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Chromatographic characterization of nitrobenzylthioinosine binding proteins in band 4.5 of human erythrocytes: purification of a 40 kDa truncated nucleoside transporter

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DEAE-column-purified band 4.5 polypeptides of human erythrocyte membranes are mostly glucose transporters with nucleoside transporters as a minor component. The purpose of the present work was to differentially identify and isolate the nucleoside transporters in band 4.5 free from glucose transporters. Equilibrium binding studies demonstrated that the band 4.5 preparation binds nibrobenzylthioinosine (NBTI), a potent nucleoside transport inhibitor, at two distinct sites, one with a high affinity (dissociation constant, K_D of 1 nM) with a small capacity, B_T (0.4 nmol/mg protein), and the other with a low affinity (K_D of 15 μM) with a large B_T (14–16 nmol/mg protein). The B_T of the low-affinity site was equal to that of the cytochalasin B binding site in the preparation. A gel-filtration chromatography of band 4.5 photolabeled with [^{3}H]NBTI and [^{3}H]cytochalasin B identified three polypeptides of apparent M_r 55000, 50000 and 40000. Of these, the 55 kDa polypeptide was specifically labeled by cytochalasin B (p55GT), indicating that it is a glucose transporter. Both the 50 and 40 kDa polypeptides were labeled with NBTI at low ligand concentrations (less than 0.1 μM), which was abolished by an excess (20 μM) of nitrobenzylthioguanosine, indicating that they are two forms (p50NT and p40NT, respectively) of the high affinity NBTI binding protein or nucleoside transporter. At higher (not less than 10 μM) NBTI concentrations, however, p55GT was also labeled with NBTI, indicating that the low-affinity NBTI binding is due to a glucose transporter. Treatment of band 4.5 with trypsin reduced the p50NT labeling with a concomitant and stoichiometric increase in the p40NT NBTI labeling without affecting the high-affinity NBTI binding of the preparation. These findings indicate that the nucleoside transporter is slightly smaller in mass than the glucose transporter and that trypsin digestion produces a truncated nucleoside transporter of apparent M_r 40000 which retains the high-affinity NBTI binding activity of intact nucleoside transporter. Both p55GT and p50 NT were coeluted in a major protein fraction, P1 in the chromatography, while p40NT was eluted separately as a minor protein fraction, P1a. All three polypeptides formed mixed dimers, which were eluted in a fraction PO. We have purified and partially characterized the truncated nucleoside transporter, p40NT. The purified p40NT may be useful for biochemical characterization of the nucleoside transporter.

Introduction

Nucleosides exert important biochemical and physiological effects on cell function, modulating immune response, neurotransmission and muscle contraction [1] and subserving as an in vivo energy source in certain animal cells [2]. In addition, nucleoside analogs represent widely used therapeutic agents for neoplastic diseases [3]. In order to exert these effects, nucleosides must first enter into the cell. Thus their transmembrane transport is an important determinant for their biological as well as therapeutic activities. Transmembrane transport of physiologically important nucleosides and their analogs is known to occur via a specific, protein-mediated mechanism [4,5]. Nucleoside transport in human and pig erythrocytes has been studied extensively in this regard [6–11].

Nitrobenzylthioinosine (NBTI) is a potent inhibitor of nucleoside transport in human erythrocyte [12–16]:

Abbreviations: HNBTI, hydroxynitrobenzylthioinosine; NBTG, nitrobenzylthioguanosine; NBTI, nitrobenzylthioinosine; SDS, sodium dodecyl sulfate.

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the uridine transport is completely inhibited by 1 μM NBTI, whereas D-glucose transport is not at all affected, indicating that the inhibition is specific to nucleoside transport in this low concentration range. NBTI binds to human erythrocytes at a class of sites with apparent dissociation constant (K_D) of 10^{-9} M and the total capacity (B_T) of $1.2 \cdot 10^4$ sites/cell [9]. The fact that the inhibition of uridine transport by NBTI is strictly proportional to this high-affinity NBTI binding has established that this NBTI binding site is nucleoside transporter.

The high-affinity NBTI binding site or the nucleoside transporter has been shown to purify in a membrane protein fraction known as band 4.5 that was originally identified as partially purified glucose transporter [17–19]. However, nucleoside transporter content in band 4.5 (based on its high-affinity NBTI binding capacity) is negligibly small (3–5%) compared with the high glucose transporter content, which accounts for 80 to 90% of the protein in this preparation [20–25]. Further purification is thus essential in order to use this preparation for the chemical and physical characterization of nucleoside transporter. A significant purification of the nucleoside transporter has been achieved by immunoadsorption chromatography using antisera specific to this protein [26]. However, no simple chemical procedure has been known to separate these two transporters in their native form [24]. Chemical differentiation of the NBTI binding component in this preparation from the cytochalasin B binding component may facilitate further purification of the nucleoside transporter in this preparation.

We have previously characterized the cytochalasin B binding component of band 4.5 polypeptides using gel-filtration chromatography [25]. We have shown that the DEAE-cellulose column-purified band 4.5 polypeptides can be separated into three fractions of different sizes, namely, PO, P1 and P1a. Based on photolabeling patterns with [^{3}H]cytochalasin B, we have shown that P1 is mostly a monomeric glucose transporter while PO is a detergent-induced aggregate of glucose transporters.

In the present report, we characterized NBTI binding components of band 4.5 polypeptides. We demonstrate that NBTI binds to band 4.5, not only at a high-affinity site, typical of its inhibition of nucleoside transport, but also at a low-affinity site whose total binding capacity is equal to that of cytochalasin B binding to the preparation. Based on [^{3}H]NBTI photoaffinity labeling pattern, we identify two high-affinity NBTI binding polypeptides of M_r 50000 (p50NT) and 40000 (p40NT), while the low-affinity NBTI-binding site is shown to be the glucose transporter. p50NT copurified in P1 with glucose transporter, while p40NT occurred separately in P1a free of glucose transporter. We demonstrate that p50NT is an intact nucleoside transporter, while p40NT is a truncated nucleoside transporter produced by endogenous proteolysis. We have purified the truncated nucleoside transporter, practically free of glucose transporter.

Experimental procedures

Preparation of band 4.5 polypeptides of human erythrocytes. Human erythrocytes isolated free of other cellular debris from freshly outdated blood-bank whole blood by repeated washing with isotonic NaCl solution were used. Band 4.5 peptides were prepared as described previously [25]. Briefly, white erythrocyte ghosts were treated with 0.2 mM EDTA (pH 12) for 30 min. The detergent extract was applied to a DEAE-cellulose column (25 × 80 mm) that was preequilibrated with 38 mM octyl glucoside in 50 mM Tris-HCl (pH 7.4) containing 2 mM dithiothreitol. The column was eluted with the same buffer. The first 100 ml flow through was diluted with the same buffer minus octyl glucoside but containing 100 mM NaCl and the mixture was centrifuged (185000 × g for 60 min) and washed to remove the detergent. Band 4.5 vesicles thus purified were suspended in distilled water to a protein concentration of approx. 1 mg/ml and stored in liquid nitrogen until use.

Gel-filtration chromatography. Bio-Sil TSK 250 columns (Bio-Rad) were used with a medium pressure liquid chromatographic system (Pharmacia). Unless otherwise stated, samples of 20–50 μg protein equivalent of band 4.5 preparation were used for each chromatography to separate P1a clearly from P1. Chromatography was carried out as described [25] with a flow rate of 1.0 ml/min. Fractions (0.5 ml each) were collected and absorbance peaks were integrated by Fraction Collector FRAC-100 (Pharmacia). Absorbance peak positions were perfectly reproducible every run throughout the studies. Apparent molecular masses of absorption and radiolabeled peaks were estimated using seven molecular mass markers on the gel-filtration chromatography as previously described [25]. A slight shift in the molecular mass calibration was noted in the present study, which gave estimation of slightly larger apparent molecular masses for component proteins.

Photolabeling of band 4.5 peptides with cytochalasin B and NBTI. Band 4.5 peptide preparations (0.5 to 1.0 mg) were equilibrated at room-temperature for 30 min with either [^{3}H]cytochalasin B (10^{-7} M) and CE (10^{-5} M) or [^{3}H]NBTI (10 nM unless otherwise stated) in Tris buffer (50 mM Tris-HCl, pH 7.4) solution containing 50 mM dithiothreitol in a final volume of 1.0 ml. Samples were irradiated on ice (1–2° C) for 60 s using a 450 W mercury arc lamp (Conrad-Hanovia, Newark, NJ) at a distance of 6.5 cm from the silica sleeve. A filter (C57-54, Corning Glass) was used to cut off radiation with wave length less than 280 nm. Irradiated vesicles were washed extensively four times with 20 ml of 50 mM Tris buffer containing CB (10 μM) or NBTG (20 μM) to remove

unreacted radioligands. Contamination of unreacted radioligand in vesicle suspensions was less than 2% of the total radioactivity in the suspension. The photolysis condition used here is similar to those previously reported [17,25], but the UV source is different from that of our previous work with cytochalasin B labeling [25].

Equilibrium binding of cytochalasin B and NBTI to band 4.5 peptides. The amounts of ligand bound to band 4.5 peptide vesicle preparations were determined by a centrifugation method [27]. Vesicles were incubated with [^{3}H]NBTI at specified concentrations for 30 min at room-temperature and centrifuged for 60 min at 4°C at 185000 × *g*. Pellets and supernatants were recovered with minimum cross-contamination. Radioactivities of both pellets and supernatants were assayed, from which bound ligands were calculated as percentage of the total ligands in the assay mixture. Typically, binding assay mixture contained 10–30 μg band 4.5 peptides in a buffer containing 10 mM Tris-HCl (pH 7.4), 1.0% ethanol and specified amounts of ligands in a final volume of 1.0 ml. In displace experiments assay mixtures also included increasing amounts of specified additives. Equilibrium binding data were analyzed by Scatchard [28] plots after correction for nonspecific binding. Displacement data were analyzed by a reciprocal plot of the amount displaced as a function of additive concentrations.

Trypsin digestion of band 4.5 proteins. Band 4.5 in vesicles (500 μg) suspended in 10 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl were treated with trypsin (1 μg/500 μg membrane protein) for 60 min at 20°C in a dark room. Proteolysis was stopped by the addition of 50 μg phenylmethylsulfonyl fluoride and vesicles were collected after washing by centrifugation at 185000 × *g* for 60 min.

Reconstitution of purified protein into liposomes. Partially-purified p40NT (20 μg protein) was mixed with the total lipid (100 μg) extracted from human erythrocyte ghosts in 1 ml of 50 mM Tris-HCl buffer (pH 7.4) in the presence of octyl glucoside (45 mM). The detergent was removed by centrifuging (185000 × *g* for 60 min) the mixture as described [25].

Miscellaneous assays. Protein was determined by the method of Lowry [29] in the presence of 1% SDS using bovine serum albumin as a standard. Radioactivities were measured in a liquid scintillation counter (LKB Diagnostics). Sulfhydryl residues were titrated using *p*-chloromercuribenzoate as described [30].

Results

Equilibrium binding of NBTI to band 4.5

The DEAE-cellulose column-purified band 4.5 of human erythrocyte bound cytochalasin B with a dissociation constant (K_D) of $2.1 \pm 1.8 \cdot 10^{-7}$ M and specific binding activity (B_T) of 16.1 ± 1.3 nmol/mg protein (mean ± S.E. calculated from eight measurements). The preparation is thus essentially identical to the preparation that we have characterized previously [25]. Fig. 1 illustrates results of a typical equilibrium binding of

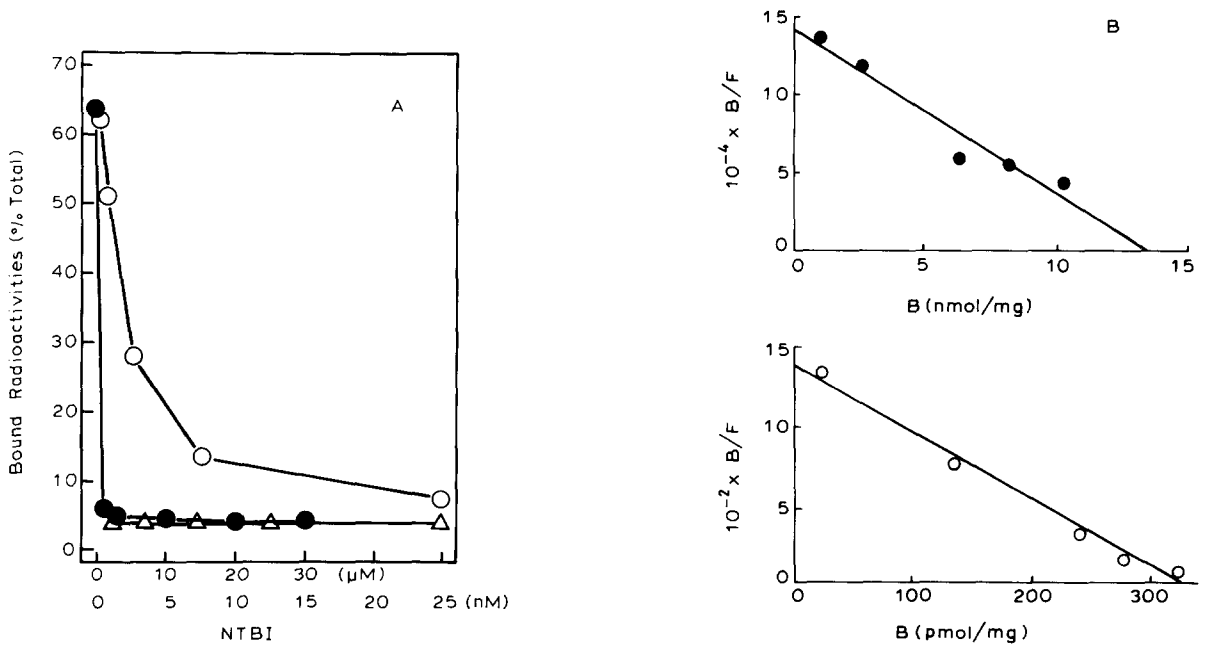

Fig. 1. Equilibrium binding of [^{3}H]NBTI to band 4.5 as a function of ligand concentrations. Panel A: Binding data were expressed as bound radioactivities in percent of the total radioactivity of binding assay mixture, and plotted against overall ligand concentration in the assay mixture. The concentrations are nanomolar (○) and micromolar in the absence (●) and in the presence (△) of 20 μM NBTI. Panel B: Scatchard plots of the data with nanomolar (○) and micromolar (●) concentrations of NBTI. Bound/free are in unit of l/mg.

NBTI to band 4.5 measured as a function of NBTI concentrations from 0.6 nM to 30 μM. Scatchard analysis of the data revealed two binding components. One was a small number of binding sites (B_T of 0.42 ± 0.16 nmol/mg protein, $n = 6$) which saturates at a nanomolar concentration range with a K_D of $2.6 \pm 2.4 \cdot 10^{-9}$ M ($n = 6$). This NBTI binding is similar to the high-affinity binding that has been described in relation to nucleoside transport inhibition [9,12,15,18]. An additional class of saturable NBTI binding was indicated with the B_T of 15.3 ± 1.8 nmol/mg protein and K_D of $6.3 \pm 2.6 \cdot 10^{-6}$ M ($n = 6$). The affinity for this binding is more than three orders of magnitude too low to be responsible for the nucleoside transport inhibition. The capacity (B_T) of this low-affinity binding was very large compared with the high-affinity binding and practically identical to the B_T for the cytochalasin B binding to this preparation (see above). The B_T for the high-affinity binding, on the other hand, is very small, being not more than 3% of the B_T for the cytochalasin B binding.

The high-affinity NBTI binding was further characterized by studying its susceptibility to displacement by various additives. In these studies, the NBTI concentration in binding assay mixture was 1 nM (or approx. 0.38 nM free-ligand concentration), where NBTI would bind exclusively (> 98%) at the high-affinity site. The binding was not affected by the presence of 500 mM D-glucose or 10^{-5} M cytochalasin B (Table I). On the other hand, the high-affinity NBTI binding was effectively displaced by various substrates and inhibitors of nucleoside transporter in a dose-dependent manner (Table I). Apparent inhibition constants K_I (Table I) calculated in these displacement experiments are similar to the inhibition constants of these ligands, with a possible exception of adenosine, for the inhibition of nucleoside transport in human erythrocytes. These findings support the concept that the high-affinity site is nucleoside transporter.

TABLE I

Displacement by various substrates and inhibitors of nucleoside and glucose transporters of bound NBTI and cytochalasin B to band 4.5 preparation

Values are apparent displacement (or inhibition) constants (K_I), estimated from the double-reciprocal plot as illustrated in Fig. 2. The assay mixture contained 10 μg of band 4.5 protein per ml. The observed increase in free ligand concentrations due to displacement was less than 30% and no detectable deviation from the linearity that simple mass action predicts in this plot was observed. Nevertheless, the values may represent an overestimation by as high as 30% due to the increase in free ligand concentrations. Each value represents mean ± S.E. of 3–6 independent experiments.

Displaced by	K_I (M) for the displacement of	
	[^{3}H]NBTI (1 nM)	[^{3}H]cytochalasin B (100 nM)
D-Glucose	$> 5 \cdot 10^{-1}$	$3.8 \pm 1.2 \cdot 10^{-2}$
L-Glucose	$> 5 \cdot 10^{-1}$	$> 5 \cdot 10^{-1}$
Adenosine	$3.3 \pm 0.6 \cdot 10^{-3}$	$8.1 \pm 1.2 \cdot 10^{-3}$
Adenine	$> 10^{-1}$	$9.3 \pm 1.8 \cdot 10^{-3}$
Uridine	$1.6 \pm 0.8 \cdot 10^{-2}$	$4.2 \pm 0.8 \cdot 10^{-2}$
NBTI	$2.8 \pm 0.7 \cdot 10^{-9}$	$1.1 \pm 0.6 \cdot 10^{-5}$
NBTG	$4.5 \pm 1.2 \cdot 10^{-8}$	$4.1 \pm 1.3 \cdot 10^{-5}$
HNBTI	$4.2 \pm 1.2 \cdot 10^{-8}$	$1.8 \pm 0.6 \cdot 10^{-4}$
Cytochalasin B	$> 10^{-5}$	$2 \pm 1.8 \cdot 10^{-7}$

Data illustrated in Fig. 2 demonstrate that NBTI at micromolar concentrations displaces cytochalasin B

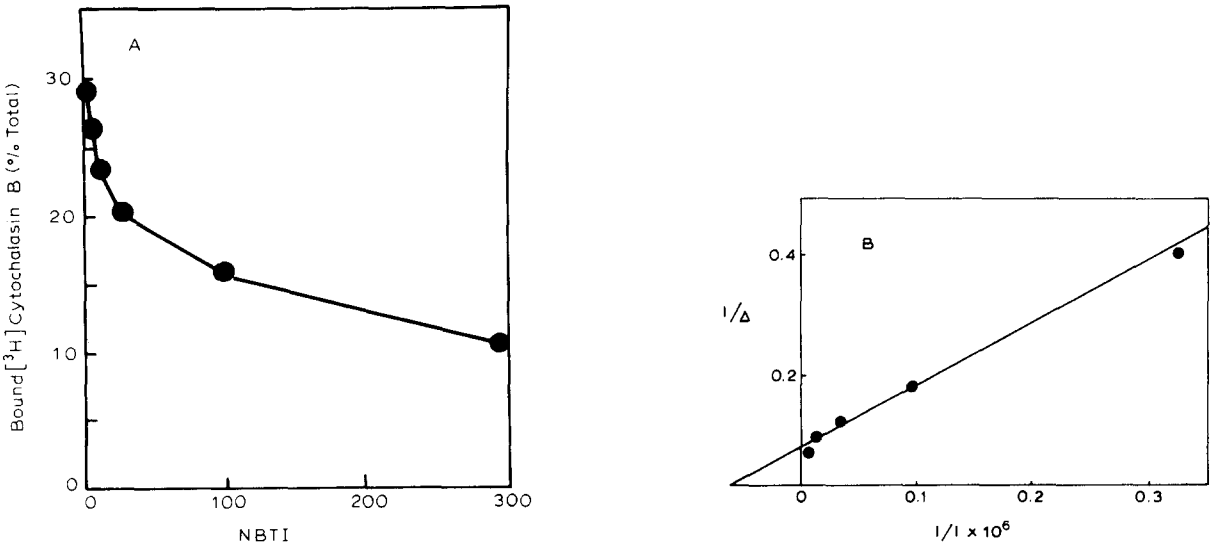

Fig. 2. Effects of NBTI on cytochalasin B binding to band 4.5 as a function of NBTI concentrations. Percent displacements (panel A) and a double-reciprocal plot of the displacement (panel B) of [^{3}H]cytochalasin B by NBTI are shown. Δ and (I) signify % displacement and NBTI concentration, respectively. The straight line represents the best fit to data, from which a K_i value of $16 \cdot 10^{-6}$ M was estimated. Binding assay mixture contained 10 μg band 4.5 peptides protein whose specific cytochalasin B binding activity was 15.1 nmol/mg protein, and a fixed amount of [^{3}H]cytochalasin B (10^{-7} M) with an increasing amount of NBTI in 10 mM Tris-HCl (pH 7.4) containing 1% ethanol in a total volume of 1 ml. There were significant changes in free NBTI concentrations due to displacement, which causes an overestimation of the K_i value as high as 30%.

binding from band 4.5. It should be noted that this NBTI effect was insignificant at NBTI concentrations as high as 1 μM, where the high-affinity binding sites were completely saturated. This indicates that the high-affinity NBTI binding was not involved in the displacement of cytochalasin B. NBTG and HNBTI also displaced cytochalasin B but with lower affinities than NBTI. Nucleoside transport substrates such as adenosine and uridine also displaced the cytochalasin B bound to the preparation with affinities (Table I) which are similar to their reported affinities as nucleoside transport substrates. They are, in fact, comparable to the affinity of D-glucose to the cytochalasin B binding site.

Gel-filtration chromatographic identification of two high affinity NBTI binding proteins, p50NT and p40NT

In order to identify the high-affinity NBTI binding protein, we photolabeled band 4.5 polypeptides with [^{3}H]NBTI and separated them by gel-filtration chromatography. Unless stated otherwise, the chemical concentration of NBTI in the photoreaction mixture was kept at 10 nM or lower to ensure that only the high-affinity sites are labeled. To differentiate NBTI binding protein from cytochalasin B binding protein [25], we also ran in parallel gel-filtration chromatography of band 4.5 photolabeled with [^{3}H]cytochalasin B.

The gel-filtration chromatography separated band 4.5 polypeptides into three protein fractions, PO, P1, and P1a, with apparent molecular masses of 90, 55 and 40 kDa, respectively (Fig. 3A). These molecular masses are slightly larger than our previous estimates [25] and we claim that relative, but not absolute values, are significant and were reproducible. Lipids were eluted in a separate fraction, P2 (Fig. 3A) [25]. The chromatography also revealed distinct patterns of radioactivity distribution among these fractions (Fig. 3B). Cytochalasin B labeling was mostly found in P1, with a slight labeling in PO (Fig. 3B). The P1 labeling peaked precisely at the UV absorption peak. This cytochalasin B labeled, 55 kDa polypeptide (p55GT) has been identified as the glucose transporter [25]. No cytochalasin B labeling was found in P1a. This essentially reproduces our previous observations [25]. A slight, but consistent labeling was also found in P2. This P2 labeling was not observed in our previous report [25], where a 280 nm monochromator was used as the radiation source (see Experimental procedures).

In comparison with this cytochalasin B labeling pattern, the NBTI labeling pattern of these fractions was quite different (Fig. 3B): NBTI label was found in fractions P1, P1a and P2, each with a significant intensity, but very little in PO. The most evident difference was in P1a, which showed an abundant NBTI label but no cytochalasin B label. Relative intensity of the labeling was slightly higher in P1 than in P1a, while the lipid labeling at P2 varied significantly among different preparations (Table II). Although P1 fraction was labeled by both ligands, the NBTI label consistently peaked at a slightly but significantly lower molecular mass position (50 kDa) compared with the cytochalasin B labeled peak (55 kDa) which coincided with the UV absorption peak of P1 (Fig. 3B). The NBTI label in P1a peaked at M_r 40 000 Da, coinciding with the UV absorption peak of this fraction (Fig. 3). Thus we have identified in band 4.5 two high-affinity NBTI binding polypeptides, of 50 kDa (p50NT) and 40 kDa (p40NT).

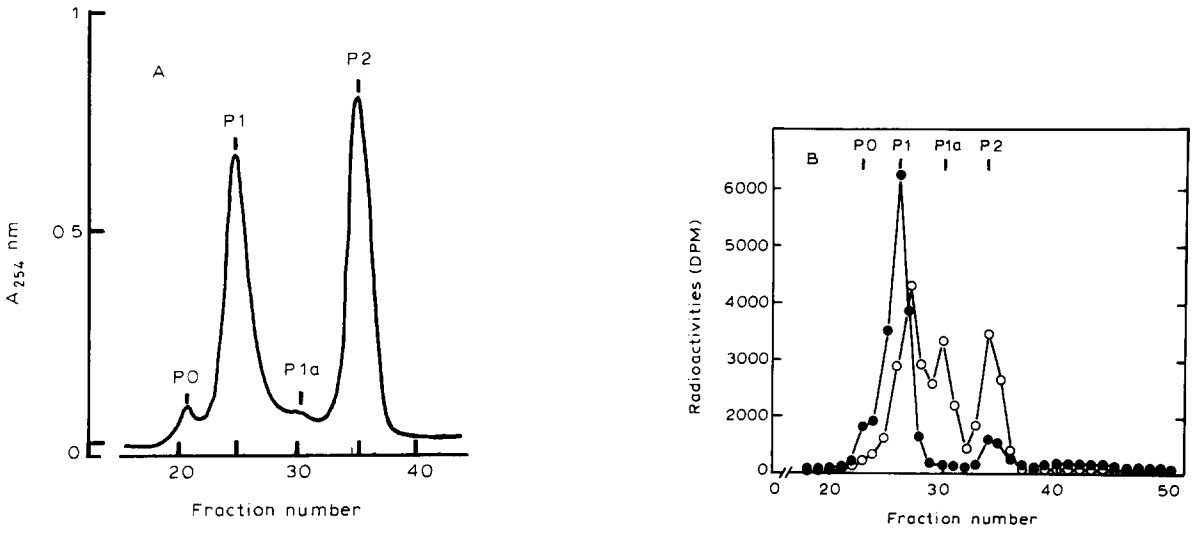

Fig. 3. Gel filtration chromatographic separation of photolabeled component peptides of band 4.5 preparation. (A) UV absorbance (254 nm) scan with designation of major UV absorbing fractions. (B) Radioactive distribution pattern for [^{3}H]cytochalasin B (●) and [^{3}H]NBTI (○). Superimposed are the positions of the UV absorption peaks of panel A. Band 4.5 preparation was photolabeled with [^{3}H]cytochalasin B (10^{-7} M) or [^{3}H]NBTI (10 nM) as described in the Experimental procedures. 50 μg of the labeled preparation was mixed with 100 μl of Tris-HCl buffer and 100 μl of 2% $NaDodSO_4$ spun at 185000 × g for 15 min and the resulting supernatant was applied to a Bio-Sil TSK 250 column. 0.1% SDS-phosphate buffer was used for elution with a flow rate of 1.0 ml/min, at 20 °C.

TABLE II

Relative (%) label distribution among band 4.5 component polypeptides

The band 4.5 peptide preparations were photolabeled by [^{3}H]NBTI (10 nM) or [^{3}H]cytochalasin B (10^{-7} M) and subjected to gel-filtration chromatography. Values are means ± S.E. calculated from six independent measurements. Each component UV absorptive fraction was obtained arbitrarily by pooling 0.5 ml eluent fractions specified by fraction numbers in parentheses.

Labels	PO (20–24)	P1 (25–28)	P1a (29–32)	P2 (33–37)	Others (1–19 and 38–70)	Recovery (1–70)
Cytochalasin B	11.4±1.9	63.3±4.8	3.4±0.8	8.6±2.1	13.4±1.2	100±6.1
NBTI	1.4±0.3	39.0±2.3	24.3±2.1	25.4±8.3	6.9±0.7	100±6.3

The NBTI labeling of PO was less intensive relative to the PO cytochalasin B labeling, although both peaked at the UV absorption peak position of an apparent M_r 90000 Da (Fig. 3B). We have previously shown that PO represents an aggregate of P1 induced by detergent treatment [25]. Fig. 4 illustrates the effects of detergent-incubation of photolabeled band 4.5 on NBTI labeling among different gel column fractions. Incubation of band 4.5 in 1% octyl glucoside prior to the column separation resulted in a large shift of NBTI labeling from P1 and P1a to PO. The labeling in P2 was not significantly affected by the incubation. This detergent effect was also observed with Triton X-100, although it was less extensive (not illustrated). These findings not only support our previous conclusion [25] that PO represents an aggregate of p55GT in P1, but further demonstrate that the high-affinity NBTI binding proteins p50NT and p40NT are also included in the detergent-induced aggregates of PO. The participation of P1a in aggregation was not detected in our previous cytochalasin B labeling experiments [25] simply because P1a was not labeled with cytochalasin B and UV absorption at P1a was too small to detect any change. The NBTI labeling of lipids at P2 was as extensive as those of P1 and P1a (Fig. 3B) and was not affected by the detergent-induced aggregation referred to above (Fig. 4).

The NBTI labeling of p50NT and p40NT was sensitive to the presence of several agents during the photolabeling reaction. This protein labeling was totally abolished by the presence of 2% octyl glucoside during photolysis, whereas P2 lipid labeling was enhanced many-fold (Fig. 5). The NBTI labeling of p50NT and p40NT was also reduced by more than 80% when an excess (20 μM) of NBTG, a competitive inhibitor of nucleoside transporter (Table I), was included during photolabeling (Fig. 5). The NBTI labeling in P2 again was not affected significantly in these experiments, indicating that NBTG specifically inhibits p50NT and p40NT labeling but not lipid labeling. These findings together strongly suggest that both p50NT and p40NT are related to the nucleoside transporter. Presence of 10

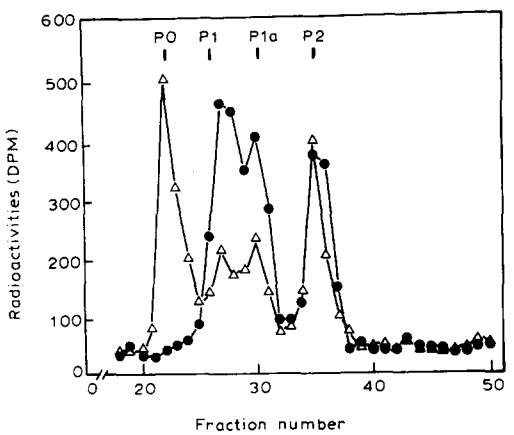

Fig. 4. Effects of detergent-incubation on relative sizes of [^{3}H]NBTI-label and component polypeptides of photolabeled band 4.5 preparation separated on gel-filtration chromatography. [^{3}H]NBTI-photolabeled polypeptides (10 μg protein) prior to (●) and after (△) a 1-h incubation in 1% octyl glucoside were separated by gel-filtration chromatography using a Bio-Sil TSK 250 column. Conditions were otherwise the same as in Fig. 3. Also indicated are the peak positions of UV absorbance scan.

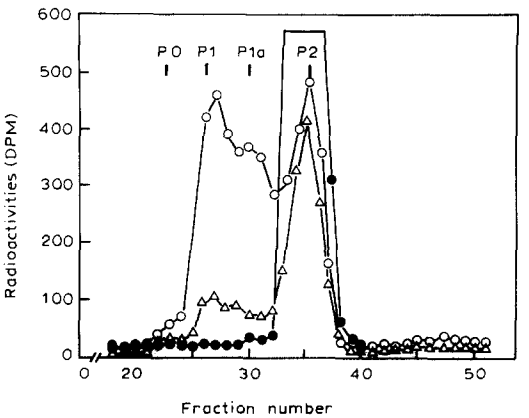

Fig. 5. Effects of octyl glucoside or NBTG present during photolabeling of band 4.5 polypeptides with [^{3}H]NBTI. Band 4.5 preparation (200 μg protein) was photolabeled with [^{3}H]NBTI (0.8 μM) in the absence (○) or in the presence of 2% octyl glucoside (●) or 20 μM NBTG (△) for 1 min at 0 °C with a 450 W medium pressure arc lamp as detailed in the Experimental procedures. Gel filtration chromatography was as described in Fig. 3. Also shown are the UV absorption peak positions. Because relatively large amounts of sample protein were applied to the column chromatographic resolution of radioactivity distribution among different component proteins was poor.

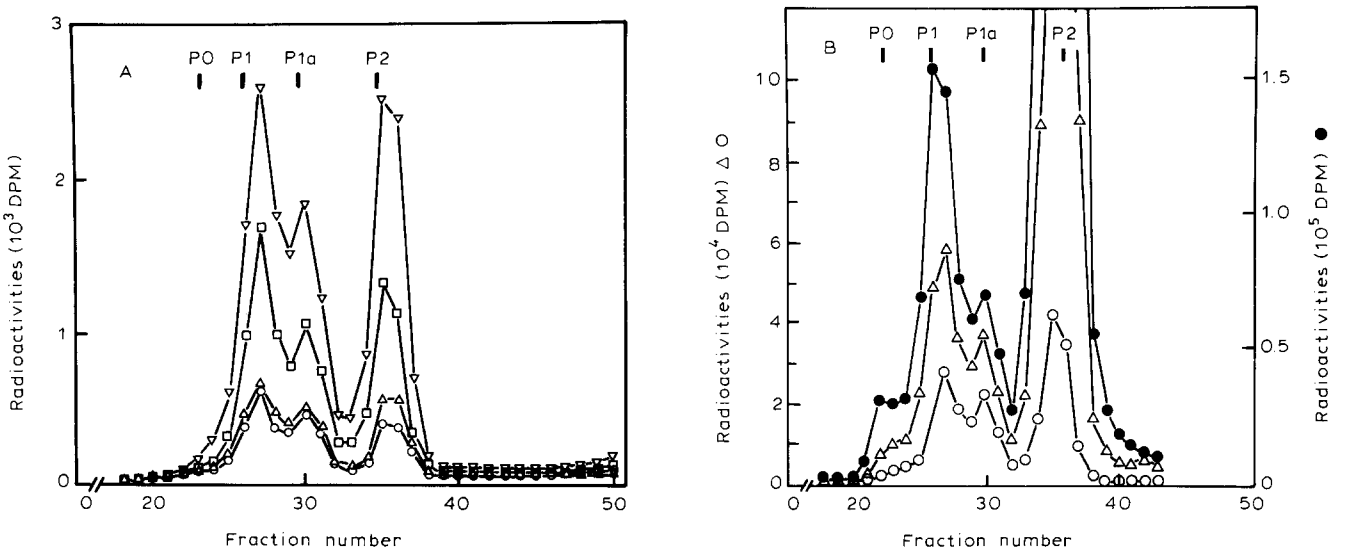

Fig. 6. Effects of NBTI concentration on photolabeling of component proteins. The band 4.5 protein preparation was labeled with an increasing concentration of [^{3}H]NBTI. Nanomolar (A) and micromolar (B) concentrations were present during labeling reaction: (A) 1 (○), 2 (□), and 4 (▽) nM NBTI. Also included are the data of 1 nM NBTI in the presence of 10^{-5} M cytochalasin B (△). The specific radioactivity of [^{3}H]NBTI was fixed at 66.6 μCi/nmol. (B) 1 (○), 10 (△) and 30 (●) μM, with a fixed specific radioactivity of 4 μCi/nmol. Please note that the data with 30 μM NBTI were presented in a reduced scale according to the right side coordinate. 50 μg protein of labeled preparation was applied in each run. Superimposed are the peak positions of corresponding UV absorbance scan.

μM of cytochalasin B during the photolabeling did not affect the NBTI labeling of p50NT and p40NT (Fig. 6) consistent with its inability in displacing the high-affinity NBTI binding to band 4.5 preparation (Table I).

Gel-filtration chromatographic identification of glucose transporter as the low-affinity NBTI binding site

NBTI labeling of each component protein fraction of band 4.5 was studied as a function of the NBTI concentrations used for photolabeling (Fig. 6). In these experiments, micromolar as well as nanomolar concentrations of NBTI were studied to differentiate the high- and low-affinity binding protein labelings. With ligand concentrations of 1 μM or lower, the NBTI labeling intensity ratio of P1 to P1a remained unchanged at approx. 1.3. At NBTI concentrations above 1 μM, however, the ratio increased significantly, reaching as high as 2.2 at 30 μM NBTI. An excess (10^{-5} M) of cytochalasin B effectively abolished this increase (not illustrated). These results indicate that at these higher NBTI concentrations, an additional NBTI labeling reaction occurs at only P1, most likely at the low-affinity NBTI binding site. It is important in this regard to also note a shift of the P1 NBTI label peak position at the higher ligand concentrations (Fig. 6). With NBTI concentrations up to 1 μM, the P1-labeling peaked at M_r 50000 (fraction 27 in Fig. 6). With 30 μM NBTI, where the low-affinity binding dominates, the P1-labeling peak shifted to M_r 55000 (fraction 26 in Fig. 6B), now coinciding exactly with the cytochalasin B labeling peak (p55GT) or the P1-UV absorption peak. The P1a-NBTI label (p40NT) peak position (fraction 30 in Fig. 6) was not affected throughout the entire concentration range tested. These findings support that the low-affinity NBTI binding site is a glucose transporter.

When 1 mg of band 4.5 polypeptides were photolabeled with 1 μM NBTI, the concentration which would give virtually a complete saturation of the high-affinity binding, approx. 170 pmol of NBTI was incorporated. This corresponds to approx. 45% of the total high-affinity binding capacity (the total nucleoside transporter) of the preparation. This indicates that photolabeling efficiency of nucleoside transporters with NBTI is considerably higher than that of cytochalasin B labeling to glucose transporter, which was typically 10–14% (unpublished data).

Relationship of the two high-affinity NBTI binding proteins, p50NT and p40NT

When [^{3}H]NBTI-labeled band 4.5 preparation was subjected to mild digestion with trypsin, the P1 (p50NT) label was drastically reduced (30–45%) while the P1a (p40NT) label was proportionately increased without any obvious change in the total labeling of band 4.5 (Fig. 7). Similar results were obtained when the preparation was digested before photolabeling with NBTI (not illustrated). This would indicate that trypsin digestion converts p50NT to p40NT without affecting the high-affinity NBTI-labeling efficiencies. The same trypsin digestion caused an appearance of a new UV absorption peak between P1a and P2 with an estimated M_r of 25000 (not illustrated). The same trypsin digestion of [^{3}H]cytochalasin B-labeled band 4.5 resulted in a reduction (more than 50%) in the p55 kDa labeling with a

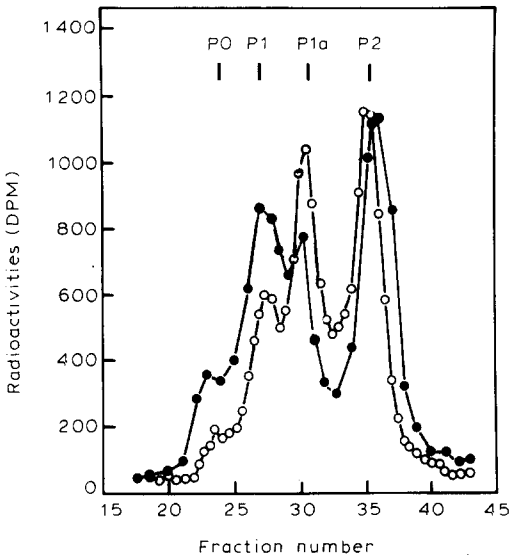

Fig. 7. Gel-filtration chromatographic separation of the high-affinity NBTI binding proteins of the band 4.5 preparation after trypsin digestion. Also shown are the peak positions of VU absorbing fractions of undigested sample. Band 4.5 preparation was photolabeled with [^{3}H]NBTI (10 nM), then digested with trypsin (○) as detailed in Experimental procedures prior to gel-filtration chromatography. No digestion (●). Comparison of total recoveries of radioactivity showed that digestion and subsequent washing caused a 10–15% loss of labeled materials. The radioactivity associated with P1 and P1a was calculated in % of total as done in Table II, respectively. In this particular set of experiments, the radioactivity distribution was reduced from 38 to 21% in P1 and increased from 26 to 40% in P1a, due to pepsin digestion.

concomitant appearance of a new label peak at an estimated M_r of 25 000 (data not illustrated). Similar effects of trypsin digestion on the cytochalasin B binding protein have been reported [16]. The trypsin digestion referred to above did not affect the high-affinity, NBTI-binding characteristics of band 4.5 in equilibrium binding studies with K_D and B_T of $2.3 \pm 1.5 \cdot 10^{-9}$ M and 0.40 ± 0.23 nmol/mg protein, respectively ($n = 4$). A trypsin digestion similar to this has been shown to reduce NBTI binding affinity in unresealed erythrocyte membranes slightly, without affecting the binding capacity [16]. These results strongly indicate that p40NT is a truncated nucleoside transporter produced by proteolysis, which retains the high-affinity NBTI binding activity of p50NT, most likely the intact nucleoside transporter.

Purification of P40NT, the truncated nucleoside transporter

Data illustrated in Fig. 3B clearly indicate that p40NT may be purified by rechromatography of a P1a fraction. Radioactivity scan of the rechromatography of P1a (pooled fractions 29 to 32 of Fig. 3B) obtained from the band 4.5 prelabeled with [^{3}H]NBTI (at 1 μM) and [^{3}H]cytochalasin B showed only NBTI label without any cytochalasin B label (Fig. 8A), indicating that it is free of p55GT. The VU scan of this rechromatography (Fig. 8B) demonstrates that the pooled P1a is 80% pure p40NT (in P1a peaking at fraction 30) with 5% p50NT (in P1 peaking at fraction 27) and 15% their dimers (in PO peaking at fraction 22). The pooled fraction did not originally contain PO: the dimers observed in the rechromatography must thus be formed during the rechromatography of mostly p40NT. The overall yield was approx. 80%. The purified p40NT preparation contains a small amount of lipids (p2 in Fig. 8B) (less than 5% of the band 4.5 lipid content). p40NT was, however, readily reconstituted into liposomes. Efficiency of this reconstitution in terms of protein incorporation was as high as 70%. In a control experiment (Fig. 8C), rechromatography of P1 fraction resulted in a preparation of glucose transporter (p55GT) contaminated by less than 1% (in protein mass) of intact nucleoside transporter (p50NT).

Discussion

Of the two different classes of NBTI binding sites that we unraveled here in the DEAE column-purified band 4.5 preparation the high-affinity binding site is the nucleoside transporter. Several lines of evidence support this conclusion: the affinity constant (K_D) of this site is in the nanomolar range, where nucleoside transport is effectively inhibited by NBTI [12]. The K_D of the high-affinity binding observed here with the purified band 4.5 preparation (2.6 nM) is slightly higher than that reported with an erythrocyte membrane preparation (less than 1 nM) [9]. This may be due simply to a purification artefact, as the binding affinity is known to be sensitive to mild experimental manipulation such as simple storage [9]. The B_T of this high-affinity binding (0.42 nmol/mg protein) is also not too different from expected total number of nucleoside transporters in this preparation [26]. The high-affinity NBTI binding was not affected by typical substrates or inhibitors of glucose transporter although readily displaced by the known substrates and inhibitors of nucleoside transporter (Table I).

Results of the present study uncovered a low-affinity NBTI binding site in band 4.5, which saturates only at micromolar concentrations. The following observations made in this study demonstrate that this low-affinity NBTI binding site is a glucose transporter: The B_T (15.3 nmol/mg protein) of this NBTI binding is practically identical to the B_T of cytochalasin B binding (16.3 nmol/mg protein) to this preparation. NBTI inhibits cytochalasin B binding to the preparation (Fig. 2) with an inhibition constant (K_I) essentially identical to the K_D for this low-affinity NBTI binding. An interaction of NBTI with glucose transporter has been indicated previously by Jarvis et al. [31]. They have shown that photolysis of human erythrocyte membranes with the

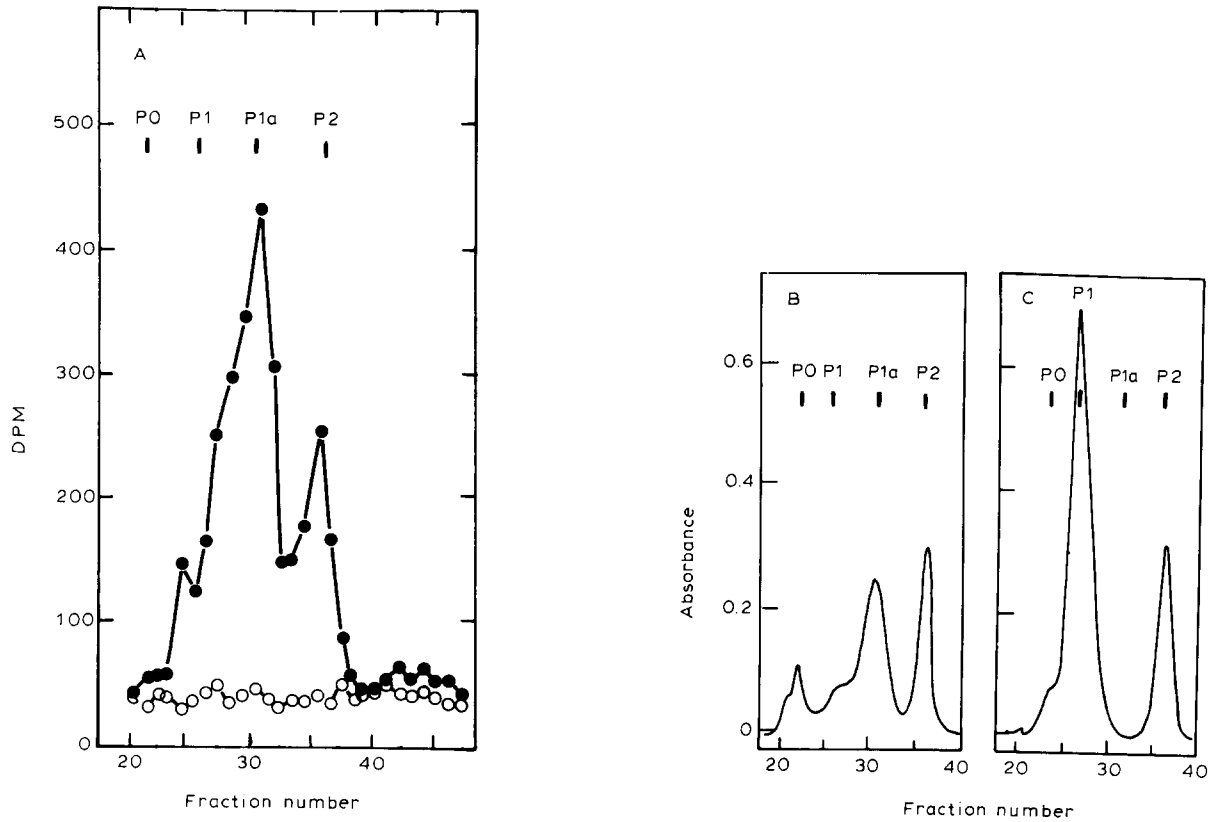

Fig. 8. Partial purification by rechromatography of P1 (p55GT plus p50NT) and P1a (p40NT) of band 4.5 preparation. 200 μg of the band 4.5 preparation was chromatographed to separate P1 (fractions Nos. 25–28) and P1a (fractions Nos. 29–32). Materials resulted from five such chromatographies were pooled and rechromatographed. (A) Radioactivity distribution, [^{3}H]NBTI label (●) and [^{3}H]cytochalasin B label (○). (B) UV absorbance (254 nm) scan for the rechromatography of P1a. (C) UV absorbance scan for the rechromatography of P1. Also shown are the peak positions of UV absorbance scan of original band 4.5 preparation.

8-azidoadenosine (10 μM) labeled band 4.5 region of $NaDodSO_4$-polyacrylamide gels and this labeling was blocked by D-glucose and cytochalasin B, suggesting that glucose transporter was labeled. They have also shown that glucose flux and glucose-sensitive cytochalasin B binding in erythrocyte membranes can be inhibited by various nucleosides [31].

Nucleosides also displace cytochalasin B binding to the preparation quite effectively (Table I). The affinities for this interaction are only slightly lower than that for the displacement of the high-affinity NBTI binding. In fact, adenosine is more effective than glucose in displacing cytochalasin B bound to the preparation (Table I). However, adenine is as effective as adenosine in this displacement (Table I), indicating that the nucleoside binding to the low-affinity site (glucose transporter) does not require any sugar moiety and may be a non-specific interaction primarily determined by hydrophobicity. This is in contrast with the observed ineffectiveness of adenine in displacing the high-affinity NBTI binding (Table I) and indicates that adenosine effect on the high-affinity NBTI binding to nucleoside transporter requires a specific molecular conformation. Thus, the observed displacement of cytochalasin B by nucleosides would not necessarily indicate the structural similarity between the glucose transporter and the nucleoside transporter of human erythrocytes.

Our results of gel-filtration chromatographic separation of component polypeptides of photolabeled band 4.5 preparation revealed several important insights into chemical differentiation of nucleoside transporter from glucose transporter. We identified p50NT and p40NT as two high-affinity NBTI binding proteins or the nucleoside transporter. The following evidence supports this conclusion. NBTG, a well known analog of NBTI, inhibited NBTI-labeling of both of these proteins (Fig. 5), while cytochalasin B had no effect (Fig. 6). The labeling was completely abolished by the presence of octylglucoside during photolysis (Fig. 5), indicating that a specific protein conformation is required for the labeling. The labeled species were susceptible to the detergent-induced protein aggregation forming PO fraction (Fig. 4). The two labeling peaks cannot be chromatographic artefacts; they were reproducibly eluted as two separate peaks upon rechromatography.

Trypsin digestion converts NBTI-labeling in the band 4.5 preparation from the p50NT peak to the p40NT peak stoichiometrically (Fig. 7). This demonstrates that p40NT is a digestion product of p50NT. The trypsin digestion where 60–70% of the original p50NT label

was converted to p40NT label (Fig. 7) did not affect high-affinity NBTI binding characteristics of band 4.5 preparations significantly. This indicates that the truncated p40NT binds NBTI with the high affinity that is indistinguishable from that of p50NT or the intact nucleoside transporter prior to digestion. 30–40% of the nucleoside transporter in freshly prepared band 4.5 is in truncated form (Table II and Figs. 3B and 4).

The results of gel-filtration chromatography shown here suggest that nucleoside transporter is slightly smaller in size than glucose transporter, with estimated molecular masses of 50 000 and 55 000 Da, respectively (Fig. 3B). The UV absorption peak of P1 coincides with the cytochalasin B label peak of p55GT, but not with the high-affinity NBTI label peak of p50NT, indicating that the protein mass in P1 is mostly p55GT and p50NT represents only a small fraction of P1. This finding is consistent with the fact that P1 is mostly glucose transporter [25] and nucleoside transporter is a minor (1–2%) component of band 4.5 polypeptides.

Results of our gel-filtration chromatography of NBTI and cytochalasin B labeled band 4.5 preparation demonstrate that the truncated nucleoside transporter, p40NT, occurs in P1a fraction free of glucose transporter (Fig. 3B). By rechromatography of P1a fraction (Fig. 8A) we have obtained p40NT free of glucose transporter. Our preliminary experiments indicated that this purified p40NT shows distinctly more cysteine and tyrosine and less tryptophan residues compared with glucose transporter. It is not yet possible to prepare the p50NT intact nucleoside transporter free of glucose transporter (p55GT) by gel chromatography alone. The purified truncated nucleoside transporter, which retains the high-affinity NBTI binding activity of intact nucleoside transporter, would be a useful preparation for further studies on the biochemistry of human erythrocyte nucleoside transporter.

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