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Characterization of Cytochalasin B Photoincorporation into Human Erythrocyte D-Glucose Transporter and F-Actin†

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ABSTRACT: The photoincorporation of cytochalasin B into the human erythrocyte glucose transporter and purified G-actin previously reported by this laboratory [Shanahan, M. F. (1982) *J. Biol. Chem. 257*, 7290–7293] was investigated. [^{3}H]Cytochalasin B photolabeled polypeptides of M_r ~43 000–73 000, as determined by polyacrylamide gel electrophoresis, in a concentration-dependent manner with maximum incorporation occurring at 5 μM [^{3}H]cytochalasin B and a half-maximum value of 0.63 μM. This incorporation, previously shown to be partially blocked in the presence of D- but not L-glucose, did not occur in the absence of photolysis and increased linearly with a photolysis time up to 30 s. The reaction was relatively insensitive to pH in the range of pH 6–9, but apparent nonspecific labeling significantly increased at pH 5. The effect of cytochalasin B photoincorporation on D-glucose uptake in intact erythrocytes was also examined. Purified chicken muscle F-actin was also photolabeled with this ligand, but at a specific activity of incorporation (pmol/mg of protein) ~50 times lower than that of the erythrocyte transporter polypeptides. D-Glucose had no effect on this incorporation while 10^{-4}M cytochalasin E completely blocked actin photolabeling. The efficiency of photoincorporation for both the transporter and F-actin was around 1%. Extraction of [^{3}H]cytochalasin B labeled membranes with Triton X-100 resulted in the selective elution of labeled polypeptides from the transporter region while cytochalasin B labeled polypeptides in the region of red cell actin remained in the extracted pellet.

Entry of monosaccharides into human erythrocytes is a facilitated diffusion process mediated by proteins located in the plasma membrane [see Jones & Nickson (1981) for review]. In recent years these transporter polypeptides have been identified by a number of laboratories using a variety of approaches. Several laboratories have isolated this transport system and reconstituted glucose transport activity in liposomes using purified transporter polypeptides. These investigators have identified the glucose transporter as a broad band of heterogeneously, glycosylated proteins of M_r 43 000–70 000 by $NaDodSO_4$[1]–polyacrylamide gel electrophoresis (Kasahara & Hinkle, 1977; Kahlenberg & Zala, 1977; Baldwin et al., 1981). Other methods employing differential labeling (Batt et al., 1976; Lienhard et al., 1977; Shanahan & Jacquez, 1978) and immunological techniques (Sogin & Hinkle, 1980; Baldwin & Lienhard, 1980) have also identified these polypeptides as the glucose transporter.

Cytochalasin B is a potent reversible inhibitor of erythrocyte monosaccharide transport (Taverna & Langdon, 1973; Lin et al., 1974; Jung & Rampal, 1977). Binding of this ligand to the transporter is blocked by D-glucose but not by L-glucose, and this effect has been used to characterize and purify the glucose transporter (Zoccoli et al., 1978; Baldwin et al., 1979; Pinkofsky et al., 1978; Gorga & Lienhard, 1981). Two laboratories have recently developed a method for irreversibly photoincorporating [^{3}H]cytochalasin B into the human erythrocyte glucose transporter (Shanahan, 1982; Carter-Su et al., 1982). Herein we describe a more detailed characterization of the conditions and kinetics of this reaction.

Experimental Procedures

Materials. Cytochalasins B and E were purchased from Aldrich Chemical Co. Electrophoresis reagents were obtained from Bio-Rad. Sequanal grade (lauryl) $NaDodSO_4$ was from Pierce Chemical Co. [^{3}H]Cytochalasin B (radiochemical purity >98%) was obtained from Amersham at a specific activity of 10.3 Ci/mmol. All other reagents were obtained from Sigma Chemical Co. Cytochalasins B and E were routinely prepared in an ethanolic stock solution (10 mg/mL) and diluted to their appropriate final concentration in buffer as described below. Silicone oil (density 1.01–1.02 g/cm^3) was obtained from Contour Chemical Co., Woburn, MA. D-[^{3}H]Glucose (30 Ci/mmol) and L-[1-^{14}C]glucose (54.8 mCi/mmol) were purchased from New England Nuclear.

Preparation of Plasma Membranes. Outdated blood was provided by the American Red Cross Regional Blood Band, Madison, WI. Washed human erythrocytes and membrane ghosts were prepared by the method of Steck & Kant (1974). For ghosts, this method was modified by using the procedure of Fröman et al. (1980) in which osmotically lysed red cells are separated from hemoglobin by gel chromatography using a Sepharose CL-6B column (9 × 30 cm) equilibrated with 5 mM sodium phosphate buffer, pH 8.0 (5P8). Ghost membranes in 5P8 buffer were quick frozen in liquid nitrogen and stored at −100 °C until use. For experimental conditions involving different buffers, the membrane samples were pel-

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline, 150 mM $NaCl_2$–5 mM sodium phosphate, pH 7.4.

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leted at 38000*g* for 20 min in a Sorvall SS-34 rotor and washed twice in the appropriate buffer. All procedures were carried out at 4 °C unless stated otherwise.

Photoincorporation of [^{3}H]Cytochalasin B. Plasma membranes were photolabeled with [^{3}H]cytochalasin B as previously described (Shanahan, 1982).

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. Electrophoresis in 7% polyacrylamide slab gels (1.5- or 3.0-mm thickness) was performed according to Laemmli (1970) unless otherwise noted. Membrane pellets were solubilized in Laemmli sample buffer without heating. Fixing, staining, and slicing procedures for the gels were performed as described previously (Shanahan, 1982; Shanahan et al., 1982). Molecular weight markers for some figures indicate standards obtained from Sigma Chemical Co., while molecular weight designations for other figures correspond to the estimated molecular weights of the major erythrocyte polypeptide bands (Jones & Nickson, 1981). The gel patterns observed were identical in the presence or absence of 5% 2-mercaptoethanol in the sample buffer.

Glucose Uptake Assay. Uptake of glucose into intact erythrocytes was a modification of the oil centrifugation method described by Eilam & Stein (1974). All assays were performed at 7 °C. Briefly, a 10-μL aliquot of PBS solution containing 100 mM D-[^{3}H]glucose and 100 mM L-[^{14}C]-glucose was added to a microfuge tube. At zero time 90 μL of a red cell suspension (50% hematocrit in PBS) was pipetted into the tube, and the solution was mixed by drawing back and forth into the micropipettor tip several times. At the desired time (10 s) 90 μL of the suspension was rapidly expelled into 100 μL of mercury–phloretin stopper solution (Eilam & Stein, 1974) at 0.4 °C, which had been layered on top of 150 μL of silicone oil (ρ 1.01–1.02 g/cm^3) in a microfuge tube. The tubes are immediately centrifuged for 30 s at maximum speed (12000*g*). The tubes were cut with a scalpel, and the tips containing the cell pellets were placed in a test tube containing 1 mL of hemolyzing solution (2% Triton X-100 in water). One milliliter of 10% Cl_3CCOOH was then added, and the samples were spun for 2 min on a desk centrifuge. A sample of the supernatant was neutralized with NaOH and taken for determination of radioactivity with Instagel (Packard) as the solvent. All samples were done in triplicates. Zero times were performed by mixing the samples with stopper solution at 0.4 °C, followed by the addition of labeled glucose and centrifugation. Cell counts were determined by assay of the hemoglobin content of lysed cells (Eilam & Stein, 1974). Uptake under these conditions is linear up to 20 s.

Photolabeling of F-Actin. Chicken muscle G-actin (Sigma) was polymerized to filamentous actin by solubilizing G-actin in 200 μL of water (0.3 mg/mL) containing 2 mM $MgCl_2$ final concentration (Grumet & Lin, 1980). In some experiments 0.7 M D-glucose was also present. Following polymerization, membranes were photolyzed for 30 s in the presence or absence of 10^{-4} M cytochalasin E and 10^{-6} M [^{3}H]cytochalasin B. After photolysis 100 μL of each sample was added to 100 μL of 2× sample buffer and appled directly to electrophoresis gels.

Membrane Extraction Procedures. Extraction of erythrocyte membrane polypeptides with Triton X-100 was performed by incubating photolabeled membranes (1 mg/mL) in 5P8 buffer containing 0.5% Triton X-100 for 30 min in an ice bath. The suspension was then centrifuged at 38000*g* for 30 min as described above. The pellets and supernatants were separated by aspiration and frozen until use for electrophoresis. The pellets were solubilized directly in sample buffer while the supernatants were mixed with an equal volume of 2× concentrated sample buffer. Samples were then applied directly to the gels and electrophoresed without further treatment.

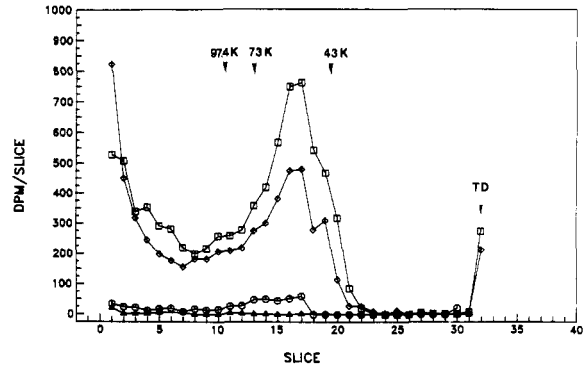

FIGURE 1: Concentration dependence of [^{3}H]cytochalasin B photoincorporation. Erythrocyte ghost membranes were preincubated in the presence of 1 M L-glucose and [^{3}H]cytochalasin B and then photolyzed for 30 s as described under Experimental Procedures. (▲) 0.01 μM cytochalasin B; (○) 0.1 μM cytochalasin B; (◇) 0.5 μM cytochalasin B; (□) 1 μM cytochalasin B. Following photolysis the membranes were washed and solubilized and 200 μg of protein applied to 7% NaDodSO$_4$–polyacrylamide gel electrophoresis slabs as described under Experimental Procedures. *Arrows* depict the location of some of the major erythrocyte membrane proteins as determined by Coomassie blue staining; 97.4K, band 3; 73K, band 4.2; 43K, band 5.

Other Procedures. Protein determinations were performed according to the modified Lowry procedure reported by Peterson (1977). For samples containing Triton X-100, the procedure of Wang & Smith (1975) was used.

Results

Concentration Dependence of Cytochalasin B Photoincorporation. The photoincorporation of cytochalasin B into the region of zone 4.5 (M_r range ~43 000–73 000) in human erythrocytes has been shown previously by this laboratory (Shanahan, 1982; Shanahan et al., 1982) and others (Carter-Su et al., 1982) to be partially blocked (up to 70%) by D-glucose but not L-glucose. Our previous experiments were performed at a cytochalasin B concentration of 0.05–0.5 μM. We have since examined this incorporation into zone 4.5 over a wider range of cytochalasin B concentrations, and the results are shown in Figure 1. The actual labeling profile on NaDodSO$_4$–polyacrylamide gel electrophoresis depicted in this figure represents photoincorporation of [^{3}H]cytochalasin B over the range 0.01–1 μM. Below 1 × 10^{-8} M cytochalasin B virtually no incorporation can be detected unless very large protein loads are placed on the gel (>400 μg of protein). Above this concentration the amount of [^{3}H]cytochalasin B photoincorporated into the zone 4.5 region increased with increasing cytochalasin B concentration. Background labeling is more evident at higher ligand concentration, and several additional labeled regions are noticeable in the higher molecular weight regions. This high molecular weight labeling may be due to nonspecific incorporation or may represent an aggregation effect which we occasionally observed in these gels and which is discussed in a later section of this paper. Figure 2 represents a composite of several experiments in which the amount of cytochalasin B incorporated into the zone 4.5 region is plotted as a function of free ligand concentration from 0.01 to 5 μM. The amount of cytochalasin B incorporated into this region of the gel under these photolysis conditions appears to approach a plateau at around 5 μM. The half-maximal value of this incorporation is 0.63 μM which is in close agreement with results obtained from cytochalasin B labeled dimethyl-

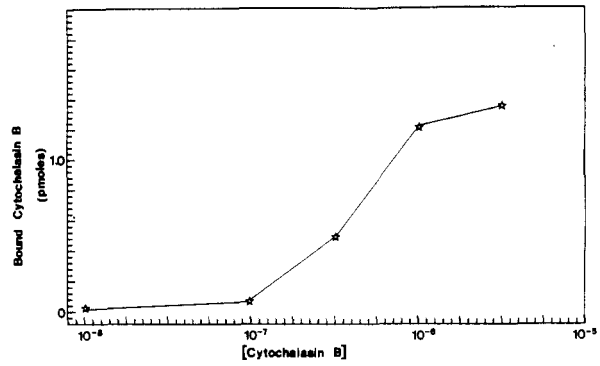

FIGURE 2: Photoincorporation of [^{3}H]cytochalasin B into the zone 4.5 polypeptides as a function of concentration. Experiments were performed as in Figure 1 with the total moles of cytochalasin B photoincorporated into the zone 4.5 region of the gels plotted as a function of the free [^{3}H]cytochalasin B concentration from 0.01 to 5 μM.

maleic anhydride extracted ghosts (Carter-Su et al., 1982).

Photolysis Time Dependence. We have previously reported that the amount of incorporation of [^{3}H]cytochalasin B into the glucose transporter polypeptides of erythrocyte ghost membranes was dependent upon the duration of photolysis. Under conditions of preincubation with [^{3}H]cytochalasin B, but in the absence of photolysis, no specific incorporation into zone 4.5 polypeptides was observed. For photolysis times up to and including 40 s, there was a linear increase of incorporation of radioligand into this region (data not shown). However, beyond 30 s photolysis time with our apparatus (1-kV high-pressure mercury lamp with Pyrex filters), cross-linking of membrane proteins increased severely. This is indicated by a large amount of Coomassie blue staining material at the top of the stacking gel and, in some cases, by the shift of a significant amount of tritium label to the top of the gel. This phenomenon is especially evident when quartz glass is substituted for Pyrex in both the lamp housing and reaction vessel. Under these conditions the amount of [^{3}H]-cytochalasin B incorporated during 3–5 s of photolysis is approximately equal to that incorporated with 20–30 s of photolysis when Pyrex is used. Unfortunately, this greater incorporation is also accompanied by a large increase in photo-cross-linking of the membrane polypeptides to each other, and a significant portion of the total membrane protein never enters the gel.[2] This effect is probably due to a greater flux of ultraviolet light transmitted by quartz, as opposed to Pyrex glass which has a very low coefficient of transparency for wavelengths below 300 nm.

Effects of pH on Photolysis Reaction. As part of our effort to maximize the efficiency of photoincorporation of ligand into the glucose transporter region of $NaDodSO_4$–polyacrylamide gel electrophoresis polypeptides, we examined this reaction under conditions of varying pH. In this case, membranes were resuspended in 10 mM sodium phosphate buffer over a range of pH 5–9. The suspensions were photolyzed in the presence of 0.5 M L-glucose and 0.05 μM cytochalasin B, and the results are presented in Figure 3. Although the experiments were performed at pH 5, 6, 7, 8, and 9, only pH 5, 7, and 9 are depicted in this figure. This was done because the gel labeling profiles were virtually identical between pH 6 and pH 9 with only slight variations between the maximum peak height and labeling patterns in the transporter region as well as all other regions of the gel. The notable exception to this is the pattern observed at pH 5. Under this condition the labeling of the

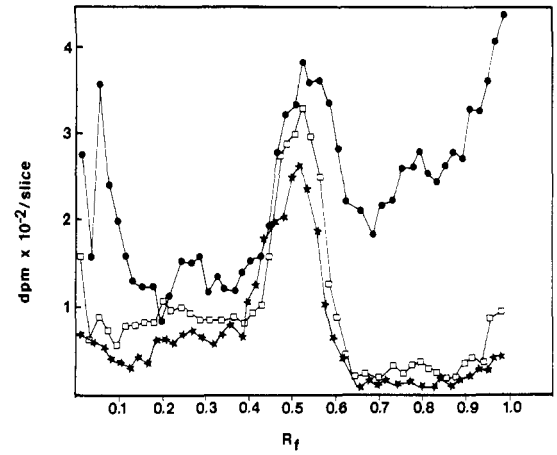

FIGURE 3: [^{3}H]Cytochalasin B photolabeling pattern as a function of pH. Ghosts were incubated and photolyzed with 0.5 M L-glucose and 0.1 μM [^{3}H]cytochalasin B in 10 mM sodium phosphate buffer under the following conditions: (●) pH 5; (□) pH 7; (★) pH 9. Ghost membranes (100 μg) were applied to 5% acrylamide disc gels and electrophoresed according to the method of Fairbanks et al. (1971). The ordinate is normalized to the tracking dye migration for each gel (R_f = 1).

zone 4.5 region is maximal, but additional peaks occur in all regions of the gel. This is especially noticeable at the top of the gel and corresponds to polypeptides in the high molecular weight region around spectrin (bands 1 and 2). The labeling profile in the lower molecular weight region of the gel is also dramatically increased relative to the other gel profiles. These experiments were performed before we discovered that the staining/destaining procedure had no effect on the photoincorporation pattern of radioligand in the major polypeptide regions of the gels. Furthermore, although membrane lipids generally migrate ahead of the tracking dye (i.e., $R_f > 1$), under some loading conditions they may trail behind the tracking dye and overlap into the regions of the lower molecular weight membrane polypeptides.[2] Thus, although this region may contain some unreacted [^{3}H]cytochalasin B, the increased labeling which is evident in this figure may represent membrane lipids which have photoreacted with cytochalasin B. Alternatively, the observation might represent proteolytic peptide fragments generated by an endogenous membrane protease(s) which is activated at low pH (Tökés & Chambers, 1975). The large increase in cytochalasin B incorporation in all regions of the gel appears to represent an increase in nonspecific photoincorporation at pH 5 and an apparent photoincorporation into most of the major erythrocyte membrane polypeptides.

Aggregation Effects. One phenomenon which we observed early in our investigations was the appearance of a large amount of labeling in the polypeptide region of the gels above a molecular weight of 100 000. This often was manifested by two or three additional distinct peaks of approximate molecular weights of 100 000 and 200 000. Furthermore, large amounts of labeled polypeptides were also detected in the stacking gels. Under the electrophoretic conditions used this would correspond to polypeptides of monomeric molecular masses of greater than 500 000 daltons. An example of this phenomenon for a resolving gel is illustrated in Figure 4.

We now believe these effects are a result of aggregation of transporter proteins due to conditions associated with solubilization and electrophoresis. The conditions which tend to promote this effect are protein loaded, high ionic strength gel systems, such as the Laemmli system, as well as the type of sodium dodecyl sulfate used in conjunction with heat treatment

[2] M. F. Shanahan, unpublished observation.

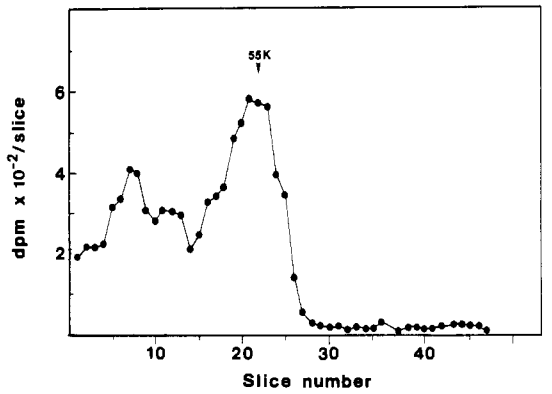

FIGURE 4: Aggregation of [^{3}H]cytochalasin B labeled polypeptides due to electrophoresis conditions. The graph indicates the distribution of [^{3}H]cytochalasin B on Laemmli gels run with dodecyl sulfate and heat-treated (100 °C, 3 min) samples. *Arrow* indicates the center of the zone 4.5 polypeptide region.

of the sample in sample buffer. Thus, when samples are not heat treated following solubilization in sample buffer, no aggregation is observed with the Fairbanks gel system (a low ionic strength buffer system). However, when buffers containing pure sodium dodecyl sulfate were used in conjunction with the Laemmli gel system (high ionic strength), aggregation occurred. When a mixture of sodium C12, C14, and C16 alkyl sulfates (Pierce Chemical Co.; sequanal grade lauryl sulfate) was used with the Laemmli gel system (Baldwin et al., 1982), no aggregation was observed. Finally, if the solubilized samples are boiled prior to electrophoresis, then varying degrees of aggregation are observed, when either gel system is used. We have recently found that with the sample buffer described by Gorga et al. (1979), which contains 3% lauryl sulfate, no high molecular weight labeled polypeptides are observed in the presence or absence of boiling the sample for 1 min. Under these conditions the labeling pattern is localized in the zone 4.5 region and is virtually identical for heat-treated vs. untreated samples in Laemmli gels. Aggregation effects have been reported for electrophoresis of other erythrocyte membrane glycoproteins such as glycophorin (Potempa & Garvin, 1976; Silverberg & Marchesi, 1978) as well as for the purified glucose transporter itself (Sogin & Hinkle, 1978; Baldwin et al., 1982). In addition, Baldwin et al. (1982) have recently reported that the purified glucose transporter exhibits anomalous migration patterns during $NaDodSO_4$ gel electrophoresis and that molecular weight estimates of the transporter vary with the percentage of acrylamide used for the gels.

Effects of Photolysis on Transport. Cytochalasin B is a potent inhibitor of glucose transport in human erythrocytes (Bloch, 1973; Taverna & Langdon, 1973). Furthermore, this inhibition can be reversed when cells are washed free of the inhibitor (Bloch, 1973; Lin & Spudich, 1974). In order to test for any irreversible residual effect on transport by cytochalasin B following photolysis, initial uptakes of D-glucose were measured in intact erythrocytes under various conditions of exposure to unlabeled cytochalasin B. It was found that a 10-s photolysis alone, in the absence of cytochalasin B, resulted in a 10–20% inhibition of glucose uptake. Longer photolysis times increased this irreversible inhibition even more, and approximately 50% inhibition of uptake was observed at 40 s. Beyond 40 s, L-glucose leak fluxes began to increase substantially, and cell hemolysis became apparent. With such a high background inhibition due to photolysis itself, it was not possible to determine any residual inhibition effects which may have been due to photoinsertion of cytochalasin B since the photolabeling was estimated to be only on the order of 5%

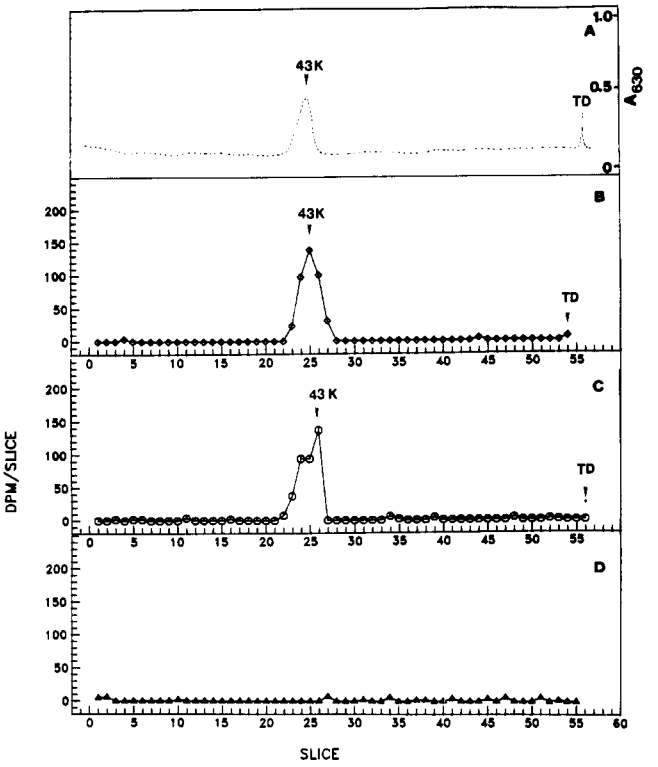

FIGURE 5: [^{3}H]Cytochalasin B photoincorporation into purified muscle F-actin. Chicken muscle G-actin was polymerized to F-actin as described under Experimental Procedures. The F-actin was preincubated for 15 min as described below and then photolyzed for 30 s in the presence of 0.5 μM [^{3}M]cytochalasin B. $NaDodSO_4$ (7.5%)–polyacrylamide slab gels were loaded with 30 μg of protein in Laemmli sample buffer. (A) Coomassie blue staining pattern obtained prior to slicing gel B below. (B) ^{3}H distribution of F-actin photolabeled with [^{3}H]cytochalasin B only. (C) F-Actin photolabeled in the presence of 0.7 M D-glucose and [^{3}H]cytochalasin B. (D) F-Actin photolyzed in the presence of 100 μM cytochalasin E and [^{3}H]cytochalasin B. TD, tracking dye front.

or less of the transporter polypeptides.

Photoincorporation of Cytochalasin B into F-Actin. The human erythrocyte membrane contains approximately 500 000 actin monomers per cell (Jones & Nickson, 1981). Moreover, purified F-actin has been shown to contain a low number of high-affinity cytochalasin B binding sites, located on the ends of filaments, which can be blocked by cytochalasin E (Flanagan & Lin, 1980). We have previously reported that one of the major peaks associated with photoincorporation of [^{3}H]-cytochalasin B into erythrocyte ghost polypeptides occurred in the region of band 5 ("red cell actin", $M_r \simeq 43\,000$–$45\,000$). Our preliminary data indicated that this region contained polypeptides associated with the glucose transporter (i.e., cytochalasin B binding sites which could be blocked by D-glucose, but not cytochalasin E), as well as actin binding sites which were sensitive to displacement by cytochalasin E but not D-glucose. In order to better distinguish the binding characteristics of these comigrating species, we investigated [^{3}H]-cytochalasin B photoincorporation into purified F-actin derived from chicken muscle. Figure 5 depicts the result of one such experiment in which soluble muscle actin, polymerized in the presence of $MgCl_2$, was photolyzed in the presence of 0.5 μM [^{3}H]cytochalasin B and subjected to $NaDodSO_4$ gel electrophoresis. Figure 5A represents the Coomassie blue staining profile of the gels. A single polypeptide staining band is evident which corresponds to the same mobility as band 5 when erythrocyte membranes are run on these gels. The results of photoincorporation of [^{3}H]cytochalasin B are presented in the remainder of Figure 5.

Table I: Comparison of Photoincorporation of [^{3}H]Cytochalasin B into Erythrocyte Ghost Membrane Proteins and F-Actin

expt	photolysis time (s)	covalently bound CB[a]	displacement by D-glucose (%)	total amt of high-affinity CB binding sites	efficiency (%)
red cell (ghosts)	20	2.02 pmol/mg of membrane protein	53	300 pmol/mg of membrane protein[b]	0.7
red cell (ghosts)	30	3.6 pmol/mg of membrane protein	65	300 pmol/mg of membrane protein[b]	1.2
F-actin	30	0.59 pmol/mg of actin	0	50 pmol/mg of actin[c]	1.2

[a] Photolabeling for ghost membranes and F-actin was performed as described under Experimental Procedures. The cytochalasin B (CB) concentration was 5×10^{-7} M in all experiments. Covalently bound CB calculated following polyacrylamide gel electrophoresis of photolabeled polypeptides. [b] Estimated value from Sogin & Hinkle (1980). [c] Estimated value from Flanagan & Lin (1980).

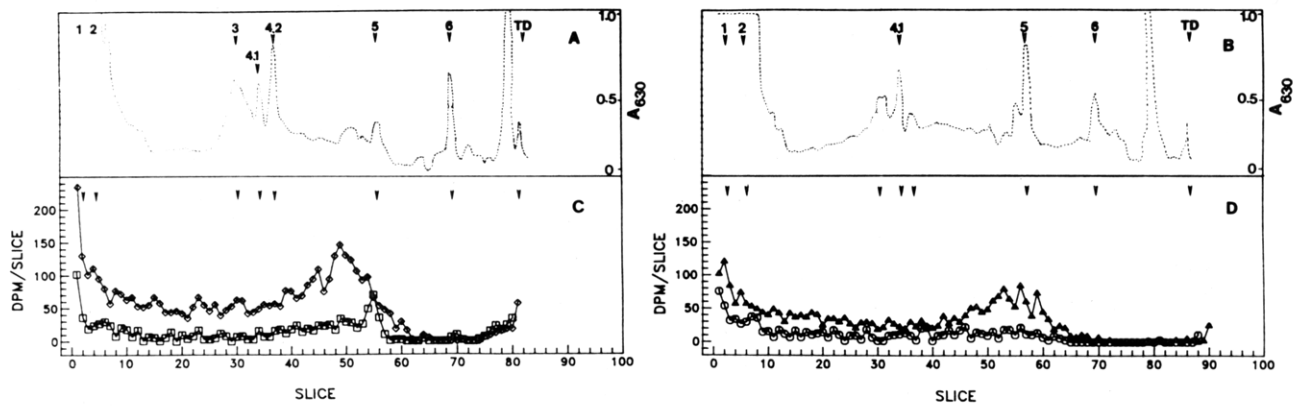

FIGURE 6: Triton extraction of membrane polypeptides prelabeled with [^{3}H]cytochalasin B. RBC ghost membranes were photolyzed with 0.5 μM [^{3}H]cytochalasin B in the presence or absence of cytochalasin E prior to extraction in 0.5% Triton X-100 (see Experimental Procedures). (A) Coomassie blue gel staining profile of the supernatant extract. (B) Gel staining profile of the extracted pellet. (C) ^{3}H distribution of the supernatant extract (◇) and pellet (□) from membranes photolyzed in the absence of cytochalasin E. (D) ^{3}H distribution of the supernatant extract (△) and pellet (○) from membranes photolabeled in the presence of 100 μM cytochalasin E.

Incubation of ligand in the absence of any glucose is shown in Figure 5B. A significant amount of photoincorporation is evident and coincides with the region of Coomassie blue staining. When 0.7 M D-glucose was included in this experiment, no difference in total ligand photoincorporation was observed (Figure 5C). However, when 10^{-4} M cytochalasin E was included in the medium (Figure 5D), all of the [^{3}H]-cytochalasin B photoincorporation was blocked. These results agree with the results of studies by Flanagan & Lin (1980) which showed that cytochalasin E blocked cytochalasin B binding to purified actin. A comparison of the stoichiometry of photoincorporation into erythrocyte zone 4.5 polypeptides and F-actin is presented in Table I. The amount of photoincorporation of cytochalasin B into membrane polypeptides (calculated as pmol of cytochalasin B bound/mg of membrane protein) is approximately 3–5 times that incorporated into purified F-actin. However, since the transporter represents only a fraction of the total membrane protein, this comparison of labeling activity with pure actin filaments is low. By use of the estimate by Jones & Nickson (1981) that the zone 4.5 region constitutes 9.8% of the total membrane polypeptide based on gel staining patterns, then estimates for the red cell transporter binding of cytochalasin B (expressed as pmol bound/mg of zone 4.5 polypeptides) would be 20.6 and 36.7 pmol/mg, respectively, for the two experiments in Table I. This corresponds to a difference of 35–60 times greater incorporation of cytochalasin B into the transporter proteins than into F-actin. Moreover, this estimate for transporter incorporation is also probably low because it is unlikely that all of the stained polypeptides migrating in this region represent glucose transport polypeptides (Baldwin et al., 1982).

Although it appears that the photoincorporation of cytochalasin B is much greater for the transporter polypeptides than for F-actin, the efficiency of incorporation appears to be about the same (Table I). On the basis of a number of observations, the estimated efficiency of photoincorporation into the glucose transporter ranges from 0.5 to around 3% depending on the conditions, while several experiments with F-actin have given results of 1–2%. This indicates that only a small number of the high-affinity cytochalasin B binding sites in both these polypeptides are irreversibly labeled during the photoreaction.

Solubilization of Photoincorporated Cytochalasin B Binding Sites. In order to further investigate the characteristics of the cytochalasin B labeled polypeptides in the erythrocyte membrane, we subjected erythrocyte ghosts which had been prelabeled with [^{3}H]cytochalasin B to differential extraction with the nonionic detergent Triton X-100. Treatment of these membranes with 0.5% Triton X-100 resulted in the selective elution of bands 3, 4.2, and 6 and zone 4.5 as revealed by $NaDodSO_4$ gel electrophoresis (Figure 6A). This extraction pattern is similar to that originally reported by Yu et al. (1973) for this detergent. The pellet containing extracted ghosts (Figure 6B) was composed predominately of polypeptides from bands 1 and 2 (spectrin), 4.1, and 5 (actin). Varying amounts of almost all the major proteins were present in both the extracted pellet and the supernatant extract, indicating that this procedure was only partially selective. These gels were further analyzed for the relative distribution of photoincorporated [^{3}H]cytochalasin B. Figure 6C is the radioactive profile of membrane polypeptides labeled with [^{3}H]cytochalasin B in the absence of cytochalasin E. The two plots represent gels from the pellet and supernatant of the same experiment. The most interesting aspect of this experiment is that almost all of the radiolabeled polypeptides were eluted by detergent and appeared in the supernatant gel. This radioactivity was located predominately in the zone 4.5 region. The increased background of radioactivity in the high molecular weight region of this gel is most likely a trailing effect arising from the zone 4.5 region. This effect was observed

consistently in the gels containing the supernatant extract, which exhibited some streaking of stained proteins that can probably be attributed to the presence of Triton X-100 in the sample buffer. The gel pattern for the extracted ghost pellet polypeptides did not show this trailing effect, and the only radiolabeled polypeptides observed were in the region of band 5, which is red cell actin (Figure 6C). Even more interesting is that in a parallel experiment in which the membranes were prelabeled with [^{3}H]cytochalasin B in the presence of 10^{-4} M cytochalasin E (Figure 6D), the gel patterns of the supernatant and extract were similar except for the region of red cell actin (band 5). In the presence of cytochalasin E the [^{3}H]cytochalasin B incorporation into band 5 of the pellet was abolished. We believe that this evidence, in conjunction with the data presented in Figure 5, indicates that red cell actin can be labeled in situ by this procedure. In addition, these experiments indicate that there are also glucose transporter polypeptides which comigrate with actin in this region. When cytochalasin E is included during the photolysis reaction, the actin labeling can be eliminated, and we have previously observed this effect with intact ghost membranes (Shanahan, 1982). When selective extraction procedures are used, it appears possible to observe separately the labeling effects on each of these different groups of high-affinity cytochalasin B binding sites. Additional selective extraction procedures such as low ionic strength, EDTA, or high pH (Steck & Yu, 1973) may also prove useful in this regard.

Discussion

The technique investigated in this paper for labeling the human erythrocyte glucose transporter has only recently been reported in literature (Carter-Su et al., 1982; Shanahan, 1982). It has also been successfully used to identify similar glucose-sensitive, cytochalasin B binding polypeptides in chick embryo fibroblasts (Pessin et al., 1982; Shanahan et al., 1982) as well as adipocytes (Shanahan et al., 1982). While confirmation that these labeled polypeptides constitute the glucose transport system in these cells has not yet been obtained by other methods, there is strong evidence that this is the case. In the red cell a number of other investigators have already identified polypeptides in the zone 4.5 region by several other labeling methods as previously mentioned in the introduction. Purification and reconstitution of this transporter has also been accomplished (Kasahara & Hinkle, 1977; Sogin & Hinkle, 1978; Baldwin et al., 1979, 1980). Furthermore, a number of investigators have convincingly demonstrated that the D-glucose-sensitive cytochalasin B binding sites are associated with the transporter polypeptides in both intact membranes (Jung & Rampal, 1977; Pinkofsky et al., 1978) and purified transporter (Sogin & Hinkle, 1978, 1980; Baldwin et al., 1979, 1980, 1982). Because of the high degree of specificity of this cytochalasin B photolabeling technique for zone 4.5 polypeptides as well as the selective sensitivity to D-glucose, we feel that the method indeed labels the glucose transporter. This is further confirmed by the fact that in the present study conditions of detergent extraction with Triton X-100, which are similar to those originally used to extract and purify the zone 4.5 transporter polypeptides (Kasahara & Hinkle, 1977; Sogin & Hinkel, 1978), totally extracted the cytochalasin B photolabeled components from the zone 4.5 region of ghost membranes.

This new procedure therefore offers many potential advantages for identifying monosaccharide transport systems in other cell tissues which are subject to inhibition of this transport process by cytochalasin B. The methodology is simple and appears to be highly selective for labeling these proteins. The technique additionally offers promising potential as a monitoring technique for purifying the glucose transporter in cells other than the erythrocyte, since our evidence indicates that less than 2% of the sites are irreversibly labeled. This suggests that in most cases functional transport activity would remain intact while enough label might still be incorporated to allow evaluation of the efficacy of any particular technique used in purification procedures, assuming that cytochalasin B would not alter the separation properties of the transporter. In this regard the method may prove useful for purifying glucose transport systems which have so far been resistant to these attempts such as the insulin-sensitive glucose transporter in adipocyte and muscle cells or fibroblasts. Thus, while the present method and immunological techniques (Wheeler et al., 1982; Lienhard et al., 1982; Salter et al., 1982) have all identified similar polypeptides in adipocyte and fibroblast membranes, as well as cardiac muscle,[2] none of these systems has yet been purified in an intact functional form. Evidence based on cytochalasin B binding (Karnieli et al., 1981; Salter & Weber, 1979) indicates that the adipocyte and fibroblast plasma membranes contain less than 10% of the number of sites found in erythrocytes (Jung & Rampal, 1977). Purification of these systems therefore has presented a much more difficult task than that of the erythrocyte.

Since this method can also be used to label both purified and membrane-bound actin, it may offer an additional potential use in other areas of cell function. Studies involving various cellular contractile systems may also be complemented when variations of this technique are used. Thus, even in the case of the erythrocyte membrane where both red cell actin and some of the transporter polypeptides of similar molecular weight are labeled with cytochalasin B, it is still possible to distinguish them from each other under appropriate experimental conditions.

The exact nature of the photoincorporation reaction of cytochalasin B with the membrane polypeptides is, as yet, unknown. As mentioned in our previous paper it could involve a tritium exchange from cytochalasin B to the membrane polypeptide, direct photoactivation and insertion of cytochalasin B itself, or photoactivation of the transporter polypeptide at some residue on or near the cytochalasin B binding site. We have not been able to detect any loss of tritium from [^{3}H]-cytochalasin B under photolysis conditions similar to membrane labeling, nor have we been able to detect any change in the TLC mobility or ultraviolet absorption spectra of [^{3}H]cytochaslin B following irradiation. More detailed studies such as photoproduct analysis are necessary in order to elucidate any photoreactive mechanism for this ligand. In addition we have not as yet been able to rule out the possibility that the transporter protein itself contains a photoactive moiety (McLaren & Shugar, 1964) which may be responsible for the photoinsertion of this ligand into membrane polypeptides. Experiments are currently in progress to investigate all three of these potential photoreactive mechanisms.

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