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Thiol-fatty acylation of the glucose transport protein of human erythrocytes

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Incubation of intact human erythrocytes with [^{3}H]palmitate labeled a protein with electrophoretic characteristics of the glucose transporter. This labeling occurred via a thioester linkage, since it was unaffected by organic solvent extraction, but was substantially removed as the hydroxamate upon treatment with neutral hydroxylamine. Immunoprecipitation of the labeled protein with a monoclonal antibody to the glucose transporter confirmed its identity.

Thiol-fatty acylation; Glucose transport protein; Palmitylation; Erythrocyte, human

1. INTRODUCTION

Thiol-fatty acylation is one of several mechanisms by which plasma membrane proteins may be covalently linked to lipid [1,2]. This process is post-translational, and occurs even in mature erythrocytes, in which acylation by [^{3}H]palmitate has been reported for ankyrin [3], the anion transporter [4], the Rh(D) antigen [5], and an unidentified $M_r = 53$–55000 protein [5,6]. A major criterion for such acylation is failure to remove [^{3}H]palmitate by organic solvent extraction or upon separation of membrane proteins and lipid during sodium dodecyl sulfate-polyacrylamide gel electrophoresis [1,2,7]. The presence of a thioester rather than a carboxylic ester or amide is shown by lability of the linkage to treatment with neutral hydroxylamine, associated with the formation of [^{3}H]palmitohydroxamate [1,2,7].

The human erythrocyte glucose transporter is a likely candidate for thiol-fatty acylation, since it is an extremely hydrophobic multi-spanning membrane protein [8], which contains six cysteine residues [8], only five of which are reactive with *N*-[^{3}H]ethylmaleimide under strong denaturing conditions [9]. Furthermore, Staufenbiel [6] has shown that [^{3}H]palmitate labeling of erythrocyte ghosts occurs in the broad band 4.5 transporter region (nomenclature of Fairbanks et al [10]) on polyacrylamide gel electrophoresis. Because of the potential contribution of such a linkage to anchoring of a protein in the membrane, or to its functional regulation [1,2,11], the possibility that the glucose transporter is thiol-fatty acylated was investigated in human erythrocytes.

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2. EXPERIMENTAL

Human erythrocytes were obtained and prepared as described previously [12] using buffer containing 10 mM Hepes, 135 mM NaCl, 2.2 mM KCl, 2.1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.5 mM KH_2PO_4, 10 mg/ml bovine serum albumin, and 5 mM D-glucose, pH 7.4. Incubations were initiated by adding 25 μCi of [9,10-^{3}H]palmitic acid (30 Ci/mmol, New England Nuclear) to each ml of 50% cells. The labeled palmitic acid was dissolved in ethanol, the final fraction of which never exceeded 1%. Incubations were carried out for 2 h at 37°C with gentle agitation and terminated by 3 centrifugation washes in 20 vols of the same buffer. Labeled cells were lysed immediately, leaky ghost membranes prepared [10], and these depleted of extrinsic membrane proteins as described by Gorga and Lienhard [13]. Purified glucose transporter was prepared as described by Baldwin et al. [14]. The labeled glucose transport protein was immunoprecipitated from purified band 4.5 as described by Tai and Carter-Su [15], using monoclonal antibody 7F7.5 coupled to Protein A-Sepharose CL-4B [16].

Radiolabeled protein was subjected to slab-gel sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described [17,18]. Following electrophoresis, the gels were treated by one of several methods. Some lanes were sliced into 1.9 mm sections for radioactive counting [18]. Others were extracted with chloroform/methanol (2:1) to remove noncovalently attached [^{3}H]palmitate [3]. Additional lanes were incubated with 1 M $NH_2OH \cdot HCl$, pH 6.6, for 2 h at 23°C to cleave thiol esters [19], and then sliced for radioactive counting. For determination of hydroxylamine cleavage products from the glucose carrier region, following electrophoresis the band 4.5 region was identified by the location of prestained standards (Bethesda Research Laboratories, Gaithersburg, MD), cut from an entire gel, and treated as above with hydroxylamine. The hydroxylamine-cleavage products were isolated as

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described by Schlesinger et al. [20]: the hydroxylamine buffer was extracted three times with equal volumes of chloroform/methanol (2:1), the extracts were dried under nitrogen, the residue was redissolved in chloroform/methanol (1:1), and a sample was mixed with 50–100 μg of palmitohydroxamic acid, prepared as described by Heusser [21]. Thin-layer chromatography was carried out on an aliquot of this solution using silica gel G plates developed in toluene/chloroform/acetone (1:1:1). Carrier hydroxamate was detected by spraying with 0.7% $FeCl_3$ in methanol/ether/water (45:45:10) containing 0.75% HCl [21]. Radioactivity was quantitated by scraping 0.5 mm sections of the gel lanes from the plate, adding 0.5 ml of water, and counting in 5 ml of Poly-Fluor (Packard Instrument Co.). In this thin-layer solvent system the R_f of palmitohydroxamate was 0.2, and that of [^{3}H]palmitic acid was 0.5.

3. RESULTS

Purified glucose transport protein from cells incubated with [^{3}H]palmitate showed labeling of a broad peak in the band 4.5 region corresponding to that expected for the glucose transporter [14]. Although not clearly separated from the tracking dye in the 8% acrylamide gel of Fig. 1, labeling of a band in the $M_r = 28$–32000 region was also prominent in 10% gels. This region did not correspond to band 7, which could be seen in adjacent Coomassie brilliant blue-stained gel lanes (not shown). Neither peak was appreciably decreased by extraction of the gel with chloroform/methanol (not shown).

The nature of the linkage between [^{3}H]palmitate and the labeled band 4.5 protein was determined by treatment with hydroxylamine at pH 6.6, which cleaves thioesters, but not carboxylic esters or amides [1,2].

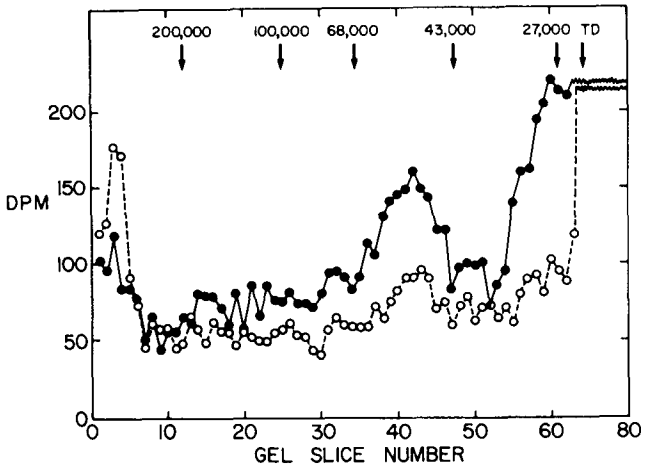

Fig. 1. Electrophoretic pattern of [^{3}H]palmitate-labeled purified band 4.5 and effects of neutral hydroxylamine treatment. Band 4.5 was purified from [^{3}H]palmitate-labeled erythrocytes and incubated in 50 mM Tris containing 1 mM EDTA and 150 mM NaCl, pH 7.4, without (closed circles) or with (open circles) 1 M hydroxylamine, for 2 h at 23°C. The incubation was terminated by a 15-fold dilution and centrifugation for 1 h at 100000×g. Electrophoresis of 100 μg of untreated, and 200 μg of hydroxylamine-treated band 4.5 pellets was performed across several lanes of an 8% acrylamide gel.

Hydroxylamine treatment of purified band 4.5 before electrophoresis resulted in loss of radioactive label from all the major peaks, including band 4.5 (Fig. 1). This loss was even greater than directly apparent in Fig. 1, since twice the amount of hydroxylamine-treated band 4.5 protein was applied to the electrophoretic gel. Similarly, when purified band 4.5 was subjected to polyacrylamide gel electrophoresis, subsequent treatment of the intact gel with hydroxylamine resulted in the same cleavage pattern and loss of radioactivity (not shown). Hydroxylamine treatment decreased the band 4.5 radioactivity by 50–70% in the several experiments performed. The nature of the hydroxylamine-cleavage products was studied by cutting the band 4.5 region from a similar gel, treating it with neutral hydroxylamine, extracting the resulting supernatant with chloroform/methanol (2:1), and identifying the radiolabeled products by thin-layer chromatography. In a typical chromatogram, 60–70% of the radioactive extract was identified as palmitohydroxamate and a small amount of palmitic acid (not shown). Taken together, these results indicate that most of the labeling in the band 4.5 glucose transporter region of electrophoretic gels involves thioester linkages.

Thiol-fatty acylation of the glucose transporter was confirmed by its immunoprecipitation from a purified band 4.5 preparation derived from cells which had been labeled with [^{3}H]palmitate. As shown in Fig. 2, immunoprecipitation with monoclonal antibody 7F7.5, which in this preparation should bind only the glucose transporter [15], did result in a labeled peak in the band 4.5 region upon subsequent electrophoresis of the immunoprecipitate. Immunoprecipitation with a control monoclonal antibody did not yield a labeled band 4.5 peak (Fig. 2).

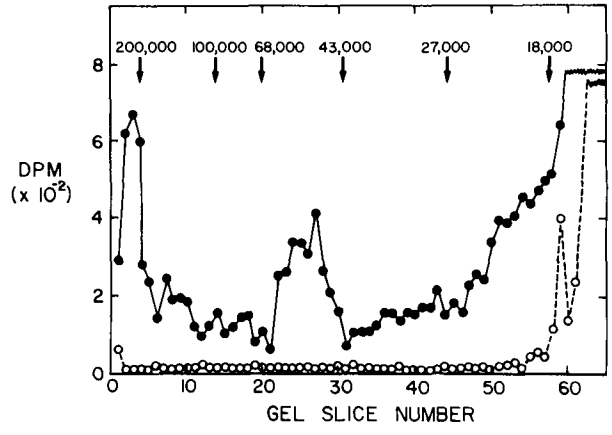

Fig. 2. Immunoprecipitation of [^{3}H]palmitate-labeled glucose transporter. A purified band 4.5 preparation from erythrocytes which had been labeled with [^{3}H]palmitate was immunoprecipitated with monoclonal antibody 7F7.5 (closed circles) or a non-specific monoclonal antibody (open circles), and 20 μg were subjected to electrophoresis on a 10% acrylamide gel.

4. DISCUSSION

The major finding of this work is that the glucose transport protein of human erythrocytes is thiol-fatty acylated. By analogy with other proteins [1,2,4,5], this feature may contribute to its hydrophobic nature, its tendency to aggregate in denatured form, and to difficulties encountered in its purification [14,22,23]. Although the purified band 4.5 prepared as described herein consists of about 70–80% glucose transporter relative to other proteins retained in the endogenous lipid micelles [14], it is known to be contaminated with the nucleoside transporter in the $M_r = 43$–66000 range, and with band 7 [14,15]. Since the [^{3}H]palmitate-labeled peak in the $M_r = 43$–66000 glucose transporter region could reflect labeling of the former, proof that the glucose transporter was indeed labeled required its specific immunoprecipitation. This was accomplished with monoclonal antibody 7F7.5, which has been shown by Tai and Carter-Su [15] to differentiate between the two transporters.

In purified band 4.5, a second [^{3}H]palmitate-labeled peak was observed on electrophoretic gels. This band varied in apparent molecular weight with the acrylamide concentration of the gel, usually appearing as a peak and shoulder, ranging from $M_r = 28$-32000 (Fig. 1). This region contains the Rh antigens, and the Rh(D) antigen is known to be fatty acylated under these conditions [5]. The Rh proteins, like the glucose transporter, are also quite hydrophobic and may be retained in the purified band 4.5 preparation. The peak was not present in electrophoreses of immunoprecipitated glucose carrier (Fig. 2).

The cysteine(s) in the glucose transporter involved in thiol-ester formation were not determined in the present study. Deziel et al. [9] have shown that of the six cysteines in the erythrocyte glucose transport protein [8], only five are susceptible to labeling by *N*-ethylmaleimide under denaturing conditions. The resistant cysteine is in the $M_r = 19000$ nonglycosylated tryptic fragment [9], which also contains an exofacial sulfhydryl reactive with impermeant maleimides [24,25]. In other thiol-fatty acylated proteins, a common location for the involved cysteine is on a cytoplasmic segment relatively near the membrane [1,2]. This location apparently serves the function of tethering the segment to the membrane. In the $M_r = 23$–40000 glycosylated tryptic fragment of the glucose transporter, hydropathy analysis suggests that Cys-207 is located very close to the lipid bilayer on the large cytoplasmic loop [8], so that this may be a plausible site for thioester formation. Additional proteolytic digestion studies are indicated to further localize the labeled sulfhydryl(s).

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