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Localization of a Reactive Exofacial Sulfhydryl on the Glucose Carrier of Human Erythrocytes†

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ABSTRACT: Tryptic digestion studies of the human erythrocyte glucose carrier have shown that a reactive and transport-sensitive exofacial sulfhydryl is located in the carboxy-terminal half of the molecule, corresponding to Cys^{347}, Cys^{421}, or Cys^{429}. In the present studies, the erythrocyte glucose carrier labeled on the exofacial sulfhydryl with bis(maleimidomethyl) ether-L-[^{35}S]cysteine was chemically cleaved, either at tryptophans by *N*-bromosuccinimide or at nonalkylated cysteines by 2-nitro-5-thiocyanobenzoic acid. The resulting fragments were separated by linear gradient polyacrylamide gel electrophoresis, and the labeled fragments were identified by their apparent molecular weight, and by immunoblotting with antibodies to specific regions of the carrier protein. All of the labeled fragments were recognized by an antibody to the carboxy terminus of the carrier, but not by an antibody to a cytoplasmic loop on the C-terminal half of the carrier. The labeled exofacial sulfhydryl was assigned to Cys^{429}, since this is the only residue of the three possibilities which is beyond the expected cleavage sites of the two reagents in the carrier sequence. These results concur with the predictions of hydropathy analysis and will be relevant for studies of how modification of this sulfhydryl affects carrier function, particularly since several other known carrier isoforms lack a corresponding cysteine.

The facilitative glucose carrier of human erythrocytes has an exofacial sulfhydryl which is reactive with a variety of sulfhydryl reagents which either do not penetrate the cell membrane (Batt et al., 1976; Roberts et al., 1982; May, 1987, 1989a) or do so very slowly (May, 1989b). In addition to its exposure on the cell surface, this group has several features which make it potentially useful in the study of structure–function relationships of the carrier: (1) it is exposed when the carrier is in a conformation ready to accept substrate from without, but not from within the cell (Krupka, 1985; May, 1989a,b), (2) it is not required for substrate binding (Krupka & Devés, 1986; May, 1989a), (3) its reaction may or may not affect the ability of the carrier to subsequently change conformation depending on the sulfhydryl reagent used (May, 1989a,b), and (4) its reaction with low concentrations of *N*-ethylmaleimide has no apparent effect on transport at all (May, 1989c).

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Of the six cysteines in the deduced amino acid sequence of the HepG2/human erythrocyte glucose carrier (Mueckler et al., 1985), only five appear to be reactive with *N*-ethylmaleimide, even in the denatured protein (Deziel et al., 1985). Protective (Deziel et al., 1985) or differential labeling (May, 1989a,b) of intact cells with a cell-impermeant sulfhydryl reagent, followed by tryptic digestion of the carrier from its cytoplasmic surface, has localized the exofacial sulfhydryl to the M_r = 19000 tryptic fragment, which contains cysteines at positions 347, 421, and 429 (Mueckler et al., 1985). Hydropathy analysis predicts that, of the three, only Cys^{429} has an exofacial location on the outwardly exposed loop nearest the C-terminal (Mueckler et al., 1985). However, the uncertainties of such predictions have recently been emphasized (Lodish, 1988).

The present studies were performed to further localize the reactive cysteine in the carrier sequence, since this will help to define the transmembrane loop structure of the carrier and possibly the sites of attachment of other probes which label this tryptic fragment, including cytochalasin B (Cairns et al., 1987; Holman & Rees, 1987), derivatives of bis(mannose) propylamine (Holman & Rees, 1987), forskolin (Shanahan et al., 1987), and ATP (Carruthers, 1989).

Experimental Procedures

Materials. Both ^{35}S-labeled and unlabeled bis(maleimidomethyl) ether–L-cysteine (Cys-Mal)[1] were prepared as previously described (May, 1989b). Amersham International supplied the [^{35}S]cysteine at a specific activity of 1.1 Ci/mmol. All other reagents were from Sigma or Aldrich Chemical Co., except as noted. The antibodies used were monoclonal antibody 7F7.5 (Tai & Carter-Su, 1988), monoclonal antibodies G3 and CG1 (Allard & Lienhard, 1985), and M379 from Dr. Samuel W. Cushman (a polyclonal rabbit antiserum directed against the carboxy-terminal 16 amino acids of the human erythrocyte glucose carrier).

Differential Cell Labeling and Membrane Preparation. Freshly drawn human erythrocytes were prepared as previously described (May, 1988) and stored for no more than 2 days before use. Differential labeling of intact erythrocytes with [^{35}S]Cys-Mal was performed by methods previously detailed (May, 1989b). Briefly, erythrocytes at a 50% hematocrit were incubated for 10 min at 37 °C with 50 μM cytochalasin B to protect the exofacial sulfhydryl. Nonspecific cell-surface sulfhydryls were then blocked by treating with 4 mM Cys-Mal for 30 min at 37 °C. Cytochalasin B and unreacted reagent were removed by three washes by centrifugation in 10 volumes of 12.5 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl. The cells were resuspended to the original volume and treated 5 min at 37 °C with 100 μM phloretin to displace residual cytochalasin B and reorient carriers outwardly. It was possible to use phloretin for this purpose because in binding to the outward-facing carrier, it does not directly affect the reactivity of the exofacial sulfhydryl, but actually enhances labeling by exposing more carrier sulfhydryls (May, 1989a). Labeling with radioactive Cys-Mal was carried out for 30 min at 37 °C, with concentrations and specific activities as noted. The reaction was terminated by a 10-fold dilution in the phosphate-buffered saline and three washes by centrifugation in the same volume. Cells were hypotonically lysed and white ghosts prepared by the method of Fairbanks et al. (1971), with subsequent preparation of protein-depleted ghosts according to the procedure of Gorga and Lienhard (1981). Purified band 4.5 was prepared by the method of Baldwin et al. (1982).

Immunoprecipitation of Cys-Mal-Labeled Carriers. Immunoprecipitation of radiolabeled carriers was performed according to the method of Tai and Carter-Su (1988) as follows: Protein-depleted erythrocyte ghosts prepared from cells that had been differentially labeled with [^{35}S]Cys-Mal were suspended to a protein concentration of 2 mg/mL in 50 mM Tris-HCl containing 1 mM EDTA, pH 7.4. Solid octyl glucoside was added to the membranes to a final concentration of 2% (w/v), followed by incubation for 20 min on ice, dilution with 3 volumes of 1% octyl glucoside in the same buffer, and centrifugation at 100000*g* for 60 min. The supernatant was decanted, and a 3–5 mL volume was incubated with 0.5 mL of antibody-coupled protein A–Sepharose (Sigma) for at least 2 h on ice with gentle stirring. Antibodies were covalently coupled to protein A–Sepharose by using dimethyl pimelidate (Schneider et al., 1982) and the beads prepared in 1% (w/v) octyl glucoside/50 mM Tris-HCl/1 mM EDTA (Tai & Carter-Su, 1988). Upon completion of the incubation with solubilized membranes, the beads were washed three times by centrifugation in 10 volumes of octyl glucoside buffer. After the last wash, 1.2 volumes of 0.1 mM sodium citrate buffer, pH 3.6, were added to the tightly packed beads to elute bound antigen. The beads were again tightly packed by centrifugation, and the supernatant saved for electrophoresis. The antibody-coupled beads were rejuvenated as previously described (Tai & Carter-Su, 1988).

Polyacrylamide Gel Electrophoresis. Samples were prepared and sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as previously detailed (May, 1988b) according to Laemmli (1971). Exponential gradient polyacrylamide gels were performed according to the method of Hennessey and Scarborough (1989), with minor modifications. Samples were treated as previously described (May, 1988b). Glycerol was not used in the gel, the ratio of acrylamide to bis(acrylamide) was 200:1 (w/w), and a linear gradient of 12–20% acrylamide was poured from the bottom of the glass plate sandwich (10 cm × 16 cm × 1.5 mm). Electrophoresis was carried out at 26–30 mA/gel until samples were in the running gel, and then at 35–38 mA/gel until the tracking dye had reached the bottom of the gel (6–8 h). Prestained low molecular weight standards (Bethesda Research Laboratories, Bethesda, MD) were run in lanes adjacent to samples. Following electrophoresis, the gels were either sliced into 1.9-mm segments and counted for radioactivity as previously described (May, 1988b) or frozen at −70 °C until immunoblotting.

Immunoblotting and Autoradiography. Following electrophoresis, a gel was either frozen or electrotransferred directly. Before the transfer, it was soaked or thawed for approximately 10 min in transfer buffer [25 mM Tris, 192 mM glycine, 0.1% sodium dodecyl sulfate, and 15% (v/v) methanol] and then assembled in a transfer sandwich between Whatman chromatography paper (0.18 mm thickness) and poly(vinylidene difluoride) microporous membrane (Immobilon, Millipore), which had been prewetted in methanol (Garfin & Bers, 1989). Electrotransfer was performed in a Bio-Rad Trans-Blot apparatus at 28 V (120–140 mA) for 14 h in order to transfer low molecular weight proteins, and at 70 V (420–640 mA) for an additional 2 h to allow high molecular weight proteins to transfer (Otter et al., 1987).

The membrane was dried on chromatography paper, rewetted with methanol, cut into strips, and blocked for 20 min with 20% fetal calf serum in 10 mM Tris containing 149 mM NaCl, 7.7 mM NaN_3, and 0.05% Tween-20, pH 8.0. The

[1] Abbreviations: Cys-Mal, bis(maleimidomethyl) ether–L-cysteine reaction product; NTCB, 2-nitro-5-thiocyanobenzoic acid.

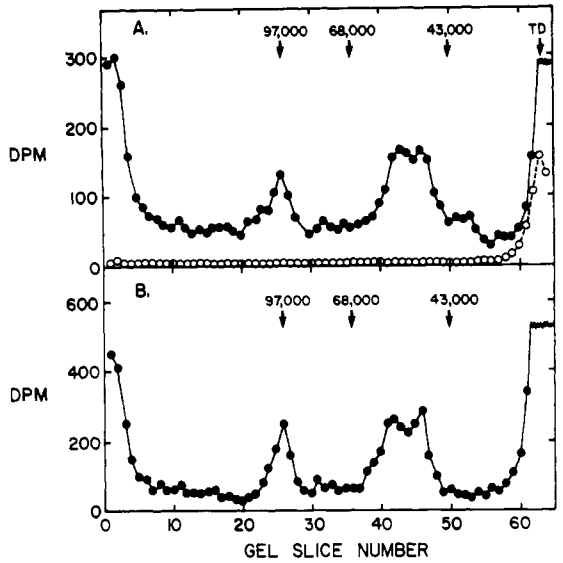

FIGURE 1: Immunoprecipitation of the [^{35}S]Cys-Mal-labeled erythrocyte glucose carrier. Erythrocytes were differentially labeled with [^{35}S]Cys-Mal (0.2 mM, 75 Ci/mmol) and prepared for immunoprecipitation as described under Experimental Procedures. (A) Immunoprecipitation by monoclonal antibodies 7F7.5 (solid symbols) and CG1 (open symbols). (B) Immunoprecipitation with monoclonal antibody G-3 (Allard et al., 1985). In each instance, 15–20 μg of immunoprecipitated protein was applied across four gel lanes and electrophoresis performed along with the indicated prestained molecular weight markers in adjacent lanes.

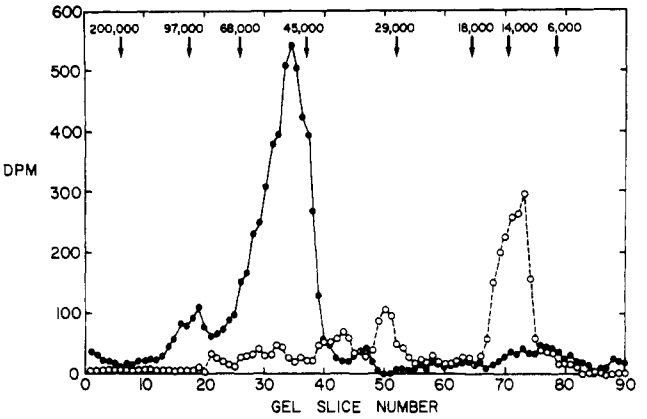

FIGURE 2: Cleavage of [^{35}S]Cys-Mal-labeled carrier with *N*-bromosuccinimide. Protein-depleted ghosts derived from cells that had been differentially labeled with [^{35}S]Cys-Mal (0.3 mM, 190 Ci/mol) were subjected to the cleavage procedure described by Holman and Rees (1987) using 500 μg/mL *N*-bromosuccinimide, except that samples were (1) desalted by dialysis against 50 mM Tris containing 1 mM EDTA, pH 7.4, at 5 °C for 48 h using 3500 molecular weight cutoff tubing (Spectrapor-3, Spectrum Medical Industries) and (2) lyophilized before electrophoresis. Dried samples were dissolved directly in electrophoresis sample buffer, and 50 μg of protein was applied to each of four gel lanes. Following electrophoresis, these were sliced for radioactive counting. The closed circles and solid lines represent control smaples carried through the cleavage procedure but not exposed to *N*-bromosuccinimide, and the open circles and dashed lines represent samples that were treated with *N*-bromosuccinimide. The locations of prestained molecular weight markers run in adjacent lanes are noted.

blocking solution was removed, and the membranes were washed three times in the blocking buffer without fetal calf serum. Membrane strips were incubated with the indicated primary antibody in the blocking buffer containing 20% fetal calf serum for an hour at room temperature. The antibody solution was removed and saved for future use, and the filter was washed three times with the blocking buffer in the absence of fetal calf serum. The membrane strips were then incubated for 1 h with alkaline phosphatase conjugated either to protein A (for detection of polyclonal IgG antibodies) or to goat anti-mouse IgG (for detection of monoclonal antibodies) (Boehringer-Mannheim). The alkaline phsphatase solution was removed, the strips washed three more times, and bands detected with a color development reagent kit containing 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue tetrazolium (Bio-Rad). Autoradiography of the blotted and stained poly(vinylidene difluoride) membranes was carried out for 6–8 weeks at −70 °C using Kodak AR X-ray film.

Results and Discussion

In order to use immunoblotting to detect cleavage fragments from the glucose carrier, it was first necessary to confirm that carrier-specific antibodies could recognize the carrier protein when modified on the exofacial sulfhydryl by [^{35}S]Cys-Mal. Therefore, immunoprecipitation of the Cys-Mal-labeled carrier was performed with several anti-carrier antibodies. The results with two carrier-specific monoclonal antibodies are shown in Figure 1. In protein-depleted erythrocyte ghosts prepared from cells that had been differentially labeled with [^{35}S]-Cys-Mal, monoclonal antibodies 7F7.5 (Tai & Carter-Su, 1988) and G3 (Allard & Lienhard, 1985) both immunoprecipitated a labeled peak in the band 4.5 region on electrophoretic gels, along with some higher molecular weight material, which may reflect aggregation due to the conditions of immunoprecipitation (Tai & Carter-Su, 1988). Similar results were observed with the C-terminal-specific polyclonal antibody (not shown). On the other hand, a nonspecific monoclonal antibody (CG1; Allard & Lienhard, 1985) did not immunoprecipitate a labeled band in the carrier region of electrophoretic gels (Figure 1). These results show that exofacial sulfhydryl alkylation with Cys-Mal does not prevent binding of anti-carrier antibodies directed to cytosolic regions of the octyl glucoside solubilized carrier protein.

Cleavage of the Cys-Mal-Labeled Carrier by N-Bromosuccinimide. This reagent rapidly cleaves a peptide chain on the carboxy-terminal side of tryptophan residues (Ramachandran & Witkop, 1967). In urea-solubilized protein-depleted ghosts which had been prepared from cells previously differentially labeled with [^{35}S]Cys-Mal, *N*-bromosuccinimide cleavage according to Holman and Rees (1987) resulted in the fragmentation pattern shown in Figure 2. In the control sample, the M_r = 45 000–66 000 band 4.5 region containing the glucose carrier was almost exclusively labeled, with little labeling of the M_r = 100 000 band 3 anion carrier (Figure 2). Cleavage of the broad band 4.5 peak by *N*-bromosuccinimide was nearly complete and resulted in labeling of a small M_r = 30 000 peak and of a larger peak and shoulder in the M_r = 10 000–16 000 region. In some experiments two distinct peaks were resolved in the latter region (not shown). It is unlikely that the labeled M_r = 10 000–16 000 band(s) derives (derive) from the band 3 anion transporter, since this protein was labeled to a relatively minor extent under these conditions (Figure 2), and since no cysteine-containing fragments of this size are retained in the cell membrane following *N*-bromosuccinimide cleavage of band 3 (Drickamer, 1977).

In Figure 3 the immunoblots taken from the same experiment are shown. Immunoblotting with the polyclonal C-terminal-specific antibody detected a faint band at M_r = 30 000 and a broad band in the M_r = 10 000–16 000 region (lane b), both of which corresponded to bands labeled by Cys-Mal on autoradiography (lane a). On the other hand, monoclonal antibody 7F7.5, which is known to react with the M_r = 19 000 tryptic carrier fragment at a site other than on the cytoplasmic tail (Tai & Carter-Su, 1988), blotted several larger fragments

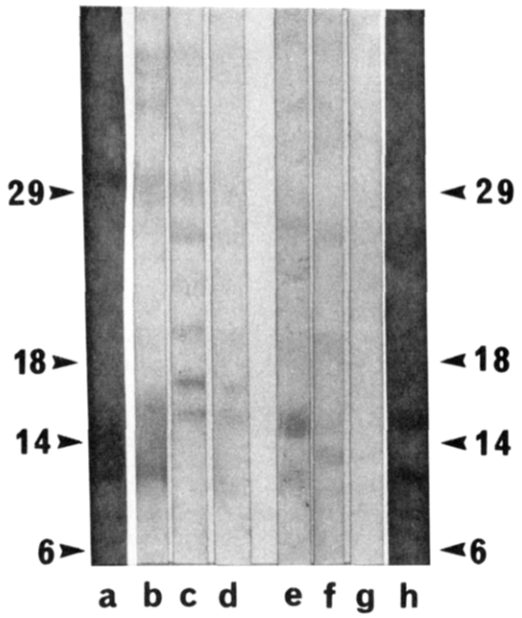

FIGURE 3: Immunoblots and autoradiograms of Cys-Mal-labeled carrier cleavage fragments. Electrophoretic gel sections taken from the same experiments shown in Figures 2 and 4 were subjected to electrotransfer, immunoblotting, and autoradiography as described under Experimental Procedures. Lanes a–d correspond to electrotransferred *N*-bromosuccinimide-cleaved fragments, and lanes e–h correspond to NTCB-cleaved fragments electrophoresed on the same polyacrylamide gel. Lanes a and h are autoradiograms; lanes b and e are immunoblots with the C-terminal-specific antibody; lanes c and f are immunoblots with monoclonal antibody 7F7.5; and lanes d and g are immunoblots with a nonspecific control monoclonal antibody. The locations of low molecular weight standards are indicated in thousands.

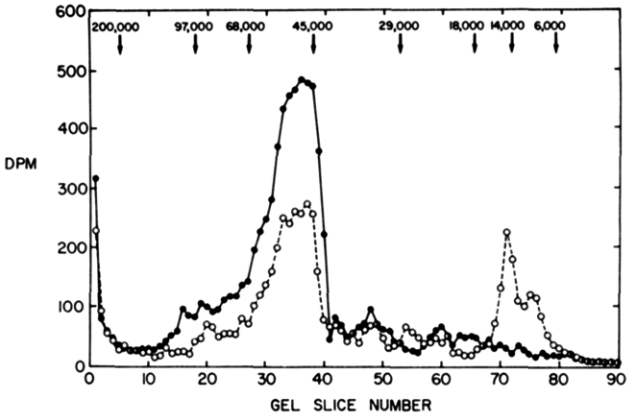

FIGURE 4: Cleavage of [^{35}S]Cys-Mal-labeled carrier with NTCB. The results shown are from the same labeling experiment as that described in Figure 2. The NTCB cleavage procedure for labeled protein-depleted ghosts described by Holman and Rees (1987) was followed exactly except that the final samples were desalted and lyophilized before electrophoresis as described in the legend to Figure 2. The closed circles and solid lines represent samples carried through the cleavage procedure, but not exposed to NTCB, and open circles and dashed lines represent samples cleaved with NTCB.

Table I: Fragmentation of the Cys-Mal-Labeled Glucose Carrier[a]

reagent	expected fragments	major Cys-Mal-labeled fragments obsd	immunoblotting M379	immunoblotting 7F7.5
N-bromo-	187–492 (33.6)	30	30	30
succin-	187–412 (24.7)			26
imide	187–388 (22.2)			23
	187–363 (19.7)			20
	364–492 (13.8)			
	389–492 (11.4)	10–16	10–16	
	413–492 (8.9)			
NTCB	207–492 (31.4)			
	207–418 (24.4)			
	207–420 (23.4)			
	207–346 (15.7)			12–15
	347–492 (15.7)	15	15	
	347–420 (7.7)			
	347–429 (8.7)			
	421–492 (8)	10	10	

[a] On the basis of the expected cleavage sites of *N*-bromosuccinimide and NTCB, the relevant sequences of the carrier fragments (Mueckler et al., 1985) and their calculated molecular masses in kilodaltons (in parentheses) are listed in the second column. In the third column, the Cys-Mal-labeled cleavage fragments observed in Figures 2–4 are assigned to carrier sequences, on the basis of their apparent molecular weight, and the recognition pattern observed in the immunoblots and autoradiograms of Figure 3. In the fourth and fifth columns are shown the immunoblot patterns and their corresponding sequence assignment for each of the two antibodies used.

of apparent M_r = 30000, 26000, 23000, and 20000 (lane c). Two sharp bands were also detected by this antibody in this experiment at M_r = 15000 and M_r = 17000. However, since a nonspecific monoclonal antibody detected the same two bands (lane d), and since they were not observed in all experiments, they were considered to be artifactual. These results show that the Cys-Mal-labeled *N*-bromosuccinimide fragments are contiguous with the C-teminus of the carrier, and although other *N*-bromosuccinimide fragments are present, they are not significantly labeled.

Cleavage of the Cys-Mal-Labeled Carrier by 2-Nitro-5-thiocyanobenzoic acid (NTCB). Cleavage by NTCB under the present conditions occurs on the N-terminal side of cysteines (Jacobson et al., 1973), although not adjacent to an alkylated cysteine. Whereas NTCB cleavage of the carrier protein was incomplete (Figure 4), two prominently labeled species of M_r = 10000 and 15000 were observed. Immunoblotting showed that these fragments were detected by the C-terminal-specific antibody (Figure 3, lane e), a finding confirmed by autoradiography of the poly(vinylidene difluoride) membrane (Figure 3, lane h). Since NTCB cleavage of the purified band 3 anion carrier produces no identifiable fragments less than M_r = 24000 (Drickamer, 1976), and since band 3 labeling in this experiment was again minimal (Figure 4), it is very unlikely that any of the Cys-Mal-labeled fragments detected in these experiments originated in band 3. As also shown in Figure 3, lane f, antibody 7F7.5 detected a band at M_r = 12000–13000 and a fainter band at M_r = 15000 in this and other gels. The former did not correspond to a labeled band or to any band detected by the C-terminal-specific antibody, although a faint band in this region was blotted by a nonspecific monoclonal antibody (Figure 3, lane g). Taken together, these results indicate again that the two bands labeled by Cys-Mal contain the cytoplasmic C-terminus of the carrier, since they correspond to bands detected by the C-terminal-specific antibody, but not to those detected by 7F7.5.

Assignment of Cleavage Fragments and Localization of the Exofacial Sulfhydryl. In Table I are listed the expected relevant cleavage locations of the carrier by *N*-bromosuccinimide and NTCB, along with molecular weight assignments of the Cys-Mal-labeled and immunoblotted fragments. Additionally, shown in Figure 5 is a diagram of the glucose carrier as currently envisioned (Mueckler et al., 1985) with the relevant amino acids and anticipated cleavage sites noted. *N*-Bromosuccinimide should cleave at four relevant sites: Trp^{186}, Trp^{363}, Trp^{388}, and Trp^{412}, whereas the relevant potential sites for NTCB are Cys^{207}, Cys^{347}, Cys^{421}, and Cys^{429}. The broadness of the Cys-Mal-labeled and C-terminal antibody-immunoblotted M_r = 10000–16000 band produced by *N*-

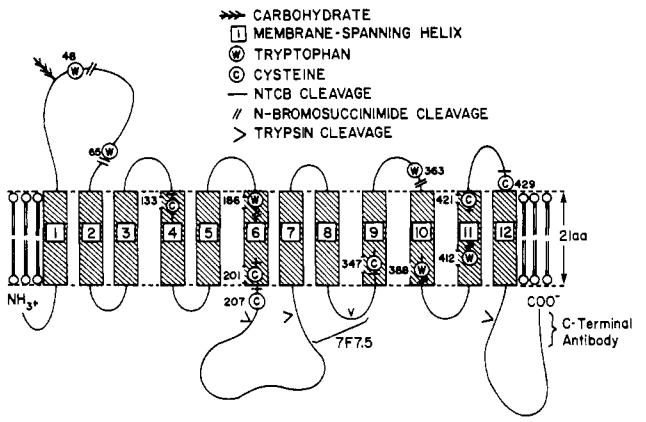

FIGURE 5: Structure of the erythrocyte glucose carrier. The transmembrane structure is that of the glucose carrier originally proposed by Mueckler et al. (1985) with anticipated cleavage sites and regions of antibody recognition. The cytosolic membrane face is oriented downward in the diagram.

bromosuccinimide cleavage (Figures 2 and 3) was considered to reflect a mixture of the three C-terminal cleavage fragments noted in Table I. Since all Cys-Mal-labeled fragments less than 30 kDa resulting from cleavage with either agent were immunoblotted by the C-terminal-specific serum, but not by monoclonal antibody 7F7.5, Cys^{347} is not the exofacial sulfhydryl.

The results of NTCB cleavage as interpreted in Table I also strongly suggest that Cys^{429} rather than Cys^{421} is the labeled exofacial sulfhydryl. This conclusion derives from the fact that Cys-Mal labeled a fragment of about 10 kDa which was assigned to a fragment (421–492) detected by the C-terminal antibody and not by monoclonal antibody 7F7.5 (Table I). Had Cys^{421} contained the labeled exofacial sulfhydryl, no *labeled* fragment of this size would have been detected by the C-terminal-specific antibody. This is because NTCB would have cleaved only at Cys^{429} (not at an alkylated Cys^{421}) and produced an unlabeled C-terminal fragment (see also Figure 5).

Although the conclusion that Cys^{429} is the erythrocyte glucose carrier sulfhydryl labeled by impermeant or poorly permeant sulfhydryl reagents must be confirmed by amino acid sequence determinations, these results demonstrate the resolving power of the present approach. The critical factors were the ability to label the protein on a single amino acid, to cleave it into relatively few fragments separable by exponential gradient gel electrophoresis, and to use immunoblotting with site-specific antibodies to identify labeled and unlabeled fragments of the known carrier amino acid sequence. Assignment of fragments to a specific carrier sequence was aided by the ability to show exact correspondence between labeled and immunoblotted proteolytic fragments in the poly(vinylidene difluoride) membrane, the former detected by autoradiography of energetic β particles from ^{35}S, and the latter detected with the use of an immunostaining procedure.

Localization of the exofacial sulfhydryl to Cys^{429} confirms the original prediction for an exofacial location of this residue, which was based on hydropathy analysis of the carrier sequence (Mueckler et al., 1985). Of the two other major carrier isoforms, the liver/islet (Thorens et al., 1988; Permutt et al., 1989) has, but the so-called "insulin-regulatable" (James et al., 1989) carrier lacks, a corresponding cysteine. Our previous demonstration (May, 1985) that the impermeant sulfhydryl reagent glutathione-maleimide-I (Batt et al., 1976) inhibited insulin-stimulated glucose transport in isolated rat adipocytes may have been caused in part by reaction with the erythroid/brain carrier also present in that cell type (James et al., 1989), or by interference with the insulin receptor–carrier coupling mechanism. The ability to selectively modify the erythroid/brain carrier on an exofacial sulfhydryl in the presence of other carrier subtypes may be useful in determining the relative amounts of different carriers functioning in the plasma membrane.

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Registry No. L-Cys, 52-90-4; glucose, 50-99-7.

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Differential Scanning Calorimetric Studies of Ethanol Interactions with Distearoylphosphatidylcholine: Transition to the Interdigitated Phase†

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ABSTRACT: It is well established that ethanol and other amphipathic molecules induce the formation of a fully interdigitated gel phase in saturated like-chain phosphatidylcholines (PC's). We have previously shown that the induction of interdigitation in PC's by ethanol is dependent upon the alcohol concentration, the lipid chain length, and the temperature [Nambi, P., Rowe, E. S., & McIntosh, T. J. (1988) *Biochemistry 27*, 9175–9182]. In the present study, we have used high-sensitivity differential scanning calorimetry to investigate the transitions of distearoylphosphatidylcholine between the noninterdigitated and the interdigitated phases. The enthalpy of the L_{β}' to $L_{\beta}I$ transition is approximately half that of the L_{β}' to P_{β}' transition which occurs in the absence of ethanol. The reversibility of these transitions has also been investigated by employing both heating and cooling scans in order to establish the most stable phases as a function of temperature and ethanol concentration. It has been demonstrated that the transition to the interdigitated phase is reversible as a function of temperature. Kinetic studies on the reverse transition ($L_{\beta}I$ to L_{β}') demonstrate that this transition can be very slow, requiring weeks to reach completion. The rate depends upon temperature and ethanol concentration. The slow phase changes mean that the lipid can exist for long periods of time in a phase structure which is not the most stable state. The biological significance of this type of lipid behavior is the implication that the phase structure of biological membranes may depend not only on the most stable phase structure of the lipids present but also on the synthetic pathway or other kinetic variables.

Interdigitated lipid phases are among the most recently recognized stable phase states for lipids (McDaniel et al., 1983; McIntosh et al., 1983; Ranck et al., 1977; Huang et al., 1983; Slater & Huang, 1988). In 1983, we showed that ethanol had a biphasic effect on the melting temperature of disaturated like-chain phosphatidylcholines (PC's)[1] (Rowe, 1983) which was subsequently shown to be caused by induction of interdigitation in the PC by ethanol (Simon & McIntosh, 1984; Simon et al., 1986). In like-chain PC's, it is induced by a variety of additives including glycerol, methanol, ethylene glycol, benzyl alcohol, chlorpromazine, tetracaine, ethanol, thiocyanate ion (McDaniel et al., 1983; McIntosh et al., 1983; Cunningham & Lis, 1986; Slater & Huang, 1988), and the *n*-alcohols up to heptanol (Rowe & Nelson, 1990). In the absence of additives, dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC) go into the interdigitated phase at increased hydrostatic pressure (Braganza & Worcester, 1986; Prasad et al., 1987). The ether analogue of DPPC, dihexadecylphosphatidylcholine (DHPC), exists in the interdigitated state under normal pressures in the absence of any additives (Ruocco et al., 1985; Kim et al., 1987; Laggner et al., 1987), as does 1,3-DPPC (Serrallach et al., 1983). Phosphatidylglycerol (PG) with like chains also goes into the interdigitated phase in the presence of a variety of additives, as well as small proteins such as myelin basic protein and polymyxin (Ranck & Tocanne, 1982; Boggs & Rangaraj, 1985). PG also becomes interdigitated in the presence of Tris buffer (Wilkinson et al., 1987). Mixed-chain PC's also exhibit interdigitated phases if one acyl chain is approximately twice as long as the other (Huang et al., 1983; Hui et al., 1984; Xu & Huang, 1987; Mattai et al., 1987). Lyso-PC's interdigitate (Mattal & Shipley, 1986; Hui & Huang, 1986), and *N*-acylsphingomyelins have recently been shown to exist in a interdigitated state depending upon the length of the variable acyl chain relative to the invariant sphingosine chain (Levin et al., 1985; Maulik et al., 1986). The apparent prevalence of this type of lipid stucture suggests the possibility that it may have some biological relevance.

Most of the research which has been reported regarding the interdigitated phases concerns their structural characterization by X-ray diffraction, and the various methods of detecting the

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[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DSC, differential scanning calorimetry; DSPC, distearoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DHPC, dihexadecylphosphatidylcholine.