GLUT 1: Identification of exofacial lysine-residues

Richard A. J. Preston and Stephen A. Baldwin

Departments of Biochemistry and Chemistry and of Protein and Molecular Biology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, U.K.

Introduction

D-Glucose is an important nutrient for many mammalian cell types and indeed some tissues, such as the brain, are almost entirely dependent upon it as an energy source. In most mammalian cells, uptake of this hydrophilic sugar molecule across the hydrophobic core of the plasma membrane occurs by the passive process of facilitated diffusion, catalysed by members of a small family of related sugar transporters. The first of these transporters to be cloned was the glucose transporter of human erythrocytes and the blood-brain barrier (GLUT 1 isoform) and this remains the best characterized member of the family [1]. Interestingly DNA-sequencing studies have shown recently that, whereas the mammalian passive glucose transporters are not related to the active, sodium-linked glucose transporters of mammalian small intestine and kidney, they do exhibit sequence similarity both to passive and to active, proton-linked sugar transporters in other organisms. These include transport proteins from higher plants, green algae, protozoans, yeasts, cyanobacteria and eubacteria [2].

Abbreviations used: GLUT, glucose transporter; NHS-LC-biotin, sulphosuccinimidyl-6-(biotinamido)hexanoate.

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Characteristically the members of this large sugar-transporter family have 12 hydrophobic segments of sequence, which are predicted to form membrane-spanning α-helices [1, 2]. Direct evidence for the highly α-helical nature of the proteins has come from circular-dichroism [3] and i.r. spectroscopic [4] studies of the purified human erythrocyte glucose transporter (GLUT 1). The existence of a 12-helix structural motif is also supported by examining the aligned sequences of the proteins. These reveal the presence of insertions and deletions only in those regions of the sequences which are predicted to form extramembranous loops connecting the putative transmembrane helices. Studies employing vectorial proteolytic digestion and site-directed antibodies as topological probes have probed evidence for the cytoplasmic location of the C-terminus of GLUT 1 [5, 6]. Similarly evidence has been obtained for the cytoplasmic location of a large, hydrophilic loop connecting putative helices 6 and 7 of GLUT 1 and for the extracellular location of the loop connecting helices 1 and 2, which includes the glycosylation site in this glycoprotein [5, 7]. Thus the sugar transporter is predicted to span the membrane 12 times, with both the N- and C-termini on the cytoplasmic face of the membrane [1]. However, there is currently little further direct evidence for such a predicted topology and the aim of the present study was to provide such evidence, by identifying exofacial lysine residues within the primary structure of GLUT 1. This was achieved by chemically modifying intact human erythrocytes with the membrane-impermeant, amino-group-specific biotinylating reagent sulphosuccinimidyl-6-(biotinamido)hexanooate (NHS-LC-biotin). Following purification of GLUT 1 from the cell membranes, the sites of biotinylation were investigated by proteolytic digestion and identification of the biotinylated fragments on Western blots using streptavidin reagents.

**Methods**

Human erythrocytes obtained from fresh blood were washed and then resuspended at a haematocrit of 25% in 200 mM Hepes, pH 8.1. Exofacial biotinylation was achieved by incubation with 1 mM NHS-LC-biotin, at 0°C, for 1 h, unless otherwise indicated in the text. Excess reagent was then quenched by adding a tenfold molar excess of glycine and membranes were prepared by hypotonic lysis. GLUT1 was purified from the biotinylated membranes using the procedure of Cairns et al. [8]. Limited proteolytic digestion of the biotinylated transporter in 50 mM sodium phosphate, 100 mM NaCl and 1 mM EDTA, pH 7.4 was achieved by adding 5% (w/w) diphenylcarbamyl chloride-treated trypsin at 0, 2 and 4 h. After a total of 6 h incubation at 25°C, the digestion was terminated by adding bovine-lung aprotinin, to give a final concentration equal to that of trypsin by weight. Samples were then electrophoresed on 12% SDS/PAGE gels and subjected either to staining with Coomassie Blue or to electrophoretic blotting onto nitrocellulose, using previously described procedures [9]. Biotinylated proteins were detected on the blots either colorimetrically, after incubation with an alkaline-phosphate conjugate of streptavidin, or using a peroxidase conjugate of streptavidin and an enhanced chemiluminescence (ECL) detection kit, as described by the manufacturers (Amersham).

**Results and discussion**

To establish the optimal conditions for exofacial biotinylation of the cells, human erythrocytes were treated with NHS-LC-biotin for periods up to 2 h, as described in the Methods section. Membranes were then prepared and their relative biotin contents assessed by slot-blotted onto nitrocellulose, colorimetric staining using streptavidin-alkaline phosphatase and then reflectance densitometry. Figure 1 shows that the incorporation of biotin into the membranes reached a maximum after approx. 1 h incubation with the biotinylating reagent and so

![Figure 1](image-url)
this period was used for all subsequent labelling experiments. The membrane-impermeability of the reagent was assessed by comparing the pattern of labelling of membrane proteins seen when intact erythrocytes were treated with the reagent with that resulting from biotinylation of unsealed erythrocyte membranes under the same conditions. In intact cells no labelling of the major peripheral protein spectrin, which lies on the cytoplasmic surface of the membrane, was observed (Figure 2b). In contrast this protein was strongly labelled in unsealed membranes, confirming the impermeability of the reagent (Figure 2a).

The major protein band labelled by the reagent in intact cells appeared to be the anion transporter, which migrates with an apparent $M_r$ of 100000 on SDS/PAGE gels, although faint labelling of a broad band of apparent $M_r$ 50000, equivalent to that of the glucose transporter, was apparent (Figure 2a). Exofacial biotinylation of GLUT1 was confirmed by purification of GLUT1 from the membranes using standard procedures, which yielded a biotinylated protein that migrated with a mobility corresponding to the authentic glucose transporter on Western blots (Figure 2c). This work was supported by the Biological Membranes Initiative of the SERC in the form of a grant to S.A.B. and a studentship to R.A.J.P. We are deeply indebted to Dr C. C. Widnell for his advice and guidance in the early stages of this work.


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