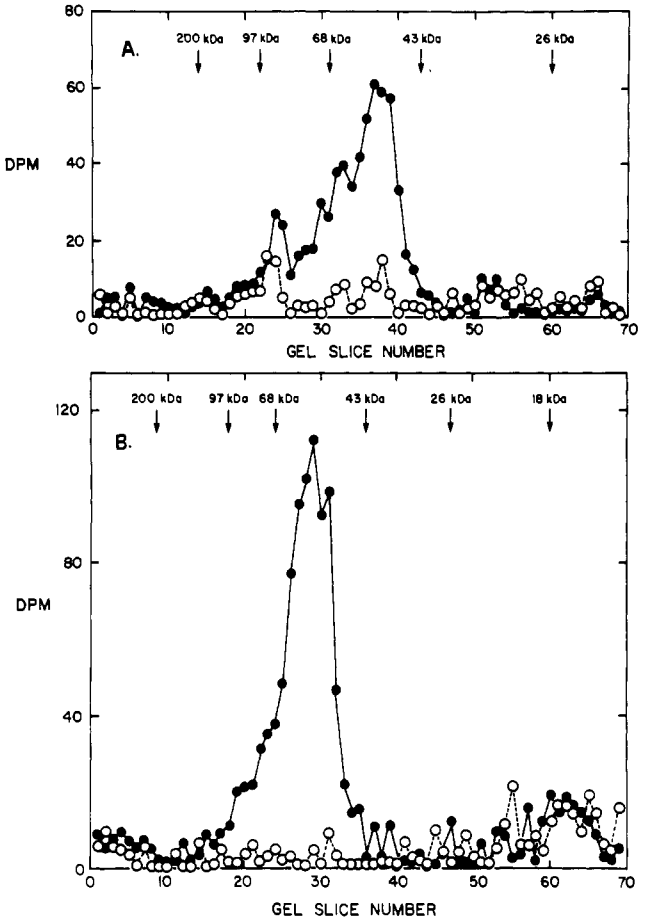

FIGURE 6: Double differential labeling with maltose or phloretin enhancement. (A) One milliliter of cells at a 50% hematocrit were incubated with 50 μM cytochalasin E (O) or B (●) for 5 min at 37 °C, followed by addition of 2 mM 2-GlcNMG for 30 min at the same temperature. Unreacted reagent and cytochalasin were removed by two centrifugation washes in 10 mL of PBS at 37 °C containing 20 mg/mL bovine serum albumin, followed by two additional washes in PBS alone. The cells were adjusted to the original volume and incubated for another 10 min at 37 °C with 100 mM sucrose (O) or 100 mM maltose (●) followed by addition of 1 mM 2-[^{3}H]-GlcNMG (6 μCi/μmol) for 30 min, also at 37 °C. The reaction was terminated by three washes in 5 mL of PBS prior to ghost preparation and electrophoresis. (B) The procedures were identical with those in (A) except that in the final incubation cells previously treated with cytochalasins E and B received either no addition (O) or 100 μM phloretin (●), respectively, before labeling with 0.14 mM 2-[^{3}H]-GlcNMG (60 μC/μmol).

(Figure 1). The estimate of maximal carrier number is also similar to the maltose-accentuated (and presumably maximal)

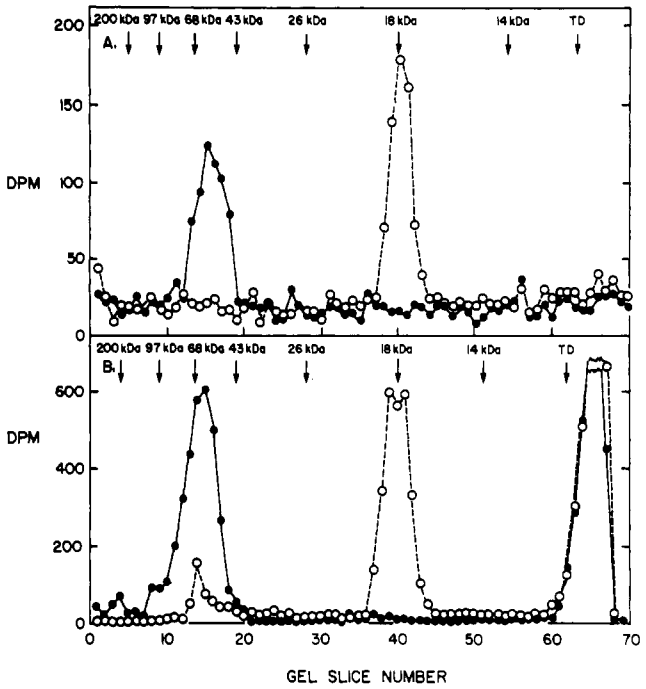

FIGURE 7: Tryptic digestion of labeled ghost membranes. (A) Band 4.5 was labeled in intact cells by 0.6 mM 2-[^{3}H]GlcNMG by using 100 μM phloretin in the last step of the double differential labeling procedure described in the legend to Figure 6. Leaky ghosts were prepared and divided into two fractions at 2–4 mg/mL. One fraction (●) was incubated for 60 min at 37 °C in 5 mM phosphate buffer, pH 8, followed by addition of soybean trypsin inhibitor (3000 units/mL) and one 22-volume wash by centrifugation prior to electrophoresis. The other fraction (O) was incubated for the same period with 1200 units/mL trypsin before addition of the trypsin inhibitor and the wash. (B) Leaky erythrocyte ghosts were photolyzed as previously described (May, 1988a) in the presence of 100 μM cytochalasin E and 0.5 μM [^{3}H]cytochalasin B. Ghosts were treated without (●) or with trypsin (O) exactly as described for (A). Electrophoresis was performed with 12% acrylamide gels.

value of 350 000 calculated from another experiment at a relatively high 2-[^{3}H]GlcNMG concentration of 4 mM.

Double Differential Labeling of the Hexose Carrier. In an attempt to improve the specificity of 2-[^{3}H]GlcNMG for the hexose carrier in intact cells, advantage was taken of the ability to manipulate carrier orientation and thus exofacial sulfhydryl exposure by using reversible asymmetrically binding transport inhibitors. In this method cells were preincubated with cytochalasin B (vs cytochalasin E) to protect the exofacial sulfhydryl during reaction of nonspecific surface sulfhydryls with unlabeled 2-GlcNMG. Following several washes to remove these agents, cells were incubated with inhibitors known to pull the carrier to an outward-facing orientation before they were finally labeled with 2-[^{3}H]GlcNMG. Figure 6A shows the results of a typical experiment with maltose in the last orientation step, while Figure 6B shows the results using phloretin, which has been shown by Krupka (1985b) to bind selectively to the outward-facing carrier. Controls, which were not protected by cytochalasin B or enhanced by maltose or phloretin, showed little labeling at all. On the other hand, highly selective labeling of band 4.5 was obtained in the differentially labeled cells, both with maltose (Figure 6A) and even more so with phloretin when performed at a low 2-[^{3}H]GlcNMG concentration (Figure 6B).

Tryptic Digestion of 2-[^{3}H]GlcNMG-Labeled Membranes. The selective labeling of the band 4.5 carrier protein in the double differential labeling protocol allowed investigation of the membrane protein pattern of tryptic digests of leaky ghosts prepared from cells that had been labeled with 2-[^{3}H]-

GlcNMG. In Figure 7A it is apparent that essentially all of the 2-[^{3}H]GlcNMG-labeled band 4.5 protein was converted by tryptic digestion to a sharp peak migrating in the M_r 18 000 region on the electrophoretic gel. This fragment is identical in mobility with that generated by tryptic digestion of ghosts in which band 4.5 had been previously photolabeled with [^{3}H]cytochalasin B (Figure 7B). No evidence of labeling in any other tryptic fragments is apparent in either gel.

Discussion

Maleimide derivatives of 2- and 6-GlcN were the most potent of several electrophilic derivatives tested as irreversible inhibitors of 3-*O*-methylglucose transport into erythrocytes, both having EC_{50} values just under 1 mM (Figure 1). Their potency for inhibition of 3-*O*-methylglucose influx was slightly greater than that observed previously for maltose–maleimide (May, 1988b) and considerably greater than reported for *N*-ethylmaleimide or impermeant maleimides (Batt et al., 1976; Roberts et al., 1982). At least part of this increased potency appeared to be related to initial binding of the sugar moiety to the exofacial substrate binding site on the carrier in the manner of an affinity label (Groman et al., 1977; Mullins & Langdon, 1980). This was most evident with 6-GlcNMG, which inhibited 3-*O*-methylglucose uptake with an apparent K_i of 2.5 mM under conditions of low temperature and short incubation times, designed to minimize its covalent reaction with the carrier. The affinity of 2-GlcNMG in the absence of covalent reaction was less than that of 6-GlcNMG, in agreement with previous work by Barnett et al. (1975), who found that substituents other than hydroxyls on the first and second carbons of glucose decreased affinity for the exofacial substrate binding site in erythrocytes. Further evidence for a reversible binding step prior to covalent reaction was obtained for 6-GlcNMG, which showed saturation in its rate of transport inactivation with increasing reagent concentrations (Figure 2). An apparent affinity constant of 1.5 mM calculated from the data is comparable to the apparent K_i of 2.5 mM derived in the absence of a covalent reaction. Previously maltose–maleimide was found to have anomalous kinetics of inhibition, possibly related to its chemical impurity (May, 1988b).

Another feature indicative of an affinity label was that the dose–response relationship of irreversible transport inhibition by 2-GlcNMG paralleled its extent of band 4.5 labeling (compare Figures 1 and 5). However, contrary to behavior expected of an affinity label, the competitive but nontransported inhibitor maltose did not interfere with transport inhibition and carrier labeling by 2-GlcNMG in intact cells, rather it potentiated both effects under the proper conditions (Table II and Figure 4B). If there is inhibition by maltose of a reversible association of reagent and the exofacial substrate binding site, it fails detection under the conditions employed, being overwhelmed by the effect of maltose to change carrier orientation to a reactive outward-facing conformation. This caveat makes it difficult to describe these reagents as affinity labels in the strict sense.

The *N*-maleoylglycyl esters of GlcN appeared to react exclusively with the exofacial carrier sulfhydryl. Their exofacial reactivity derives from the observation that they remained mostly extracellular, which was surprising given their monosaccharide structure. It is possible that the bulky *N*-maleoylglycyl group interfered sterically with the transport process, although aryl azide derivatives of 2- and 6-GlcN were readily transported by both erythrocytes (May, 1986; Weber & Eichholz, 1985) and adipocytes (Trosper & Levy, 1977). It seems more likely that the maleimide reacted with an exposed nucleophile with high efficiency prior to the translocation step and prevented further transport activity. Moreover, the exposed nucleophile was very likely a sulfhydryl, given the marked proclivity of maleimides for reaction with sulfhydryls under these conditions (Abbott et al., 1986) and the finding that the irreversible inhibition of transport by 6-GlcNMG was largely prevented by pretreatment with DTNB, which, in contrast to the maleimide, could later be removed by incubating and washing in a thiol-containing buffer (Table I). Since DTNB appears to react with the exofacial carrier without restricting carrier orientation (May, 1988c), its protection from transport inhibition by 6-GlcNMG suggests either steric hindrance or more likely reaction with the same sulfhydryl group.

The maleimide derivatives were found to respond to changes in carrier orientation according to the one-site model of transport in a fashion identical with that observed for tetrathionate (Krupka, 1985a), impermeant maleimides (Batt et al., 1976; Roberts et al., 1982), and maltose–maleimide (May, 1988b). Pretreatment with cytochalasin B was protective, whereas pretreatment with maltose accelerated transport inactivation by both 2- and 6-GlcNMG (Table II). As proposed by Krupta and Devês (1986), this indicates reaction with an exofacial sulfhydryl on the outward-facing carrier conformation, but not one that overlaps with the substrate binding site. Conversely, reaction of the exofacial sulfhydryl with 6-GlcNMG prevented [^{3}H]cytochalasin B binding to the endofacial carrier to an extent similar to that seen for transport inhibition (Figure 3). The same effect was observed for cytochalasin B binding to ghosts that had been prepared from cells treated with glutathione–maleimide–I (May, 1988a) and probably reflects a permanent outward reorientation of the carrier, resulting in a decrease in available cytochalasin B binding sites.

The reaction of 2-[^{3}H]GlcNMG with intact erythrocytes involved exofacially exposed membrane proteins. Most labeling occurred in the broad band 4.5 region and appeared to correspond to the hexose carrier, since labeling of this area was suppressed by cytochalasin B (Figure 4A) and enhanced slightly by maltose under nonsaturating conditions (Figure 4B), as expected from the transport-inhibition studies (Table II). The lack of more complete suppression of band 4.5 labeling by cytochalasin B is probably related to the irreversible nature of the interaction between 2-[^{3}H]GlcNMG and the carrier protein. The calculated number of 360 000 labeled sites/cell based on 2-[^{3}H]GlcNMG labeling (Figure 5) is greater than estimates of about 300 000 sites/cell derived from cytochalasin B binding studies (Lin & Spudich, 1974; Lienhard et al., 1977; and Figure 3), 220 000 sites/cell derived from band 4.5 photolabeling with an azidosalicylic derivative of bis(mannose)-propylamine (Holman et al., 1985), and 300 000 sites/cell derived from band 4.5 labeling with maltose–maleimide (May, 1988b). The overestimate could reflect binding to some noncarrier sites in the band 4.5 region.

The specificity of labeling of band 4.5 by 2-[^{3}H]GlcNMG in intact erythrocytes was markedly enhanced by the ability to reversibly change carrier orientation with asymmetrically binding inhibitors in a double differential labeling protocol. In this method, which is similar to that described for fluorodinitrobenzene labeling of erythrocytes (Shanahan & Jacquez, 1978), the carrier was protected by cytochalasin B during reaction of nonspecific exofacial sulfhydryls with an excess of unlabeled reagent, both of which were subsequently removed by washing. Labeling of the exofacial carrier sulfhydryl by 2-[^{3}H]GlcNMG was enhanced in the second step by increasing

its exposure during preincubation with either maltose (Figure 6A) or phloretin (Figure 6B). The importance of the second step is to increase the efficiency of labeling by a relatively low concentration of labeled reagent, which in turn minimizes the potential for nonspecific reaction.

The ability to label the carrier in a selective and stoichiometric fashion then allowed unambiguous interpretation of electrophoretic gel patterns following tryptic digestion of leaky ghosts prepared from cells labeled with 2-[^{3}H]GlcNMG (Figure 7). The original labeling of the broad-band 4.5 region shifted entirely to a sharper band in the M_r 18 000 region following trypsin treatment (Figure 7A), corresponding to that observed following similar treatment of leaky ghosts labeled with cytochalasin B (Figure 7B).

The primary amino acid sequence of the erythrocyte glucose carrier corresponds closely to that from human HepG2 cells (Mueckler et al., 1985) and from rat brain (Birnbaum et al., 1986). This conclusion is based on studies of antibody cross-reactivity (Mueckler et al., 1985; Birnhaum et al., 1986; Wang, 1987) and on comparisons of partial amino acid composition of the erythrocyte carrier (Baldwin et al., 1982) to the primary amino acid sequence of the HepG2 cell carrier derived from cDNA cloning techniques (Mueckler et al., 1985). Of the six cysteine residues in the carrier sequence (Mueckler et al., 1985), three should be in the broad M_r (average) 30000 tryptic fragment, while the other three should reside in the M_r 18000 fragment (Cairns et al., 1987; Holman & Rees, 1987). Since band 4.5 was labeled with an approximate 1:1 stoichiometry by 2-[^{3}H]GlcNMG (Figure 5) and since 2-[^{3}H]GlcNMG-labeled band 4.5 was converted exclusively to an M_r 18 000 membrane-bound fragment by trypsin (Figure 7A), it is likely that only one amino acid residue was labeled. Hydropathy analysis of the carrier sequence (Mueckler et al., 1985) suggests that the only sulfhydryl exposed on the exofacial cell surface is Cys^{429}, which would be in the M_r 18 000 tryptic fragment, just one amino acid away from entering the lipid bilayer. However, further studies are needed to identify the exofacial sulfhydryl definitively, since such predictions based on hydropathy analysis of proteins spanning the membrane multiple times may not be accurate (Lodish, 1988).

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Registry No. 6-GlcN, 576-47-6; 6-GlcNIA, 118377-56-3; 2-GlcNIA, 118455-79-1; 6-GlcNMG, 118377-57-4; 2-GlcNMG, 118377-58-5; 2-[^{3}H]GlcNMG, 118417-93-9; 2-[^{3}H]GlcN, 118398-13-3; *N*-succinimidyl iodoacetate, 39028-27-8; *N*-maleoylglycine *N*-hydroxysuccinimide ester, 55750-61-3.

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