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Evidence for an internal pool of nucleoside transporters in mammalian reticulocytes

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Comparisons of the site specific binding of nitrobenzylthioinosine (NBMPR) to intact and lysed red cells from various mammalian and avian species suggest the presence of a cytoplasmic pool of nucleoside transporters. In some species the cytoplasmic pool is about 50% of the total (mouse). On the average, the cytoplasmic pool is approx. 20% of the surface pool of NBMPR-binding sites. In sheep reticulocytes, both pools disappear in an energy-dependent manner during the maturation of the reticulocyte in vitro.

Introduction

The nucleoside transporter of red cells has been intensively studied for many years. It has been identified as a membrane spanning peptide of ≈ 55 kDa which may co-migrate with the glucose transporter on SDS-gels [1,2]. In red cells, this transporter functions in a Na^+-independent manner, and its presence can be readily assessed by the binding of the nucleoside analogue, nitrobenzylthioinosine (NBMPR) [3–5]. Although the substrate (and NBMPR) binding site is exposed at the exofacial side of the membrane [6,7], no loss of binding or transport activity occurs upon treatment of the exofacial surface of red cells or sealed ghosts with proteolytic enzymes [8,9]. These observations suggest that the substrate recognition site, although exofacial, is inaccessible to proteolytic enzymes or that exofacial cleavage does not disturb the binding site in a major way.

The level of nucleoside transport activity in red cells from a variety of species correlates remarkably well with the number of NBMPR-binding sites [10] thus providing a simple and direct assay for the quantification of the amount of transporter. It is also significant that the turnover number of the transporter is remarkably constant, despite large variability in the actual rate of transport of nucleosides amongst red cells of different species [10].

To date, few studies have addressed the question of the presence of an intracellular pool of nucleoside transporters whose transfer to and from the plasma membrane might be involved in the up or down regulation of nucleoside transport. It has now been well established that many cells contain intracellular pools of glucose transporters which may be regulated by changes in metabolic conditions [11,12]. A recent report showed that during longterm incubation of sheep reticulocytes, the number of surface NBMPR-binding sites may increase transiently, as if recruited from an internal pool [13].

In previous work we proposed that during maturation of reticulocytes and the concomitant loss of transferrin receptors, specific membrane proteins are internalized and collected into the vesicular contents of multivesicular bodies [14–16]. One of the proteins in these vesicles was identified as the nucleoside transporter by means of NBMPR-binding [17]. The latter function is almost completely lost [9] from the cell during the maturation of sheep reticulocytes and can be almost quantitatively recovered in the vesicles (exosomes) released from the red cell [18].

Since formation of exosomes apparently occurs in the intracellular compartment [16], and not by blebbing from the cell surface, an intracellular pool of nucleoside transporters in maturing red cells is implicated.

Abbreviations: NBMPR, nitrobenzylthioinosine; NaEDTA, sodium ethylenediaminetetraacetic acid; NADH, nicotinamide adenine dinucleotide (reduced); pCMBS, *p*-chloromercuriphenylsulfonate; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis.

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The following study provides evidence that a pool of intracellular nucleoside transporters does exist and that it, along with the surface localized transporters, are lost during sheep reticulocyte maturation in vitro.

Methods

Isolation of reticulocytes

Reticulocytes were prepared from phlebotomized sheep as previously described [19,20]. Whole blood was centrifuged at $7700 \times g$ for 15 min and the cell pellets were washed twice with saline to completely remove the plasma. The cells were resuspended in saline and recentrifuged at $1600 \times g$ for 1 h in 50-ml tubes. The top 20% of the layer of red cells and reticulocytes, was transferred to 15-ml corex tubes and recentrifuged at $1600 \times g$ for 50 min. The top 20% of the cell layers were rich in reticulocytes (65%–95%) and were used immediately or suspended in Eagle's minimal essential medium, supplemented with 4 mM glutamine, 200 μg/ml streptomycin, 200 units/ml penicillin and 50 mM sucrose and maintained at 4°C for use the next day.

Cell culture

Sheep reticulocyte preparations (75–80%) suspended (1–3: 100, v/v) in Eagle's minimal essential medium, supplemented with 4 mM glutamine, 5 mM adenosine, 10 mM inosine, 2 mM phosphate, 200 μg/ml streptomycin and 200 units/ml penicillin (standard culture medium) were incubated at 37°C for 3–44 h in roller bottles. The bottles were gassed with 95% O_2/5% CO_2 prior to incubation. All procedures were done under sterile condition [17,20]. To reduce the cellular ATP level, reticulocytes were cultured in standard culture medium with 2.0 mM deoxyglucose and 1 μg/ml rotenone in place of adenosine and inosine. Under these conditions ATP levels fell to $\leq$ 10% of normal in 30 min [14].

Isolation of vesicles

After 3–44 hours of culture, the cells were centrifuged at $8000 \times g$ for 8 min. The cell-free supernatant was recentrifuged at $8000 \times g$ for an additional 8 min. The cell pellets were washed twice with saline and once with 20 mM phosphate-buffered 0.15 M NaCl (PBS) and resuspended in PBS for further use. The cell free supernatant was then centrifuged at $100\,000 \times g$ for 120 min at 4°C. The supernatant was removed and the pellet (released vesicles) was collected [14,17]. Vesicles were kept frozen at −70°C for later use, or used directly after isolation.

Erythrocytes from different species

Fresh blood, drawn in heparinized tubes, was obtained from different species: namely rabbit, mouse, pig (Yucatan and White-Landrace type) and chicken. The whole blood was centrifuged at $1200 \times g$ for 5 min and the buffy coat containing the white cells was removed. The erythrocytes were washed four times with 0.9% NaCl and once with PBS (pH 7.4) with the removal of any leftover white cells. The erythrocytes were resuspended in PBS for direct use.

Chicken reticulocytes. Red blood cells of 12–14 day old embryonic chickens were almost entirely reticulocytes [21].

Membrane preparation

Plasma membranes (unsealed ghosts) were prepared by the method of Dodge et al. [22]. Cells were lysed in 30 volumes of lysis buffer (containing 5 mM phosphate buffer (pH 8.0) with 1 mM NaEDTA). The cell lysate was allowed to stand on ice for 10 min, then was centrifuged at $27\,000 \times g$ for 20 min. The supernatant was removed and the membrane pellets were washed twice more with lysis buffer, once with 5 mM phosphate buffer (pM 7.4) (5 mM P_i) and resuspended in the latter buffer to a final concentration of 4 mg protein/ml. The membranes were used directly or kept at −70°C for later use.

Preparation of total lysates

Erythrocytes (sheep reticulocytes, red cells from different species) were lysed in 10 volumes of 5 mM P_i at 0°C for 10 min. For chicken red cells, the cells were lysed in 100 volumes of 5 mM P_i and 4 mM $MgCl_2$ to make a 1% cell lysate. 100 μl of a 1% cell lysate was used.

Preparation of sealed ghosts

Sealed ghosts were prepared by the method of Hoffman [23] and Bodemann and Passow [24]. Sheep reticulocytes were initially lysed in 10 volumes of 5 mM P_i at 0°C for 5 min. Then 5 M sodium chloride was added to bring the lysate to isotonicity. To allow the ghosts to reseal, the preparation was incubated for 5 min at 0°C followed by 30 min incubation at 37°C. The ghosts were then pelleted at $15\,000 \times g$ for 20 min and washed twice more with PBS (pH 7.4). Finally, the ghosts were resuspended in PBS to the original cell volume to be used directly. Alternatively the suspension was kept on ice at 4°C for no more than two days.

NADH–cytochrome-c oxidoreductase assay

Measurements of NADH–cytochrome-*c* oxidoreductase activity in intact reticulocytes and sealed ghosts followed the procedure of Steck and Kant [25]. 10 μl of a 10% reticulocyte suspension or equivalent ghost suspension in PBS (pH 7.4) was added to 0.79 ml of PBS (pH 8.0) in a cuvette, followed by 0.1 ml of each of 2 mM NADH and cytochrome *c* (5 mg/ml) both in

PBS (pH 8.0). The reaction was followed spectrophotometrically at 550 nm at room temperature.

NADH–cytochrome-c oxidoreductase activities were also measured in intact cells and sealed ghosts with or without 0.005% saponin.

NBMPR-binding assays

(A) Filtration

All the procedures for measuring NBMPR-binding to intact cells and sealed ghosts were carried out in PBS (pH 7.4). Triplicate assays were carried out for each value. The replicates agreed within ±5%. For unsealed ghosts, the membranes were kept in 5 mM phosphate buffer (pH 7.4). Except where indicated, all the NBMPR-binding assays in the text were conducted by the filtration method. The non-specific binding to intact cells was 12 to 15% of the total binding. All values given have been corrected for nonspecific binding.

Sheep reticulocytes, red cells from different species, (sheep, pig, mouse and rabbit), sealed sheep reticulocyte ghosts or total red cell lysates (all equivalent to 10 μl of cells) were preincubated at 0°C for 15 min with shaking with or without 15 μM NBMPR in a final volume of 0.18 ml buffer. Following addition of twenty microliters of 400 nM ^{3}H-NBMPR (final concentration 40 nM), the mixture was incubated with shaking at 0°C for 30 min. NBMPR-binding to chicken red cells (mature red cells and reticulocytes) followed the same procedure, except one tenth the amount of cells was used. Unless indicated otherwise, all the NBMPR-binding assays were done at 0°C. The reaction was stopped by addition of 1 ml ice-cold buffer, followed by passage through Whatman GF/B filters under vacuum. The tubes were rinsed once with 3 ml buffer and the filters were washed once with 8 ml buffer. Filters were then dried and counted in 10 ml scintillation fluid.

To test whether lysate, free of membrane proteins, had an influence on NBMPR-binding activities of membrane preparations, the membrane free lysate was added back to washed membrane preparations and NBMPR-binding activities were compared in presence or absence of exogenous lysate. Lysate did not alter the NBMPR-binding activity. Moreover, cells lysed with different volumes of hypotonic phosphate buffer (1:5, 1:10 and 1:30) showed no apparent difference in the NBMPR-binding activities.

Quench corrections. Because the haemoglobin colour and protein on the filter may interfere with scintillation counting, the necessary quench corrections were determined by measuring the difference in ^{3}H radioactivity in aliquots of stock ^{3}H-NBMPR dried directly on filters or added to filters which contained the quantities of red cells, sealed ghosts or total cell lysates used in the experimental assays. The quenching was about 20% for filters containing an aliquot of the intact cell suspension and 7% and 10%, respectively, for samples containing sealed ghosts and total cell lysates. The presence of saponin (at 0.005%) did not alter NBMPR-binding.

(B) Methanol extraction of pellets

In addition to the above procedure, other methods have been used to measure NBMPR-binding. We compared the procedures above to others current in the literature such as the methanol extraction method described by Jarvis and Young [26]. This method depends on the extraction of washed red cell pellets with methanol. All cell associated radioactivity is extracted by the methanol.

NBMPR-binding to vesicles and membranes. ^{3}H-NBMPR-binding to vesicles and membrane preparations was determined by the poly(ethylene glycol) (PEG) precipitation method described by Hammond and Johnstone [27]. Briefly, membranes or vesicles (approx. 100 μg of protein) suspended in buffer containing 50 mM Tris, 1 mM EDTA, soybean trypsin inhibitor (50 μg/ml), and 6 μM phenylmethylsulfonyl fluoride at pH 7.1, were incubated in final volume of 1.0 ml with 40 nM ^{3}H-NBMPR with and without 15 μM unlabeled NBMPR to differentiate between specific and nonspecific binding. After an incubation of 30 min at room temperature, λ-globulin, sufficient to give a final concentration of 1.65 mg/ml was added, followed by 450 μl of 33% PEG 8000 dissolved in the buffer described above. The final PEG concentration was 10%. After vortexing the mixture and an additional 15 min incubation at room temperature, 4 ml of 8% PEG was added, followed by filtration through Whatman GF/B filters. The filters were washed once with an additional 4 ml of 8% PEG, dried, and counted. The non-specific binding was 8–10% or less. All values given have been corrected for nonspecific binding.

Trypsin treatment of cells and membranes

Trypsin treatment of sheep reticulocytes and sealed and unsealed ghosts followed the method described by Janmohamed et al. [8]. Sealed and unsealed ghosts (10% haematocrit equivalent) in PBS, or 5 mM P_i, respectively (pH 7.4) were incubated with trypsin (10 μg/ml) with or without a 10 min preincubation at room temperature with 0.005% saponin in 5 mM P_i (pH 7.4) at 4°C, 22°C or 37°C for 15 min. Proteolysis was stopped by addition of PMSF (final concentration 300 μM) and was followed by centrifugation at 27000 $\times g$ for 20 min. The pellets were washed three times with PBS or 5 mM P_i (both at pH 7.4) and containing 300 μM PMSF and then resuspended to the original packed cell volume with PBS (sealed ghosts) or 5 mM P_i (unsealed ghosts ± saponin).

Treatment with pCMBS

A 10 % reticulocyte suspension in PBS (pH 7.4) was incubated with pCMBS (1 mM, 2 mM, 4 mM) for 15 min at 22°C. The reaction was stopped by centrifugation at 15000 × *g* for 20 min and the cell pellets were washed three times more with PBS. Finally, the cells were resuspended to the original volume in PBS for the NBMPR-binding assay. Sealed ghosts or unsealed ghosts (10% haematocrit equivalent) were treated in an identical manner except that for the latter 5 mM P_i replaced the PBS.

Materials

Culture medium was obtained from GIBCO, Canada. NBMPR, pCMBS, Trypsin and PEG 8000 were obtained from Sigma Chemical Company, USA. NADH and cytochrome *c* were obtained from Boehringer Mannheim, Canada. EN^{3}HANCE and Protosol were purchased from Dupont New England Nuclear, Boston, MA. The ^{3}H-NBMPR (36 Ci/mmol) was purchased from Moravek Biochemicals, Brea, CA Other reagents, purchased from Fisher Scientific, were of reagent grade.

Results

Two methods have been used to measure NBMPR-binding to reticulocytes, a filtration method, and methanol extraction of a centrifuged cell pellet. Identical values were obtained with both methods using sheep reticulocytes. For example, in two separate experiments the filtration method gave 2.1 and 4.6 fmol NBMPR bound per 10^6 cells. The methanol extraction method gave values of 2.0 and 4.6, respectively, for the same two populations of cells. Moreover, the membrane fraction (obtained by centrifugation of a reticulocyte lysate at 31000 × *g*) contained the full complement of NBMPR-binding sites since the binding activity in the membrane fraction itself was identical to that in the total lysate indicating that all NBMPR-binding activity was membrane associated. In a typical experiment, values of 5.2 fmol/10^6 cells and 5.1 fmol/10^6 cells were obtained with membranes and total lysate, respectively. Membrane-free lysate did not interfere with binding. In most species of red cells examined, there was greater binding activity in cell lysates (or washed membranes) than in intact cells. The data in Fig. 1 compare the maximum specific binding of NBMPR to intact cells (cell surface binding), and in lysates of red cells from different species. Where a difference exists, the binding in lysates was consistently greater. The average cryptic component was 20% to 50% of the component accessible at the surface. In chicken red cells, the difference in binding between the lysate and the intact cells was not significant. It is noteworthy that, in sheep red cells, the difference in binding between cells and lysates disappeared in the course of maturation as the number of transporters diminished (Table I). These observations argue that the binding differences between intact cells and lysates represent a pool of intracellular transporters of variable size in the different species. The differences in binding persisted whether binding was carried out at

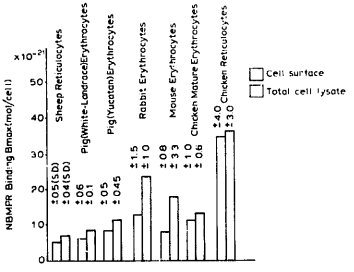

Fig. 1. Species differences in NBMPR-binding. NBMPR-binding activities in red cells from different species were measured in intact erythrocytes, or reticulocytes as well as in their osmotic, total lysates. The results are corrected for nonspecific binding and expressed as molecules per cell. The data are representative of at least two experiments. Open bars represent surface binding to intact cells and stippled bars represent the binding in osmotic lysates. Except for sheep reticulocytes, all values are means of two separate determinations ± the difference from the mean. With sheep reticulocytes the values are means ± S.D. of nine individual experiments. Each value is an average of a triplicate determination.

TABLE I

Loss of total and cell surface NBMPR-binding sites during reticulocyte maturation: effect of ATP

Sheep reticulocytes were incubated at 37°C for the periods indicated. Binding was measured in whole cells or in lysates using the filtration procedures described in Methods with 40 μM ^{3}H-NBMPR, specific activity 30 cpm/fmol. The average ATP concentration in the cells was 2.5 ± 0.5 mM ($n = 4$) and dropped to 1.5 ± 0.3 ($n = 4$) after 44 h. Rotenone and 2-deoxyglucose were used at 1 μg/ml and 2.0 mM, respectively, to lower the ATP levels to ≈ 10% of control values. A duplicate experiment is shown ± differences from the mean value. Each determination was done in triplicate. Values have been corrected for nonspecific binding.

Time of incubation	NBMPR-binding sites (fmol/10^6 cells)					
	surface binding		total binding		cryptic sites (total–surface)	
	+ATP	−ATP	+ATP	−ATP	+ATP	−ATP
Initial	5.6 ± 1.0		8.4 ± 0.1		2.8	
3 h	3.9 ± 0.6	5.2 ± 0.8	5.9 ± 0.2	7.0 ± 0.4	2.0	1.8
20 h	2.3 ± 0.1	4.7 ± 0.3	2.7 ± 0.4	5.8 ± 0.2	0.4	1.1
44 h	1.0 ± 0.7	3.4 ± 0.3	1.3 ± 0.9	4.0 ± 0.4	0.3	0.6

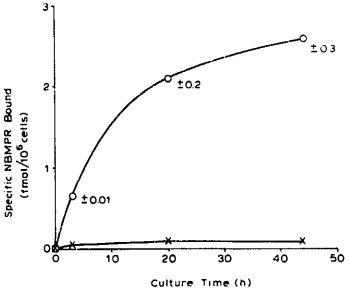

Fig. 2. Recovery of specific NBMPR-binding activity in shed vesicles (exosomes). The vesicles formed during in vitro maturation of sheep reticulocytes under ATP-replete conditions (○) and ATP-depleted conditions (×) were used to measure NBMPR-binding activity as described in Methods. The values shown are for specific NBMPR-binding activities and are expressed as fmol/10^6 cells. Each point represents the mean value from two experiments. The ± numbers indicate the spread of the mean values. Each experiment is the average (±5%) of a triplicate determination.

0°C or 22°C. For example, from four separate experiments, each consisting of triplicate assays, the average membrane/cell surface ratios were 1.45 and 1.42 at 22°C and 4°C, respectively.

The exposure of the cryptic sites could also be achieved by saponin addition to intact cells. Thus with low concentrations of saponin (0.001%) the number of apparent sites was increased and reached the level seen in lysates. High saponin concentrations (0.01%) inhibited NBMPR-binding (not shown).

The data in Table I show the energy and time-dependent loss of NBMPR-binding from the cell surface, as well as from the cryptic (intracellular) pool. The data show that with rotenone and DOG which lower ATP levels to ≤ 10% of normal, the loss of NBMPR-binding from the cell surface and the cryptic pool were diminished. Also the release of binding activity into the medium (Fig. 2) in an insoluble form (i.e. exosome formation [17]) was diminished with agents which lower ATP-levels. The intracellular pool of transporters was depleted (90%) by approx. 20 h of incubation in normal medium (Table I). With lowered ATP levels, significantly more intracellular and surface transporters (about twice as many) remained with the cell (Table I). By 40 h of incubation, few transporters remained in ATP-replete cells compared to a leftover of 50% in ATP-depleted cells. The data support the conclusion that loss of the pool of intracellular transporters, as well as surface transporters, is energy dependent (Table I). The release of NBMPR-binding activity from the cells was a mirror image of the data for loss of activity from the cells (Fig. 2).

Action of pCMBS and trypsin

Additional evidence for a pool of intracellular NBMPR-binding sites comes from studies with pCMBS and trypsin, the former causing inhibition at the cytoplasmic face and the latter digestion of the cytoplasmic domain of the transporter. In intact cells, neither pCMBS treatment nor surface digestion with trypsin altered NBMPR-binding [6,7]. (see also Tables II and III).

If the additional NBMPR sites detected in lysates and membranes were artifacts of the assay system, it might be expected that only ≈ 80% of the binding sites in membranes would be inhibited by pCMBS since it is unlikely that the artifactual binding would be sensitive to sulphydryl binding agents. Thus, using the data in Table II as an example, if pCMBS inhibited the surface binding component, but not the internal component, the residual activity would be $5.5 \times 0.1 = 0.55$ fmol/10^6 cells. The internal component would remain at 1.0 fmol/10^6 cells giving a total of 1.55 fmol/10^6 cells. The latter value is twice the observed activity of 0.75 fmol/10^6 cells. In the six experiments shown in Tables IIA and IIB the inhibition was 90% or more in every individual experiment, each of which was done in triplicate. Since an inhibition of 80% is clearly differenti-

TABLE II

Effect of pCMBS on NBMPR-binding to intact sheep reticulocyte cells and isolated membranes

Reticulocytes, lysates, membranes, and saponin treated cells were incubated ±1 mM pCMBS for 15 min at 22°C before addition of ^{3}H-NBMPR to assay binding. Each value is an average of three closely agreeing replicates (±5%). All values are for specific binding and have been corrected for controls with excess NBMPR.

	NBMPR bound (fmol/10^6 cells)	
	individual experiment	average value ±S.D.
A		
Cell surface	5.6	
Cell surface + pCMBS (1 mM)	5.5	
Lysate (total)	6.5	
Ghosts (unsealed)	6.5	
Cells and saponin	6.6	5.7 ± 1.0 [a]
Cells and saponin + pCMBS (1 mM)	0.75	0.6 ± 0.5 [a]
B		
Cell membranes	4.3	6.0 ± 2.4 S.D. [a]
+ pCMBS 1 mM	0.4	0.4 ± 0.2 S.D. [a]
2 mM	0.3	
4 mM	0.2	

[a] Represents the mean ± S.D. of values from three experiments each of which was obtained from an assay done in triplicate.

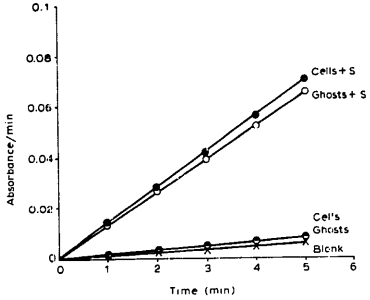

Fig. 3. Sealed ghosts retain selective impermeability. Intact cells, sealed ghosts, and cells and ghosts exposed to saponin (0.001%) were tested for the ability to reduce exogenous cytochrome-*c* with NADH. The reaction mixture contained 2 mM NADH and 5 mg/ml cytochrome-*c* (all at pH 8.0) in phosphate-buffered saline. The change in absorbance at 550 nm was monitored. ●——●, cells + saponin; ○——○, ghosts + saponin; ⊖——⊖, intact cells or sealed ghosts; × —— ×, blank, no tissue added.

ated from one of 90% (as seen below in studies with trypsin) it is concluded that virtually all the activity is pCMBS sensitive.

Since lysates and ghosts (sealed or unsealed) showed the same number of NBMPR-binding sites, it appears that in the ghosts the permeability barrier of the intact cell to NBMPR had been lost. Studies with NADH–cytochrome-*c* oxidoreductase, however, show that the sealed ghosts have maintained their selective permeability (Fig. 3)

Moreover, resealed ghosts remain insensitive to trypsin. In membranes and unsealed ghosts, the inhibition of NBMPR-binding by trypsin treatment was about 80% (Table III). Irrespective of the temperature used during the trypsin treatment, inhibition of binding did not appear to exceed ≈ 80% (Table III). In none of the five experiments cited in Table III did the inhibition exceed 81%. The fact that some NBMPR-binding activity remained despite proteolysis of the cytoplasmic surface suggests strongly that the residual peptide(s) retained binding activity, albeit greatly reduced. This would not be entirely surprising since the NBMPR-binding occurs at the exofacial domain of the transporter. The data confirm [8,9] that the cytoplasmic, but not the surface domain of the transporter reacts with pCMBS and is subject to digestion by trypsin. Since the extent of inhibition, particularly by pCMBS, was in excess of that expected for inhibition of surface sites alone, the cryptic, intracellular pool of transporters must also have been inhibited.

TABLE III

Action of trypsin on NBMPR-binding in cells and ghosts

After trypsin treatment, PMSF was added to give a final concentration of 300 μM. The cells or membranes were washed thrice with 20 mM PBS or 5 mM P_i (for membrane preparations) both of which contained 300 μM PMSF. The washed preparations were used to assay NBMPR-binding at 0°C. All values are the means of triplicate assays which agreed with ±5% of the mean value.

Condition	NBMPR bound (fmol/10^6 cells)	
Expt. 1		
Cells	4.7	
+ trypsin	4.8	
Ghosts (sealed)	6.7	
+ trypsin	6.8	
Ghosts and saponin	7.0	
+ trypsin	1.4	
	NBMPR bound (fmol/10^6 cells)	
	single experiment	pooled data ± S.D. ($n = 4$)
Expt. 2		
Membranes	4.3	5.7 ± 0.5
+ trypsin (10 μg/ml)		
15 min at 4°C	0.72	1.1 ± 0.2
15 min at 22°C	0.90	
15 min at 37°C	0.90	

Discussion

The nucleoside transporter of the mammalian red cell has long been known as a transmembrane protein whose density in the membrane may be deduced from the number of NBMPR-binding sites. The turnover number for this function remains remarkably constant among species of red cells but the number of transporters per cell varies over an order of magnitude [10].

Sheep red cells are known to lose nucleoside transport activity as well as NBMPR-binding activity during the transition between the reticulocyte and the erythrocyte stage [9]. Although the loss of the aforementioned activity has been known for some years, the fate of the lost protein has been the subject of study in only a few communications [13,17]. Recently we have quantified [18] the loss of the NBMPR-binding sites and have shown that the exosomes produced during reticulocyte maturation contain the majority (≥ 75%) of the lost activity. Since exosome formation was shown [16] to require internalization of the surface proteins, such as the transferrin receptors, prior to their release, we devised an experimental approach to assess the presence of a cytoplasmic pool of the nucleoside transporter which appeared to follow the same route for externalization as the transferrin receptor. It was anticipated that the pool would diminish with cell maturation although some surface localization of the transporter might be retained into the mature stage of the

red cell. The amount of retained transporter is known to vary amongst different strains of sheep [28,29]. In the studies described, we observed that the amount of NBMPR-binding to the surface of intact sheep reticulocytes was consistently less than the binding observed with unsealed membranes prepared by osmotic lysis or saponin treatment. Such observations suggested the presence of 'cryptic' sites which were inaccessible to NBMPR at the surface of sealed, intact cells. Since identical numbers of binding sites were obtained in an aqueous lysate, with saponin treatment (containing the proteins from both the membrane and cell cytosol) in resealed ghosts or cytosol-free washed membranes, it is likely that even the putative intracellular binding sites are membrane bound.

Given that the pool of intracellular NBMPR-binding sites was small relative to the total number of sites and different assays were used to measure binding to cells and to membranes, we considered the possibility that the cryptic sites might arise from assay artifacts. Based on the following reasons, we have concluded that the additional numbers of binding sites measured in membranes represent a pool of cytoplasmic transporters and are not due to artifacts of measurement.

(1) The difference between total and surface binding disappeared with maturation of the sheep reticulocyte. This would not be expected for an artifact but is consistent with a maturation process.

(2) The difference between total and surface binding was not present in red cells from all species. For example, chicken cells showed the same NBMPR-binding in intact reticulocytes and lysates whereas in the rabbit cells the differences between intact cells and lysates were substantial. The significance of the substantial numbers of intracellular sites in mouse and rabbit red cells is unknown. It is possible that in these species intracellular transporters may be recruited to the surface under conditions requiring nucleoside transport.

(3) Metabolic inhibitors decrease the rate of loss of the surface binding sites as well as the 'cryptic' sites. Artifacts of the binding assay would not be expected to show energy dependent loss in activity. According to our proposal of membrane remodelling associated with red cell maturation [15,16], energy is required to process proteins for externalization during the final differentiation of the red cell into the erythrocyte.

An earlier study by Blostein and Grafova [13], presented some evidence for a cytoplasmic pool of nucleoside transporters. Under their conditions of incubation, the surface component of the nucleoside transporter could be transiently increased, presumably by externalization of the cytoplasmic pool. The presence of cytoplasmic pools of plasma membrane proteins is well known in other systems. Proteins, such as the glucose transporter [11,12] and the transferrin receptor [30–33], have been widely studied both as surface proteins and as proteins in intracellular compartments. In the case of the transferrin receptor a non-recycling pool had earlier been identified in the sheep reticulocyte [34]. The latter pool may be the precursor of the cell-free exosome.

With the nucleoside transporter, the nature of intracellular pool or pool(s) has not yet been identified. Nor has NBMPR-binding been detected by cytochemical means in multivesicular bodies. However, the exosomes released during maturation of the reticulocyte contain significant levels of the transporter [17,18]. Prior to their release, the exosomes are found in sac-like intracellular multivesicular structures. It is reasonable to conclude that these sacs contain the intracellular NBMPR sites.

It should be mentioned that in contrast to the transferrin receptor, no evidence was obtained for recycling of NBMPR sites. No difference in binding of NBMPR was seen at 0°C and 37°C in intact cells under the present conditions. With proteins undergoing rapid endocytosis, there is usually a marked increase in cell associated ligand at higher temperatures. The increase with temperature reflects the relative distribution of the ligand at the cell surface versus the intracellular compartment. The lack of response of NBMPR-binding to temperature suggests that either (a) recycling for this transporter is a very slow process and not detectable in our time frame or (b) that binding of NBMPR freezes the carrier in the plasma membrane and no recycling is detected. Further work is required to address these alternatives.

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