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Radiation inactivation of the human erythrocyte nucleoside and glucose transporters

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The human erythrocyte nucleoside and glucose transporters, identified previously as band 4.5 peptides (apparent M_r 66000–45000) on SDS-polyacrylamide gels, have been characterized in situ by radiation inactivation analysis. Target size analysis of lyophilized membranes indicates an apparent M_r of 110000 ± 12000 and 124000 ± 11000 for the nucleoside and glucose carriers, respectively. These data suggest that both transporters exist in the membrane as dimers.

The transport of sugars and nucleosides across the human erythrocyte membrane occur via separate facilitated diffusion systems which are distinct functional [1,2]. Nevertheless, the two transporters have many similar properties. In particular, band 4.5 polypeptides (apparent M_r 66000–45000) have been shown to be involved in both the transport of hexoses and nucleosides (for references, see Ref. 3). However, radiation inactivation analysis has suggested that the apparent in situ M_r of the glucose transporter is significantly different from that of the nucleoside transporter. Both high-affinity nitrobenzylthioinosine (NBMPR) binding and uridine transport activities were inactivated as a single exponential function of the radiation dose, indicating an in situ target size of approximately 122000 [4,5]. In contrast, the target size of the glucose transporter, based upon D-glucose sensitive cytochalasin B binding and 3-*O*-methyl-D-glucose fluxes, was estimated to be 220000–180000 [6,7]. In the present paper, we have compared directly the apparent target sizes of the nucleoside and glucose transporters in human erythrocytes by radiation-inactivation of NBMPR and D-glucose sensitive cytochalasin B binding activities, respectively. The two carriers had similar apparent M_r values in the range 110000–124000.

Blood from healthy volunteers was collected into heparin and haemoglobin-free erythrocyte 'ghosts' and alkali-stripped 'ghosts' were prepared as previously described [8,9]. Portions of membrane suspensions, 0.7 ml in volume, (equivalent to 0.7 ml of packed erythrocytes) in 5 mM sodium phosphate (pH 8.0) were freeze-dried in 6 mm-tubes and immediately sealed under vacuum. In some experiments dithiothreitol (5 mM) was added before lyophilization. Samples were irradiated with high-energy electrons using the Phillips-MEL SL 75/20 20 MeV linear accelerator at Addenbrooke's Hospital, Cambridge at a dose rate of 2.0

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Abbreviations: NBMPR, nitrobenzylthioinosine (6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine); SDS, sodium dodecyl sulphate.

Mrad/min, at 10–20°C maintained with air/solid CO_2 cooling [4]. Calibration of the radiation dose was performed with perspex dosimetry [10]. After irradiation, membranes were reconstituted with 0.7 ml distilled water and assayed for NBMPR and cytochalasin B binding activities. Estimates of M_r were obtained from the D_{37} (the radiation dose at which 37% of the original activity remains) and the empirical equation:

$$M_r = 6.4 \cdot 10^5 / D_{37}$$

where D_{37} is in Mrad [11]. D_{37} values from simple first-order inactivation plots were obtained by linear regression analysis of log[activity (%)] versus radiation dose (Mrad).

In the first series of irradiation experiments, alkali-stripped ghosts were used because these membranes retain D-glucose sensitive cytochalasin B binding sites while most of the high-affinity cytochalasin B binding sites not displaceable by high concentrations of D-glucose are absent [1]. Membranes depleted of extrinsic proteins also retain high-affinity NBMPR binding activity [9]. Fig. 1 shows the results of a typical experiment in which the fraction of specific inhibitor binding sites for NBMPR and cytochalasin B remaining (expressed as a percentage of maximum binding in the control) is plotted against the radiation dose on a logarithmic scale. The data give a good fit to a single straight line indicating that the binding activities decay as a monoexponential function of radiation dose. Calculation of the size of the binding complexes resulted in an estimate of the apparent M_r of 113 000 and 111 000 for the NBMPR-binding complex and cytochalasin B-binding complex, respectively. The pooled results from four separate experiments demonstrated that there was no significant difference in the in situ apparent M_r of the nucleoside and glucose transporters as measured by specific NBMPR and cytochalasin B binding, respectively (see Table I). The inclusion of the thiol reducing agent dithiothreitol had little effect on the apparent M_r estimate for NBMPR-binding activity (Table I). In contrast, the presence of dithiothreitol inhibited D-glucose-sensitive cytochalasin B binding to alkali-stripped ghosts. Previous results have also demonstrated that high concentrations of dithiothreitol compete

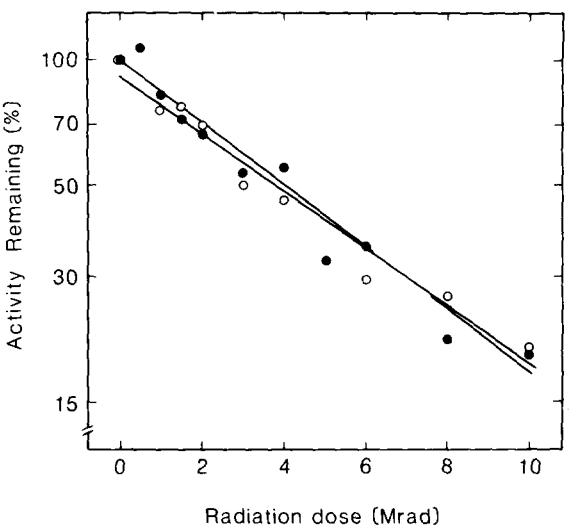

Fig. 1. Effect of radiation dose on high-affinity NBMPR-binding and D-glucose-sensitive cytochalasin B-binding activities in alkali-stripped human erythrocyte membranes. Equilibrium binding of NBMPR and cytochalasin B to erythrocyte membranes was assayed by a centrifugation method using a Beckman airfuge. Membrane suspensions (30 μl) were incubated with saturating concentrations of either [^{3}H]NBMPR (initial concentration 30 nM) in the presence and absence of 5 μM nitrobenzylthioguanosine (NBTGR) or [^{3}H]cytochalasin B (initial concentration 2.0 μM) in the presence of either 0.6 M D-sorbitol or D-glucose. Incubations were terminated 30 min later by centrifugation at 130000 × g for 10 min. The incubation time to achieve 50% equilibrium of inhibitor binding was less than 1 min for both [^{3}H]cytochalasin B and [^{3}H]NBMPR at the concentrations used in the present study (data not shown). Portions of the supernatant (200 μl) were retained for radioactivity determinations and the remaining supernatant removed before assaying the membrane pellet for ^{3}H activity by liquid scintillation counting. Unbound inhibitor in these pellets was estimated using [^{3}H]inulin and represented approx. 1.4% of the radioactivity associated with the supernatant. This value was subtracted from the total ^{3}H activity associated with the pellet to give the bound radioactivity. Specific binding is defined as the total binding minus the binding component observed in the presence of the competing ligands NBTGR and D-glucose. To correct for possible loss of protein during reconstitution of the freeze dried membranes, specific inhibitor binding activities were expressed as pmol/mg protein at each radiation dose (protein determined by the method of Lowry et al. [12]). The fractional survival of specific [^{3}H]NBMPR binding (○) and D-glucose-sensitive [^{3}H]cytochalasin B binding (●) is expressed on a semilogarithmic plot as a function of increasing doses of radiation. The least-squares slopes for the NBMPR-binding line and the D-glucose-sensitive cytochalasin B-binding line are 0.98 and 0.97, respectively. Values are the means of duplicate estimates.

reversibly with cytochalasin B for binding to a partial purified preparation of the glucose transporter [13]. Dithiothreitol may also react chemically with cytochalasin B [14]. In contrast, dithiothreitol has no effect on nucleoside transport [4].

During the preparation of alkali-stripped ghosts, peripheral proteins are removed from the erythrocyte membrane. It is possible that in situ the glucose and nucleoside transporters are attached to some of these peripheral proteins and therefore we measured the target size of the high-affinity NBMPR binding component and the D-glucose sensitive cytochalasin B binding component of the original erythrocyte ghosts which were used to prepare the alkali-stripped membranes. Table I shows that the average M_r of the nucleoside and glucose transporters measured from radiation inactivation studies of ghosts was similar to the apparent molecular weights estimated using alkali-stripped ghosts. In contrast to its effect on alkali-stripped membranes, dithiothreitol did not inhibit the binding of cytochalasin B in this case. The reason for this difference in behaviour for the two membrane preparations is unknown. In two out of the four erythrocyte ghost preparations, the inactivation curves for NBMPR-binding activity did not follow a single exponential relationship but were biphasic, while the inactivation curves for cytochalasin B binding were linear. The biphasic plots were converted to linear plots when the same membranes were irradiated in the presence of dithiothreitol, a free-radical scavenger (see also Ref. 4). Therefore, it seems likely that NBMPR-binding activity is particularly susceptible to free-radical products formed from residual oxygen present during irradiation and that such products may contribute to the loss of NBMPR-binding activity at low irradiation doses.

TABLE I

MOLECULAR WEIGHT ESTIMATES BY RADIATION INACTIVATION OF THE GLUCOSE AND NUCLEOSIDE TRANSPORTERS IN HUMAN ERYTHROCYTE MEMBRANES AND ALKALI-STRIPPED GHOSTS

Freeze-dried membrane suspensions in the presence and absence of 5 mM dithiothreitol were irradiated with increasing doses of radiation (0–10 Mrad) and the glucose and nucleoside transport activities assayed by D-glucose sensitive cytochalasin B binding and high-affinity NBMPR binding, respectively, as described in Fig. 1. The percentage inhibitor binding activity remaining was plotted on a logarithmic scale against radiation dose and the target size determined from the D_{37} value. The number of experiments is shown in parentheses. The values for the target size represent the mean ± S.E. N.A., no activity detected.

Sample	Target size (10^{-3} M_r)	
	NBMPR binding	D-glucose-sensitive cytochalasin B binding
Ghosts	104 ± 15 (4) [a]	119 ± 9 (4)
Ghosts + dithiothreitol (5 mM)	102 ± 16 (3)	123 ± 15 (3)
Alkali-stripped membranes	111 ± 5 (4)	130 ± 7 (4)
Alkali-stripped membranes + dithiothreitol (5 mM)	123 ± 5 (3)	N.A.

[a] In two of the experiments the inactivation curves did not follow a single exponential relationship and therefore were analysed as the sum of two exponential functions. The first exponential component represented 31% of the specific binding activity and gave an apparent $M_r > 10^6$ and thus only the target size of the second exponential function has been reported.

In conclusion, the present results demonstrate that the nucleoside and glucose transporters have similar apparent M_r values in situ of 110 000 ± 12 000 and 124 000 ± 11 000, as estimated by radiation inactivation of NBMPR and cytochalasin B binding, respectively. The present target size determination for the nucleoside transporter is in close agreement with previous estimates which measured the inactivation of uridine transport fluxes and NBMPR binding using glucose-6-phosphate dehydrogenase (M_r 101 000 [15]) and acetylcholinesterase (M_r 80 000 [16]) as internal standards [4,5]. However, our estimate of 124 000 for the in situ apparent M_r of the glucose transporter is significantly lower than the previous estimates of 180 000–220 000 [6,7]. The reason for the discrepancy between the present results and those of Jung and his colleagues [6,7] are unclear, although the apparent M_r estimates of 180 000–220 000 were based on single experiments with no indication of error.

There is considerable evidence demonstrating that distinct band 4.5 polypeptides (apparent M_r on SDS-polyacrylamide gels of 45 000–65 000) are involved in the transport of sugars and nucleosides

(for references, see Ref. 3). Thus, it seems probable that both the nucleoside and glucose carriers exist in the membrane as dimers.

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