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LABELLING OF THE HUMAN ERYTHROCYTE GLUCOSE TRANSPORTER WITH ^{3}H-LABELLED CYTOCHALASIN B OCCURS VIA PROTEIN PHOTOACTIVATION

MARK DEZIEL [b], WILLIAM PEGG [a], ESTHER MACK [b], ASER ROTHSTEIN [b] and AMIRA KLIP [a,*]

Departments of [a] Neurology and [b] Cell Biology, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8 (Canada)

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Irradiation of human erythrocyte membranes with ^{3}H-labelled cytochalasin B results in specific photolabelling of the glucose transporter. The action spectrum of photolabelling has a maximum at approx. 280 nm, whereas the absorption spectrum of cytochalasin B is maximal at 210 nm. By irradiating with narrow-bandwidth light centered at 280 nm for 2 h, 8% of the transporters become covalently labelled and 47% of the remaining cytochalasin B-binding sites are obliterated. We conclude that photolabelling driven by narrow-bandwidth irradiation proceeds via photoactivation of an aromatic amino acid residue on the transporter molecule, and when compared to wide-bandwidth irradiation, permits more efficient incorporation of the label without causing additional photodamage to the remaining transporters.

Glucose uptake into mammalian erythrocytes and most other non-epithelial cells occurs through facilitated diffusion which is inhibited by the mold metabolite cytochalasin B [1–4]. The similarity in the K_i of inhibition of hexose transport and the K_d of cytochalasin B binding (0.1 to 0.3 μM) to intact erythrocytes [5], isolated ghosts [5] and purified transporter [6], and the 1 : 1 stoichiometry of binding of this alkaloid to the transporter [7] have led to the widespread use of ^{3}H-labelled cytochalasin B in the estimation of the number of transporters in the plasma membrane of several cell types (see Ref. 8). Recently, a method for the covalent photo-incorporation of ^{3}H-labelled cytochalasin B into the hexose transporter of human erythrocytes has been described [9,10] and has confirmed its identity as band 4.5, a polypeptide of mol. wt. 45 000 to 60 000 [6,11]. This method has been adopted to identify the transporter in membranes of other cells [12–15] in which the complex protein composition had previously prevented the isolation and identification of the transport protein. In those studies [9,10,12–14], the unfiltered light of a mercury arc lamp was used to drive the reaction, and it was presumed that cytochalasin B was the photoreactive species. Such broad-bandwidth irradiation produced very low yields of photolabelling (0.3 to 3%) and resulted in a substantial loss of glucose transport capacity due to irradiation [16]. The recent observation of successful photolabelling of the glucose transporter of rat skeletal muscle by irradiation with narrow-bandwidth light centered at 280 nm [15] suggests that cytochalasin B may not be the photoreactive species. The present study was undertaken to establish the identity of the photoactivated species, in order to gain information on the mechanism of photolabelling. Furthermore, experimental conditions are defined which result in an increased yield of photolabelling while minimizing photodamage to the remaining unlabelled transporters.

Erythrocyte membrane ghosts were prepared by

* To whom correspondence should be addressed.

the method of Dodge et al. [17], and peripheral proteins were stripped off by treatment with 0.1 mM EDTA in 10 mM NaOH [18]. Protein was determined by the method of Lowry et al. [19]. The stripped ghosts were resuspended at a concentration of 0.5 mg protein/ml in 0.1 M sodium phosphate (pH 7.5) containing, where indicated, 0.2 M D-glucose. [^{3}H]Cytochalasin B (15 Ci/mmol, New England Nuclear) was added without dilution from ethanolic stocks to a final concentration of 1 μM; final ethanol concentration was 2% (v/v). After 30 min of dark incubation, membranes were irradiated in the sample chamber of an Aminco Bowman spectrofluorometer equipped with a 150 W xenon arc lamp. Wavelengths were selected with the excitation monochromator, with the slit removed. After irradiation for corrected time periods (see legend to Fig. 1), membranes were kept dark and sedimented by centrifugation at $13000 \times g$ for 9 min in an Eppendorf centrifuge. Membrane pellets were resuspended in 100 μl of 10 mM sodium phosphate (pH 7.5), dissolved in a SDS-based solubilizer and subjected to gel electrophoresis according to Laemmli [20]. The separated proteins were transferred to nitrocellulose filters by electroblotting [21]. Sample lanes were cut from the filters, sliced and dissolved in methanol. Radioactivity was determined by liquid scintillation counting, at an efficiency of 33%.

In order to identify the reactive species in the photolabelling of the erythrocyte hexose transporter with ^{3}H-labelled cytochalasin B, the absorption spectrum of cytochalasin B and the action spectrum of photolabelling were compared. Fig. 1 panel A shows the absorption spectrum of 10 μM cytochalasin B in water, ethanol and diethyl ether. Absorption was maximal at 214 nm in diethyl ether, 205 nm in ethanol and 192 nm in water. The absorption maxima around 210 nm may be ascribed to the keto-enolic group of this molecule. The molar extinction coefficient of cytochalasin B measured in H_2O, ethanol and diethyl ether at peak absorption was 55000, 22000 and 37500 $M \cdot cm^{-1}$, respectively. In contrast, at 280 nm it was below 500 in all solvents. The expected contribution of the phenyl ring of cytochalasin B to the absorption spectrum in the 260–280 nm region was not observed even in very concentrated (1 mM) ethanolic solutions of the chromophore. Even though the absorption spectrum of ^{3}H-labelled cytochalasin B bound to the transporter or the polarity of this microenvironment cannot be measured at present, red shifts in chromophore absorption by solvent polarity are usually circumscribed to a few nanometers [22]. Hence, the consistent absorption pattern observed in several solvents of markedly different polarity suggests that a similar absorption of the alkaloid could be expected when bound to the transporter. Shown alongside in Fig. 1A is the action spectrum of photolabelling of band 4.5 (M_r 45000 to 66000) of extraced membranes in the wavelength range of 235 nm to 310 nm. Incorporation of ^{3}H-labelled cytochalasin B was maximal at 280 nm, with decreased labelling at longer and shorter wavelengths, including those which overlap the cytochalasin B absorption spectra.

Panel B shows the profile of ^{3}H-labelled cytochalasin B incorporated into erythrocyte membrane proteins as a result of irradiation at 280 nm. As seen in previous studies with broad-bandwidth ultraviolet light [9,10], the label was incorporated almost exclusively in the band 4.5 region of the electrophoretic profile. Labelling was substantially diminished by addition of D-glucose, as was seen with unfiltered light. Hence, labelling resulting from irradiation with narrow-bandwidth light appears indistinguishable from that resulting from broad bandwidth irradiation.

The action spectrum of photolabelling of Fig. 1A showed a clear maximum at 280 nm, a spectral region typically attributed to the aromatic amino acid residues of proteins. Hence, photolabelling of the transporter with ^{3}H-labelled cytochalasin B appears to proceed via activation of an aromatic amino acid residue on the protein molecule rather than through photoactivation of the ligand seen with most photoaffinity labels. The hexose transporter contains numerous aromatic amino acids [7,11] including tryptophan residue(s) whose fluorescence is modulated by binding of cytochalasin B [23]. Such tryptophan residue(s) could conceivably participate in the photolabelling reaction. Photoactivation of the ligander rather than of the ligand has been reported to result in the covalent incorporation of [^{3}H]AMP to a receptor protein [24]. More recently it has been reported that the glycine receptor in rat spinal cord is photolabelled

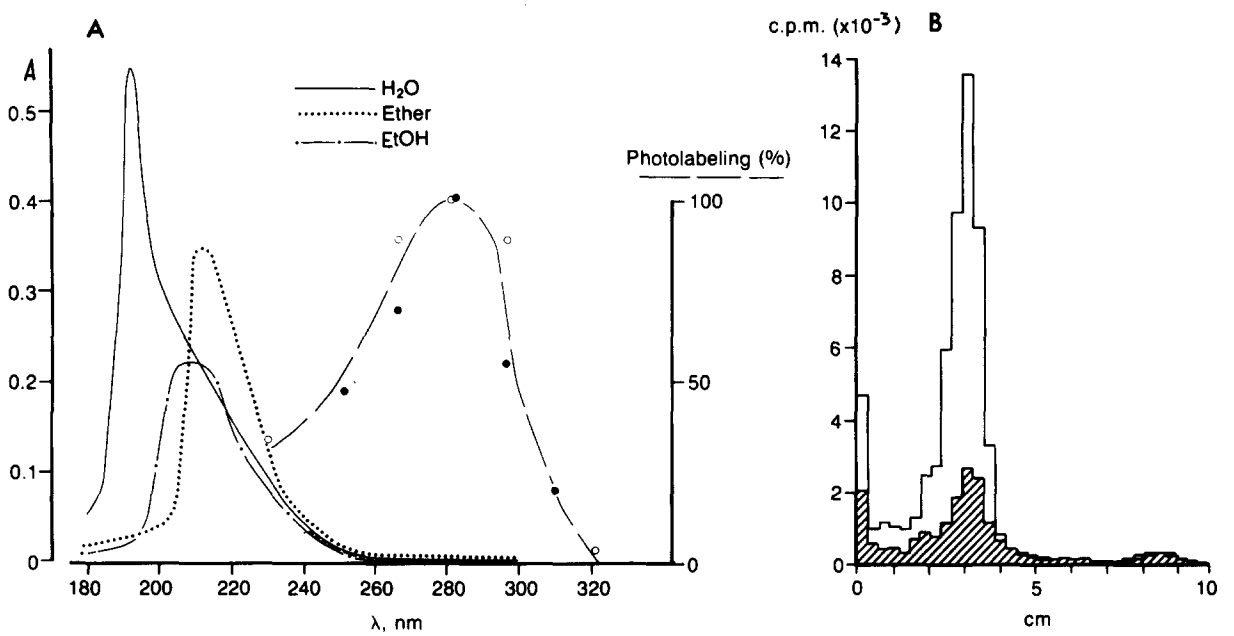

Fig. 1. Absorption spectrum of cytochalasin B and action spectrum of transporter photolabelling. (Panel A) Absorption spectra. Absorbance of 10 μM solutions of cytochalasin B in various solvents, measured with a Gilford 250 spectrophotometer. Action spectrum: Incorporation of ^{3}H-labelled cytochalasin B into band 4.5 by irradiation with narrow-bandwidth light centered at the indicated wavelengths; (○) and (●) are data from separate experiments, normalized to the percentage of maximal photolabelling in each experiment. The duration of irradiation was adjusted to compensate for the different radiant output of the monochromator at each wavelength (output spectrum supplied by Aminco-Bowman), so that the ultimate photon dose was equivalent for each sample. (Panel B) Profile of ^{3}H-labelled cytochalasin B incorporated into membrane proteins in the absence (open) and presence (shaded) of 0.2 M D-glucose.

with ^{3}H-labelled strychnine possibly through protein photoactivation [25]. Hence, this may constitute a means of labelling a variety of proteins, eliminating the need to introduce photoreactive groups into the labelling ligands.

A second objective of this study was to determine experimental conditions that would result in increased yield of photolabelling while minimizing the photodamage to the protein that occurs in light exposure. The efficiency of labelling at 280 nm is shown in Table I. During 2 h irradiation, 51 pmol of ^{3}H-labelled cytochalasin B was specifically incorporated into band 4.5 (expressed per mg protein stripped ghosts). This corresponds to 8.3% of the total cytochalasin B binding sites measured by equilibrium binding. The yield could not be improved by performing the photoreaction at 4°C. Damage sustained by the transporters during irradiation was assessed by measuring the remaining capacity of the membranes of equilibrium binding of cytochalasin B. During irradiation, 47% of the unlabelled sites were inactivated. In contrast, in an analogous experiment using unfiltered light from the xenon arc lamp, only 2.4% of the sites were labelled, and 88% of the cytochalasin B binding sites were inactivated (data not shown). These data indicate that narrow-bandwidth irradiation results in a nearly 3-fold increase in the efficiency of labelling compared to that seen by us and others [9,10] with broad-bandwidth irradiation. The reduction observed in cytochalasin B binding capacity of the unlabelled transporters (47%) is similar to the reduction in glucose transport observed after photolabelling with broad-bandwidth light [16]. Equilibrium binding of cytochalasin B may be a more direct means than glucose transport to assess photodamage because binding does not depend on the maintenance of membrane integrity needed for transport, which could be light-sensitive.

TABLE I

PHOTOLABELLING AND PHOTODAMAGE TO THE HEXOSE TRANSPORTER

The concentration of cytochalasin B was 1 μM during photoreaction and equilibrium binding.

Treatment	Bound cytochalasin B (pmol/mg protein)	
	Covalent incorporation [a]	Equilibrium binding [b]
Photoreaction 2 h, 280 nm	51	300
No irradiation	-	615

[a] [^{3}H]Cytochalasin B bound to band 4.5, expressed per mg protein stripped ghosts.

[b] Irradiated samples (and control samples incubated with ^{3}H-labelled cytochalasin B in the dark) were washed once with 10 ml of 0.1 M sodium phosphate (pH 7.5) containing 1 mg/ml albumin, and twice without albumin, in order to remove excess cytochalasin B. Equilibrium binding of ^{3}H-labelled cytochalasin B was measured as described by Lienhard et al. [26] following centrifugation at 40000 r.p.m. in a Beckman 42.1 rotor. [^{14}C]Urea was used to estimate fluid trapping within the membrane pellet. After compensation for counting spillover and fluid trapping, the differential binding of cytochalasin B in the presence and absence of D-glucose was determined.

The increased yield of photolabelling observed at 280 nm enhances the potential usefulness of this technique. Firstly, the increased yield of labelling decreases the lower limits of detection through photolabelling. More importantly, although stoichiometric labelling of the transporter was not achieved, the increased efficiency of labelling does increase the probability that the labelled fraction of transporters will be representative of the entire transporter population. This is required in order to establish a confident correlation of different amounts of photolabelling observed in varying physiological conditions [12] with changes in the number of functional glucose transporters.

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