The human erythrocyte nucleoside and glucose transporters are both band 4.5 membrane polypeptides

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Human erythrocyte membranes and partially purified nucleoside transporter (band 4.5 and 7) were photoaffinity labelled with ^3H -labelled 6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine under equilibrium binding conditions. Band 4.5 was the major site of radiolabelling in both preparations. These experiments provide additional evidence to implicate band 4.5 polypeptides in nucleoside permeation, proteins previously shown to be involved in hexose transport.

Cytochalasin B-sensitive glucose transport and NBMPR-sensitive nucleoside transport in human erythrocytes are catalysed by functionally similar yet separate carrier mechanisms (10^5 and 10^4 transporters per cell, respectively) (Widdas, 1980; Young & Jarvis, 1983). Despite considerable controversy over the last few years, it is now firmly established that the glucose transporter is a band 4.5 membrane polypeptide with an apparent M_r on SDS/polyacrylamide gels of 45000–65000 (for references see Shanahan, 1982; Carter-Su *et al.*, 1982).

The first indication that the nucleoside transporter might also be a band 4.5 polypeptide came from partial purification studies using reversible high-affinity NBMPR binding activity as an assay for nucleoside carrier protein (Jarvis & Young, 1981). Triton X-100 extraction and ion-exchange chromatography on DEAE-cellulose, a procedure developed by Kasahara & Hinkle (1977) for isolation of the glucose transporter, resulted in a 13-fold purification of NBMPR binding activity from human erythrocyte 'ghosts'. D-Glucose-sensitive binding of cytochalasin B co-purified with high-affinity NBMPR binding activity under these conditions, and the active protein fraction consisted mainly of band 4.5 with a trace of band 7. This preparation is also capable of catalysing NBMPR-sensitive uridine transport when reconstituted into phospholipid liposomes (C. M. Tse & J. D. Young,

unpublished work). Recently, we have developed a method for photoincorporating [^3H]NBMPR into the nucleoside transporter of the human erythrocyte (Young *et al.*, 1983). In the present report we demonstrate that the partially purified nucleoside transporter preparation can also be covalently labelled with [^3H]NBMPR, providing additional evidence to implicate band 4.5 polypeptide(s) in nucleoside translocation.

Materials and methods

Cell and membrane preparation

Fresh human blood was collected into heparin and the erythrocytes were washed three times with a medium containing 140 mM-NaCl, 5 mM-KCl, 20 mM-Tris/HCl (pH 7.4 at 22°C), 2 mM-MgCl₂ and 0.1 mM-EDTA (disodium salt). The buffy coat was discarded. Haemoglobin-free erythrocyte 'ghosts' and partially purified nucleoside transporter were prepared as previously described (Jarvis & Young, 1981). Reversible high-affinity [^3H]NBMPR binding capacities of the two preparations, measured by equilibrium dialysis, were 45 and 1320 pmol/mg of protein, respectively, representing a 29-fold purification of carrier protein.

Photoaffinity labelling

Samples of erythrocyte 'ghosts' and partially purified nucleoside transporter (1.35 and 0.05 mg of protein/ml, respectively) were equilibrated with a saturating concentration of [^3H]NBMPR (75 nM) in the presence and absence of 20 μM -NBTGR and exposed to high-intensity u.v. light from a 450 W mercury arc lamp (Conrad-Hanovia Inc., Newark, NJ, U.S.A.) for 45 s at 4°C in the presence of 50 mM-dithiothreitol as free-radical scavenger. The

Abbreviations used: NBMPR (nitrobenzylthioinosine), 6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine; NBTGR (nitrobenzylthioguanosine), 2-amino-6-[(4-nitrobenzyl)thio]-9- β -D-ribofuranosylpurine; SDS, sodium dodecyl sulphate; ABA, *N*⁶-(*p*-azidobenzyl)adenosine.

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samples were contained in conventional 3 ml silica spectrophotometer cuvettes (10 mm light path) and stirred continuously during photolysis. Membranes were washed free of noncovalently bound ^3H before electrophoresis (Young *et al.*, 1983), while partially-purified carrier/ ^3H -labelled photoproduct mixtures were added directly to gel sample buffer. SDS/polyacrylamide-gel electrophoresis was carried out in slab gels as described by Thompson & Maddy (1982) except that samples were not heated. The ^3H content of 2 mm gel slices was determined by liquid-scintillation counting in toluene containing 0.4% (w/v) Omnifluor and 3% (v/v) Protosol (New England Nuclear).

Chemicals

[G- ^3H]NBMPR (sp. radioactivity 17 Ci/mmol and >98% radiochemically pure) was purchased from Moravsek Biochemicals, Brea, CA, U.S.A. Molecular weight standards for SDS/polyacrylamide-gel electrophoresis were obtained from Bio-Rad Laboratories. All other materials were obtained as described in Jarvis & Young (1981).

Results and discussion

NBMPR is a specific high-affinity probe of the erythrocyte nucleoside transporter (Young & Jarvis, 1983). Binding of NBMPR to the carrier mechanisms is, however, reversible and inhibited by detergent (Jarvis & Young, 1981). To overcome these limitations we developed ABA as a photoaffinity probe of the carrier (Young *et al.*, 1983). Photoactivation of site-bound [^3H]ABA in the presence of dithiothreitol as a free-radical scavenger resulted in covalent attachment of ^3H -labelled photoproduct to the cell membrane, with selective incorporation of radioactivity into the band 4.5 region of SDS/polyacrylamide gels. Parallel experiments established that NBMPR itself could be photoactivated by exposure to u.v. light, again producing selective labelling of band 4.5 polypeptide. 'Protein-depleted' membranes were used in these experiments to minimize the possibility of non-specific labelling of extrinsic membrane protein.

In Fig. 1 we extend this initial photoaffinity labelling study by showing the results of an experiment in which human erythrocyte membrane 'ghosts' and a preparation of partially-purified nucleoside transporter (band 4.5 and band 7) were exposed to high-intensity u.v. light in the presence of a saturating concentration of [^3H]NBMPR under equilibrium binding conditions. Band 4.5 was the major site of labelling in both preparations, and ^3H incorporation into this region of the gel was abolished when photolysis was carried out in the presence of nonradioactive NBTGR as competing

ligand. Two minor NBTGR-sensitive ^3H peaks are also present in both membrane preparations, one with an apparent $M_r > 90000$, the second migrating in the M_r 35000–45000 region of the gel. The former peak may correspond either to non-specific high-molecular-weight aggregates of the carrier or to a high-molecular-weight conformational state of the transporter (Jarvis *et al.*, 1980). In addition, the partially-purified extract exhibited nonspecific labelling in a position corresponding to band 7. Both samples showed substantial non-specific labelling of lipid. The specific activity of band 4.5 radiolabelling, calculated from the difference in ^3H in band 4.5 in the presence and absence of NBTGR and expressed per mg of protein, was 32-fold higher for the partially-purified extract

Fig. 1. Photoaffinity labelling of the human erythrocyte nucleoside transporter with [^3H]NBMPR

Human erythrocyte membranes (120 μg of protein: $\blacktriangle$, $\triangle$) and partially-purified transporter (2.5 μg of protein: $\blacksquare$, $\square$) were equilibrated with [^3H]NBMPR (75 nM) in the presence (open symbols) and absence (closed symbols) of nonradioactive NBTGR (20 μM) as competing ligand. Samples were irradiated and subjected to electrophoresis as described in the Materials and methods section. Numbers and arrows refer to molecular size standards ($10^{-3} \times M_r$).

compared with intact membranes. These results provide additional strong evidence to suggest that the nucleoside transporter is a band 4.5 polypeptide.

We have therefore established important molecular similarities between the erythrocyte nucleoside and glucose transporters. There are also striking functional similarities between the two carrier systems (Young *et al.*, 1983). We feel, therefore, that the present results have interesting implications for our understanding of the structure, function and molecular evolution of membrane carrier systems in higher organisms.

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