Reconstitution and identification of the major Na^+-dependent neutral amino acid-transport protein from bovine renal brush-border membrane vesicles

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Amino acid transport activity from bovine renal brush-border membrane vesicles (BBMV) was reconstituted into phospholipid vesicles composed of phosphatidylcholine/5% stearylamine. Reconstitutable transport activity was enhanced in protein fractions binding to various lectins. When solubilized BBMV were fractionated on peanut lectin, a single protein band of average molecular mass 132 kDa was obtained. When this protein fraction was reconstituted into phospholipid membrane vesicles, amino acid transport activity was obtained with properties similar to those in native BBMV with regard to amino acid specificity, although the cation specificity was different. A monoclonal antibody which reacted with the same protein removed reconstitutable amino acid transport activity from solubilized BBMV. These findings may provide the first identification of a renal amino acid-transporting protein, although confirmation of this identification by other approaches will be required.

INTRODUCTION

The Na^+-dependent transport of amino acids into mammalian cells is of major physiological importance and has been extensively studied (for reviews see, e.g., [1–3]). In most non-epithelial cell types Na^+-dependent neutral amino acid transport is catalysed by two major transport systems with overlapping specificity, which are designated System A and System ASC. These systems transport most small neutral amino acids, but have no activity towards branched-chain or aromatic amino acids. System A, but not System ASC, transports the amino acid analogue methylamino isobutyrate, and competitive inhibition by this compound is used to distinguish between these two systems. Liver plasma membranes contain a further transport system termed System N, which specifically catalyses the Na^+-dependent transport of glutamine, histidine and asparagine [4], and a transport system with similar specificity has been characterized in skeletal muscle [5]. Another system of wide specificity transporting both neutral and basic amino acids has been reported in mouse blastocytes [6]. Transport systems for basic and acidic amino acids have also been kinetically characterized in various tissues.

In brush-border membranes of renal proximal-tubule cells, neutral amino acid transport occurs via a different system from any of the above. This system has broad specificity and catalyses the Na^+-dependent transport of branched-chain and aromatic amino acids in addition to that of small neutral amino acids [7]. Methylaminoisobutyrate is not recognized by this system. A similar system of broad specificity has been reported in rabbit intestinal brush-border membrane vesicles (BBMV) [8].

In contrast with mammalian sugar-transport proteins, which have been characterized in detail, none of the above amino acid-transport proteins have yet been identified. This is largely due to the fact that no specific tight-binding inhibitor for these systems has been found. In one approach to this problem, Na^+-dependent alanine transport has been expressed in *Xenopus* oocytes after injection of rabbit renal-cortex mRNA [9]. Recently, System A activity has been expressed in oocytes after injection of rat liver mRNA [10,11]. However, no transport protein has yet been identified.

In an alternative approach, methods for the reconstitution of System A activity from Ehrlich ascites cells [12] and liver [13–15] and of system N from liver [16] have been described. Reconstitution of amino acid transport from rabbit renal BBMV [17] and bovine renal BBMV [18] has also been achieved. On the basis of protein fractionation followed by reconstitution, McCormick & Johnstone [19] have tentatively identified a component of the protein responsible for System A transport in ascites cells as a polypeptide of 120–130 kDA. Confirmation of this in other tissues expressing System A activity has not yet been forthcoming.

We have developed a rapid method for the reconstitution of amino acid transport from bovine brush-border membranes, and have shown previously that alanine-transport activity can be fractionated on DEAE-cellulose and hydroxyapatite [20]. In the present paper, this fractionation/reconstitution approach is extended to include lectin affinity chromatography. Evidence is presented that the Na^+-dependent neutral amino acid-transport system of bovine renal BBMV is a 132 kDa glycoprotein.

MATERIALS AND METHODS

Materials

Egg yolk phospholipids were prepared by the method of Dawson [21] as described previously [18]. The detergent decanoyl-*N*-methylglucamide (MEGA-10) was synthesized as described by Hildreth [22]; this detergent is also available commercially from Sigma. Concanavalin A immobilized on Sepharose 4B, wheat-germ lectin and peanut lectin were purchased from Sigma. These lectins were coupled to CNBr-activated Sepharose 4B (Sigma) (25 mg of lectin/5 ml of Sepharose) by the method of Montreuil *et al.* [23]. ^3H-labelled amino acids were purchased from Amersham International.

Isolation of membrane fractions

BBMV from fresh bovine kidney and from rat kidney were prepared by a $MgCl_2$-precipitation method [24] as described

Abbreviations used: MEGA-10, decanoyl-*N*-methylglucamide; BBMV, brush-border membrane vesicles.
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previously [18]. The vesicles were suspended in a medium containing 0.25 M-sucrose, 10 mM-Hepes (K^+ salt) and 0.2 mM-$CaCl_2$ and rapidly frozen in liquid nitrogen. Brush-border membranes from rat intestinal mucosa were prepared by a similar method. Liver basolateral-membrane vesicles were isolated by sucrose-gradient centrifugation [25].

Reconstitution procedure

This was based on a previously published procedure [18], the success of which is critically dependent on lack of oxidation of the phospholipids. In brief, egg phospholipids in chloroform were mixed with 5% stearylamine (mol/mol of phospholipid). The chloroform was removed and the lipids were suspended in 0.1 M-cyclamate/20 mM-Hepes (K^+ salts), pH 7.4. After sonication at 40 W (10 × 10 s) on ice, the suspension was clarified by centrifugation at 100000 *g* for 30 min and the pellet discarded. Bovine BBMV were solubilized in a solution containing 20 mM-Tris/HCl plus MEGA-10 at pH 7.4 (3 mg of MEGA-10/mg of protein). After stirring for 5 min, the solution was clarified by centrifugation at 100000 *g* for 30 min and the pellet was discarded.

Phospholipids and protein were mixed at a ratio of 25 μmol of phospholipid P/mg of protein and the detergent concentration was decreased by passage through a 27 cm × 1 cm column of Sephadex G-50 (coarse grade) in 0.1 M-cyclamate 10 mM-Hepes (K^+ salts), pH 7.4. The eluate from the column was diluted 5-fold with the same buffer and immediately frozen in liquid nitrogen. For transport experiments, the vesicles were thawed at room temperature and collected by centrifugation at 40000 *g* for 20 min. The pellet was resuspended in a small volume of cyclamate/Hepes (K^+ salts) by gentle suction through a pipette tip. If this procedure was performed with a solution of MEGA-10 instead of the protein solution, virtually no pellet was obtained on centrifugation.

Lectin affinity chromatography

Bovine BBMV (80–150 mg of protein) were freed of sucrose by centrifugation at 100000 *g* for 30 min and resuspended in 20 mM-Tris/HCl at pH 7.4 and 4 °C. The membranes were then solubilized and clarified as described above and applied to a column (30 mm × 15 mm) of the appropriate lectin immobilized on Sepharose 4B. After washing with 20 mM-Tris/HCl/0.25% MEGA-10 (pH 7.4) until the A_{280} of the eluate was zero, the bound protein was eluted with 100 mM-D-mannose, 100 mM-*N*-acetylglucosamine or 100 mM-D-galactose (in the presence of 20 mM-Tris/HCl and 0.25% MEGA-10) for concanavalin A, wheat-germ lectin and peanut lectin respectively. The protein fraction was then concentrated by ultrafiltration (Diaflo, Amicon) and reconstituted as above.

Monoclonal antibodies

Monoclonal antibodies FD12 and FD19 were produced from two different fusions by using the hybridoma technology introduced by Kohler & Milstein [26]. Protein fractions for immunization were prepared from the concanavalin A-binding fraction of solubilized BBMV (FD12) or from the fraction eluted by 100–200 mM-phosphate/0.25% MEGA-10 from hydroxyapatite and subsequently bound to concanavalin A (FD19). Balb/C mice were immunized intraperitoneally with protein fractions mixed with complete or incomplete Freund's adjuvant over a period of 6 weeks. At 5 days after the last immunization, spleen cells were fused with the myeloma P3-NSO/1Ag 4.1 (NSO). Culture supernatants were screened by e.l.i.s.a. against the immunizing protein fraction. Immune ascites were produced in mice from cloned hybridomas and was purified on Protein A–Sepharose. The purified antibodies were immobilized on CNBr-activated Sepharose (approx. 7 mg of antibody/ml of Sepharose) [27].

Measurement of transport in reconstituted vesicles

Transport experiments were performed at room temperature. For measurement of alanine uptake, 15 μl of reconstituted vesicles (0.1–0.4 mg of protein/ml) suspended in 0.1 M-cyclamate/10 mM-Hepes (K^+ salt), pH 7.4, were mixed with an equal volume of the same medium which also contained 0.2 M-NaCNS and 0.2 mM-L-[^{3}H]alanine (approx. 400 d.p.m./pmol). In some experiments the NaCNS was replaced with an equal concentration of KCNS or choline chloride. After the appropriate time, the transport reaction was stopped by the addition of 1 ml of an ice-cold solution containing 0.1 M-cyclamate (K^+ salt), 0.1 M-NaCl plus 10 mM-Hepes (K^+ salt), pH 7.4. The suspension was immediately filtered through a 0.45 μm-pore-size nitrocellulose filter under suction and the filter washed with 3 × 1 ml of the stop solution. Filters were dissolved in scintillator and radioactivity was measured after standing for at least 5 h.

Results are presented throughout as time courses. Each point of a particular time course represents a separate incubation with the same reconstituted preparation, and the results are shown as means ± S.E.M. of values obtained from at least three separate reconstituted preparations.

Correction for binding

The association of radioactivity with the phospholipid membrane vesicles is biphasic. The first phase is complete within the shortest possible sampling time (1 s) and presumably represents binding. The second phase occurs over a few minutes and represents transport. The amount of radioactivity bound depends on the nature of the amino acid, and to some extent on the incubation medium. In the experiments shown here, the binding was estimated by extrapolating the uptake over the first 15 s back to zero time. This binding was subtracted from all successive values to give the net uptake. The binding of alanine was 82 ± 6 pmol/μmol of phospholipid at 0.1 mM-alanine (14 experiments). For vesicles reconstituted from total BBMV protein the lipid/protein ratio was 30–40 μmol/mg, and the binding represents 40–50% of the total association of radioactivity with the vesicles at equilibrium.

Protein assays

Protein was measured by the method of Bradford [28] for samples dissolved in 2% MEGA-10 to eliminate turbidity. For reconstituted vesicles, the phospholipids interfered with the assay. This interference was corrected for by calibrating the assay for the interference produced by a standard amount of pure phospholipid/5% stearylamine. To measure the protein content of reconstituted vesicles, the phospholipid content of the vesicles was first determined as described previously [18] and this value was used to correct the reading given in the Bradford assay. At very low protein concentrations (Figs. 3, 5 and 6) this correction factor was relatively large. No other standard protein assay method proved to be any better in this respect.

Gels and Western blotting

SDS/PAGE was performed as described by Laemmli [29] and gels were silver-stained. Western blotting was performed by the method of Towbin *et al.* [30], except that the nitrocellulose was blocked with phosphate-buffered saline (0.14 M-NaCl, 2.7 mM-KCl, 1.5 mM-KH_2PO_4, 8.1 mM-Na_2HPO_4) containing 5% (w/v)

skimmed-milk powder plus 0.2% Tween-20 and washed with phosphate-buffered saline containing 0.2% Tween-20.

RESULTS

Optimization of reconstitution procedure

We have previously established a method for the rapid reconstitution of neutral amino acid transport from bovine renal brush-border membranes, using the detergent MEGA-10 [18]. This method, which is based on the procedure of McCormick *et al.* [12], has also been used to reconstitute liver amino acid transport [15] and lactate transport from red blood cells [31], and has been fully described and characterized [15,18,31]. Preliminary fractionation studies indicated that amino acid transport activity could be reconstituted from a protein fraction eluted from a DEAE-cellulose column by 50 mM-NaCl or from a hydroxyapatite column at 100–200 mM-phosphate [20]. These procedures were, however, found to be inadequate to lead to an identification of the transport protein. Before attempting further protein fractionation, it was advantageous to improve the reconstitution procedure.

Stearylamine has been shown to decrease the cation permeability of artificial phospholipid membranes by increasing the surface positive charge. Fig. 1 shows the effect of incorporation of stearylamine in the reconstitution procedure for alanine transport from kidney BBMV. The initial rate of alanine (0.1 mM) transport was increased 3-fold, and the Na^+/K^+ discrimination was also much improved. The time course of alanine uptake in the presence of 100 mM-choline chloride was identical with that in the presence of 100 mM-KCl (results not shown). The optimal concentration of stearylamine was found to be 5% (mol/mol of phospholipid). At higher concentrations the rate was unaffected, but the extent of uptake greatly decreased, presumably indicating the formation of smaller vesicles.

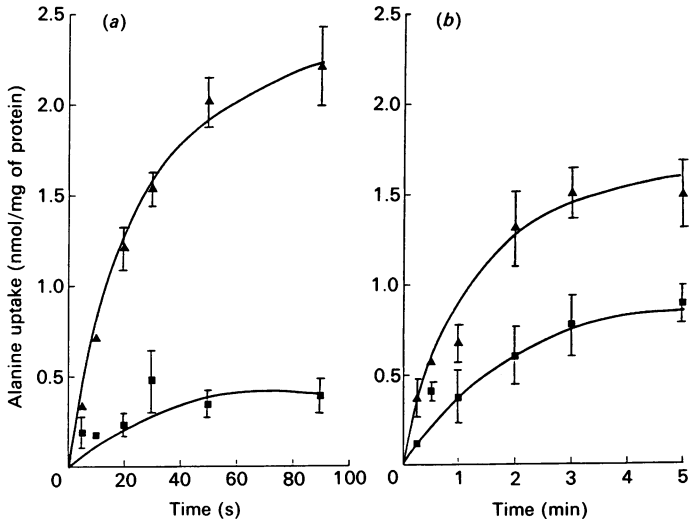

Fig. 1. Effect of incorporation of stearylamine on alanine transport in reconstituted membrane vesicles

Reconstituted vesicles were prepared from solubilized BBMV and phospholipids containing either no stearylamine (*b*) or 5% stearylamine (*a*) as described in the Materials and methods section. The alanine concentration was 0.1 mM: ▲, NaCNS; ■, KCNS. The results are means ± S.E.M. of values from four separate reconstitution experiments in each case.

Fractionation of alanine transport activity by lectin affinity chromatography

Bovine BBMV (100 mg of protein) were dissolved in MEGA-10 and passed down a column of concanavalin A immobilized on Sepharose 4B. Fig. 2 shows alanine transport in vesicles reconstituted from the initial membranes, the eluate from the column and the fraction eluted from the column by 100 mM-mannose. Alanine transport activity was largely removed from the extract by the lectin column, and the fraction absorbed on the column and then eluted with mannose had enhanced alanine transport activity. This indicates that the protein responsible for alanine transport is a glycoprotein. Similar results (not shown) were obtained with wheat-germ (*Triticum vulgaris*) lectin, which is specific for *N*-acetylglucosamine residues in glycoproteins.

To obtain further purification of the transport activity, the effect of peanut (*Arachis hypogaea*) lectin, which is specific for β-D-galactose-(1-3)-D-*N*-acetylgalactosamine residues was tested. BBMV dissolved in MEGA-10 were fractionated on peanut lectin–Sepharose. The amount of protein bound by the column was 0.19 ± 0.03 mg/100 mg of extract (7 experiments). Fig. 3 shows that reconstitution of the fraction eluted from the lectin with 100 mM-D-galactose yielded alanine transport activity which was significantly enhanced over that of the original extract and over the fraction binding to concanavalin (Fig. 2) and to wheat-germ lectin, although the protein in these vesicles could not be measured very accurately. The rate in the presence of a Na^+ gradient was not, however, significantly different from that in the presence of a K^+ gradient. Similar results were obtained in the absence of stearylamine, except that the absolute rates of transport were lower (results not shown).

The protein fractions binding to various lectins were separated by SDS/PAGE (10% gels), which were then silver-stained (Fig. 4). Peanut lectin bound a protein fraction which appeared as a single rather broad band. The average molecular mass of this protein band was 132 ± 1.8 kDa (data from four separate preparations) when measured to the centre of the band. A protein band at this value was also present in the fractions binding to wheat germ and concanavalin A. The protein pattern obtained on electrophoresis of reconstituted vesicles was identical with that of the protein fraction before reconstitution (results not shown).

Properties of reconstituted vesicles containing only the 132 kDa protein band

The properties of metabolite transport in reconstituted vesicles containing the protein fraction isolated by peanut lectin chromatography were further investigated. The number of experiments that could be done on a single preparation was greatly limited by the small amount of protein that could be obtained. From one bovine kidney only approx. 0.05 mg of protein could be obtained in reconstituted vesicles.

Fig. 5 shows that a choline chloride gradient did not support the uptake of alanine into vesicles. Although a K^+ gradient was as effective as a Na^+ gradient (Fig. 3), the uptake of alanine was greatly decreased when K^+ was present at the same concentration inside and outside the vesicles. These results indicate that the rapid rate of uptake observed in the presence of a K^+ gradient was probably due to alanine/K^+ co-transport. Neutral amino acid transport in BBMV is sensitive to inhibition by *N*-phenylmaleimide [7]. Fig. 5 indicates that uptake of alanine into reconstituted vesicles containing only the 132 kDa protein band was similarly sensitive to inhibition by *N*-phenylmaleimide.

Fig. 6 shows the specificity of transport in these vesicles. Alanine, glutamine and leucine were transported at comparable rates in the presence of a Na^+ gradient. There was, however, very little uptake of glycine under these conditions. This is consistent

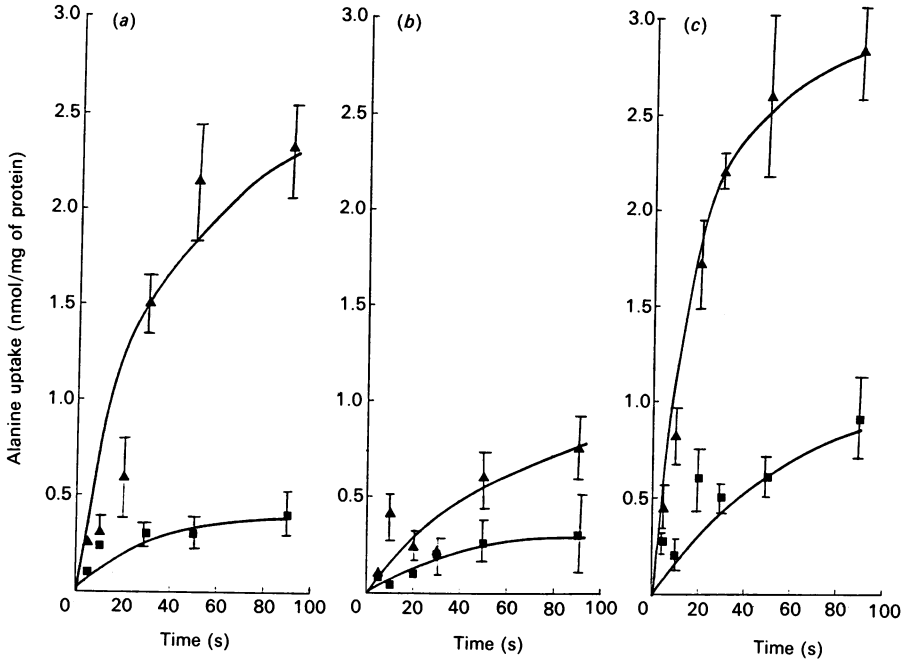

Fig. 2. Reconstitution of alanine transport from solubilized BBMV fractionated on concanavalin A

BBMV were fractionated on concanavalin A as described in the Materials and methods section. The protein fraction binding to the column was eluted with 0.1 M-mannose. Alanine transport was measured at 0.1 mM-alanine. The reconstituted fractions were (*a*) solubilized BBMV, (*b*) protein eluted with 20 mM-Tris/HCl, (*c*) protein eluted with 0.1 M-mannose: ▲, NaCNS; ■, KCNS. The results are means ± S.E.M. of values from three separate reconstitution experiments.

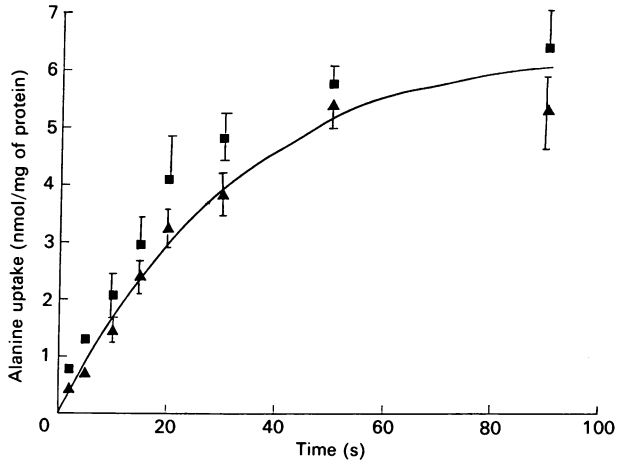

Fig. 3. Reconstitution of alanine transport from solubilized BBMV fractionated on peanut lectin

BBMV were fractionated on peanut lectin as described in the Materials and methods section. Transport of alanine (0.1 mM) in vesicles reconstituted from the fraction eluted with 0.1 M-galactose is shown: ▲, NaCNS; ■, KCNS. Results are means ± S.E.M. from four separate preparations.

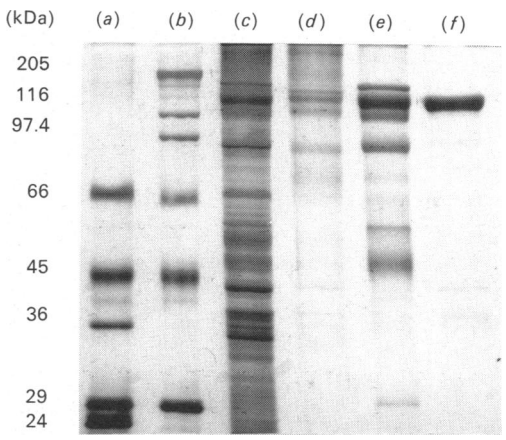

Fig. 4. Silver-stained SDS/PAGE gel of BBMV proteins bound to various lectin columns in the presence of MEGA-10

The tracks shown are: (*a*) and (*b*), molecular-mass standards (Sigma); (*c*) 5 μg of kidney BBMV; (*d*) 5 μg of protein fraction eluted from wheat-germ lectin with 0.1 M-*N*-acetylglucosamine; (*e*) 5 μg of protein eluted from concanavalin A with 0.1 M-mannose; (*f*) 2 μg of protein eluted from peanut lectin with 0.1 M-galactose.

with the specificity of the major neutral amino acid transport system in bovine renal BBMV, in which the uptake of alanine, glutamine and leucine is mutually competitive, but alanine transport is inhibited non-competitively by glycine [7]. Similar experiments (results not shown) showed that there was very little uptake of phosphate, glutamate or the glucose analogue α-methyl glucoside in the presence of a Na^+ gradient. Glucose, phosphate and glutamate are known to be transported by separate specific Na^+-dependent transport systems in renal BBMV.

Monoclonal antibodies to the 132 kDa protein

The above reconstitution experiments indicated that the protein fraction purified on peanut lectin has many of the properties of the neutral amino acid transport system in renal BBMV. While this work was in progress, monoclonal antibodies were prepared to a protein fraction obtained by fractionating BBMV succes-

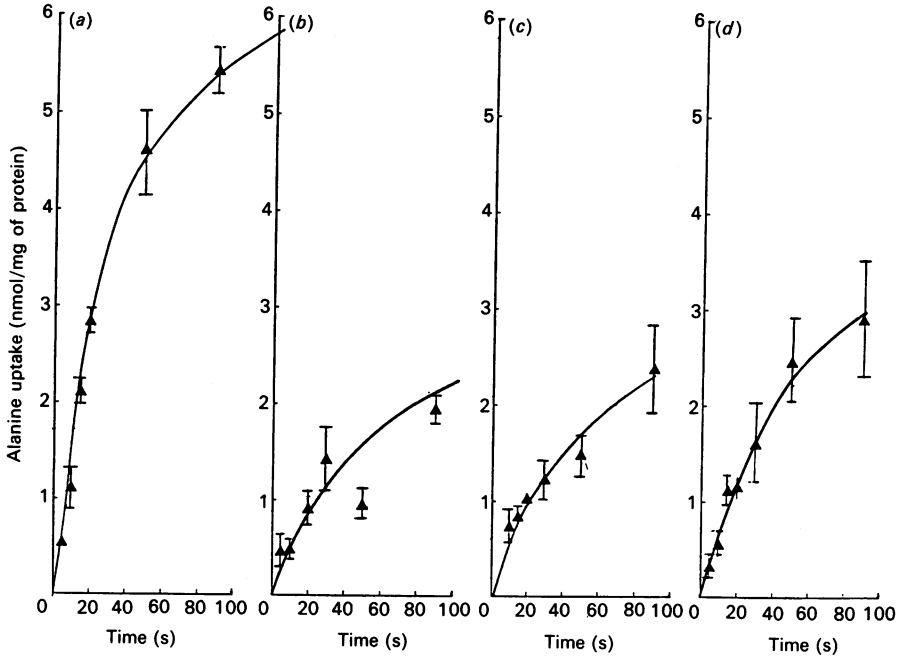

Fig. 5. Alanine transport in vesicles reconstituted from protein eluted from peanut lectin with galactose

The transport of alanine (0.1 mM) was measured in the presence of a gradient of (*a*) 0.1 M-NaCNS, (*b*) 0.1 M-choline chloride, (*c*) 0.1 M-NaCNS in the presence of 2 mM-*N*-phenylmaleimide, or (*d*) no salt gradient; 0.1 M-cyclamate + 10 mM-Hepes (K^+ salts) were present on each side of the membrane throughout.

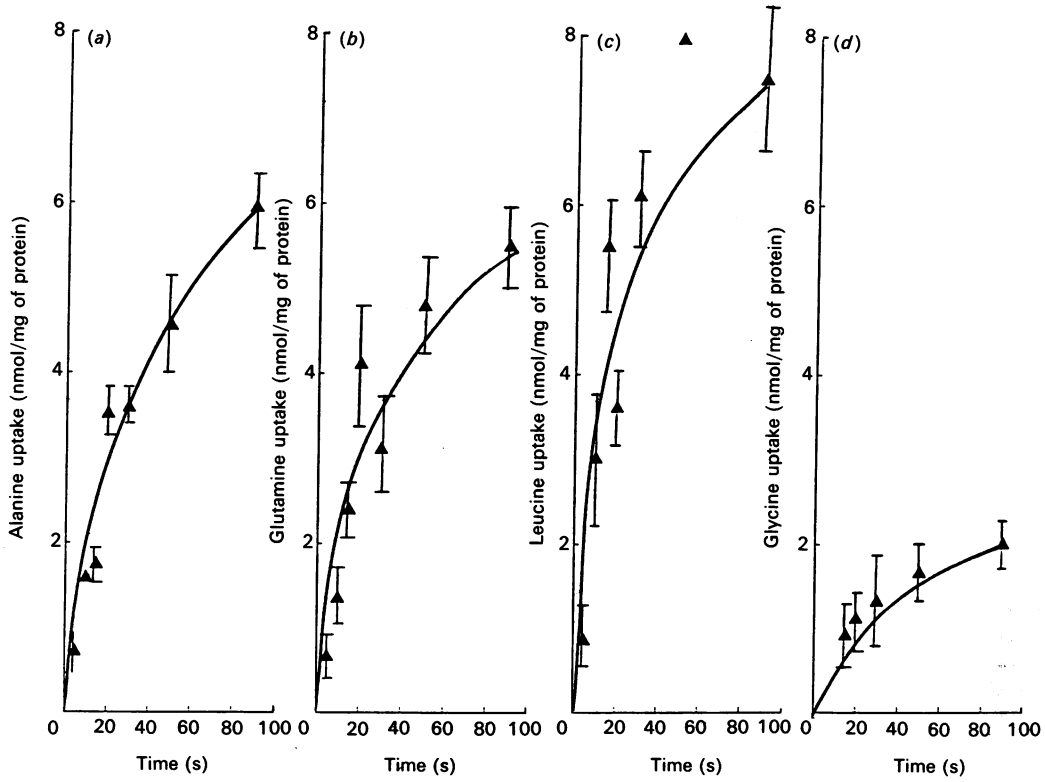

Fig. 6 Specificity of amino acid transport in vesicles reconstituted from the 132 kDa protein

Transport was measured in the presence of a gradient of 0.1 M-NaCNS. The substrates were (*a*) 0.1 mM-alanine, (*b*) 0.1 mM-glutamine, (*c*) 0.1 mM-leucine, (*d*) 0.1 mM-glycine.

sively on hydroxyapatite (taking the fraction eluted at 100–200 mM-phosphate) and then on concanavalin A. A number of antibodies were obtained, none of which had any inhibitory effect on alanine transport in BBMV. One antibody (FD19) reacted with the 132 kDa protein band which bound to the peanut lectin column (Fig. 7*a*). The antibody reacted with the

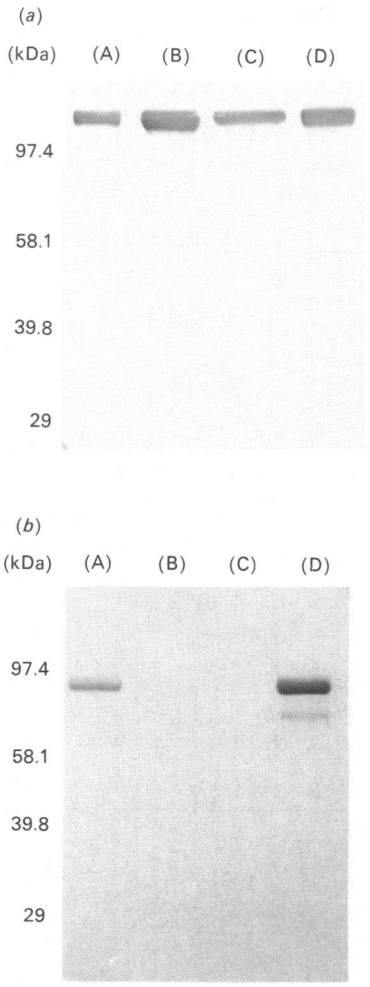

Fig. 7. Western blots of solubilized BBMV and protein fractions eluted from lectins with the monoclonal antibodies FD19 (*a*) and FD12 (*b*)

Tracks: (A) 10 μg of bovine BBMV; (B) protein binding to peanut lectin (5 μg); (C) protein binding to wheat-germ lectin (10 μg); (*d*) protein binding to concanavalin A (10 μg). The blot was calibrated with biotinylated standards (Sigma), and these standards were located with streptavidin conjugated to peroxidase.

same protein in the wheat-germ lectin and concanavalin A fractions. Another antibody (FD12) produced against a concanavalin A extract of bovine BBMV bound to a different protein and did not react with proteins binding either to wheat-germ lectin or to peanut lectin (Fig. 7*b*). This antibody was used as a control.

A 1 mg portion of solubilized BBMV was passed down a column containing the antibody FD19 immobilized on Sepharose. Fig. 8 shows that, on reconstituting the fraction which emerged from the column, little alanine transport activity was obtained, although the activity could be reconstituted from the fraction applied to the column. Reconstitution of transport activity from the protein bound to antibody FD19 was not attempted, since elution of this protein required the use of a buffer at pH 11. When the experiment was repeated with a column containing the immobilized antibody FD12, which does not react with the 132 kDa protein band, little or no alanine transport activity was removed from the extract (Fig. 9).

Further properties of the 132 kDa protein

When the 132 kDa glycoprotein band obtained by affinity chromatography on peanut lectin was incubated with the enzyme

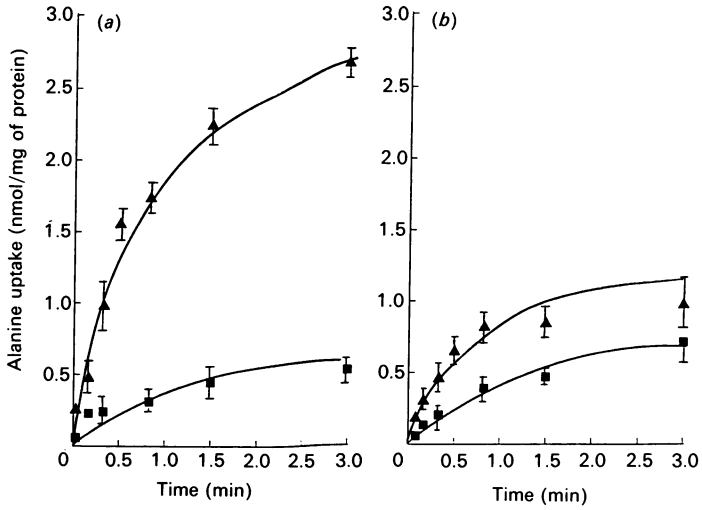

Fig. 8. Alanine transport activity in vesicles reconstituted from BBMV before and after passage through an immunoaffinity column containing the antibody FD19

Transport was measured at 0.1 mM-alanine in the presence of a gradient of (▲) 0.1 M-NaCNS or (■) 0.1 M-choline chloride. (*a*) Vesicles reconstituted from solubilized BBMV; (*b*) vesicles reconstituted from solubilized BBMV after passage through FD19 linked to Sepharose 4B. The results are means ± S.E.M. of values from four separate reconstitution experiments.

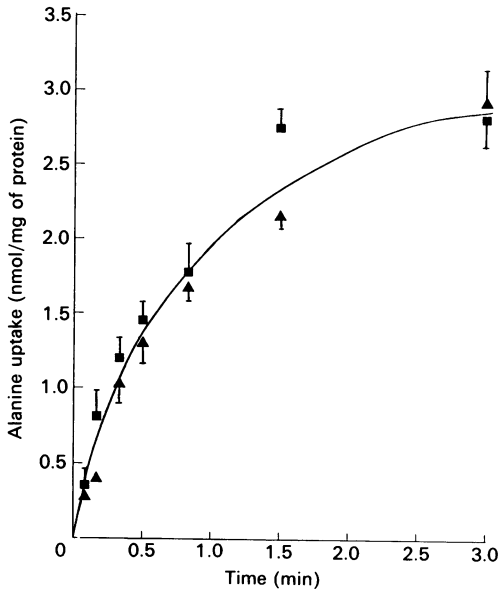

Fig. 9. Alanine transport activity in vesicles reconstituted from BBMV before and after passage through an immunoaffinity column containing the antibody FD12

Transport was measured at 0.1 mM-alanine in the presence of a gradient of 0.1 M-NaCNS: ▲, vesicles reconstituted from solubilized BBMV; ■, vesicles reconstituted from solubilized BBMV after passage through FD12 linked to Sepharose 4B. The results are means ± S.E.M. of values from three separate reconstitution experiments. No significant differences in uptake at corresponding times were observed.

endoglycosidase F, a band corresponding to 115 ± 1.2 kDa (from three separate gels) was obtained (Fig. 10); little material of intermediate molecular mass appeared. The presence of a single band after endoglycosidase treatment may indicate that the

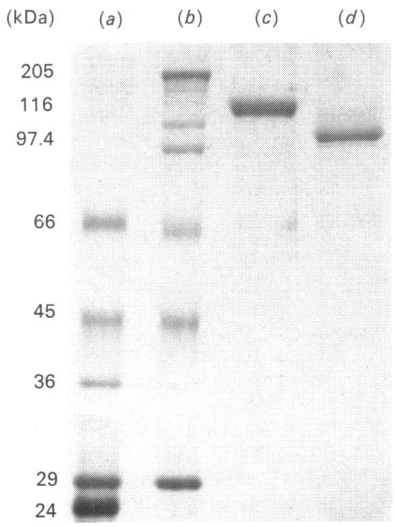

Fig. 10. Silver-stained SDS/PAGE gel of the protein obtained by chromatography on peanut lectin before and after treatment with endoglycosidase F

The 132 kDa protein obtained by peanut lectin chromatography was incubated with endoglycosidase F at 37 °C and pH 7.4 in 20 mM-Tris/HCl containing 0.25% MEGA-10 for 48 h; 2 μg of protein before and after digestion was used for electrophoresis. Lanes: (*a*) and (*b*), molecular-mass standards (Sigma); (*c*) protein before digestion; (*d*) protein after digestion.

original 132 kDa band represented a single protein which contains sugar residues equivalent to a molecular mass of 17 kDa. However, it cannot be excluded at this stage that other glycosylated proteins with similar molecular masses and sugar contents are present in this protein fraction. The monoclonal antibody FD19 reacted with the deglycosylated protein, thus indicating that it was not specific for carbohydrate residues.

Western-blotting experiments were performed to determine whether this protein was present in brush-border membranes from rat kidney or rat intestine or in basolateral membanes from rat liver. The monoclonal antibody FD19 did not react with any proteins in these membranes (results not shown).

DISCUSSION

Affinity chromatography of bovine renal brush-border membranes solubilized in MEGA-10 on immobilized peanut lectin achieves the purification of a single glycoprotein band of average molecular mass 132 kDa on SDS/PAGE. When this protein fraction is reconstituted with phospholipids, the transport properties of the vesicles obtained are similar to those of the major neutral amino acid transport system which occurs in bovine renal brush-border membranes [7]. Thus, in the presence of a Na^+ gradient, the rates of uptake of glutamine, leucine and alanine are similar. It is particularly important that the reconstituted vesicles descriminate between the transport of alanine and that of the very similar amino acid glycine. In renal BBMV glycine is know to be transported on a different system from that which transports alanine [32], and, although glycine inhibits alanine transport, this inhibition is non-competitive [7]. Alanine transport in both active BBMV and in vesicles reconstituted from the 132 kDa protein band is inhibited by *N*-phenylmaleimide.

These results suggest that the 132 kDa protein band contains at least a component of the protein responsible for the Na^+-dependent transport of neutral amino acids in renal BBMV. Important further evidence in favour of this proposition is the observation that specific removal of this protein by using an immunoaffinity column containing a monoclonal antibody also led to removal of reconstitutable alanine transport activity from the extract. It is noteworthy that this antibody was not prepared against the protein purified on peanut lactin, but against a protein fraction obtained from the transport-activity-containing fraction derived from chromatography of solubilized renal BBMV first on hydroxyapatite and then on concanavalin A. Thus the active fractions from these procedures also contained the 132 kDa protein.

The epitope of the 132 kDa protein recognized by the monoclonal antibody FD19 does not apparently occur in rat kidney or rat intestinal brush-border membrane systems. That the antibody to the 132 kDa protein does not inhibit transport in reconstituted vesicles is not surprising. Relatively few monoclonal antibodies to other transport systems have been found to inhibit transport. The epitope recognized by the antibody is presumably distant from the active site and is species-specific.

The major difference between the transport activities found in BBMV and in vesicles reconstituted from the 132 kDa protein fraction is the cation-specificity. In BBMV alanine transport is specifically Na^+-dependent, with little or no activity in the presence of a K^+ or choline gradient. The same specificity is obtained in vesicles reconstituted from protein fractions binding to concanavalin A or wheat-germ lectin. However, the reconstituted 132 kDa protein shows a different cation specificity, alanine transport occurring at approximately the same rate in the presence of either a Na^+ or a K^+ gradient, although transport activity in the presence of a choline gradient is slow. The reason for the loss of cation specificity is not clear. It is possible that the protein is re-inserted incorrectly into the membrane, or that an additional peptide required to maintain the specificity for Na^+ is lost during the lectin affinity chromatography.

There is little previous information in the literature about the possible identity of the alanine transport system from kidney brush-border membranes. Beliveau *et al.* [33] recently estimated the molecular size of the alanine- and leucine-transport proteins in rat renal BBMV by the method of radiation inactivation. Values of 274 kDa for the alanine transporter and 293 kDa for the leucine transporter were derived by this method, and it was concluded that these may well represent the same carrier protein. Given the uncertainties involved in these measurements, it is possible that the functional alanine transporter is a dimer of the 132 kDa polypeptide identified in the present paper.

In summary, it has been shown that a glycoprotein band isolated by peanut lectin affinity chromatography and having a mean molecular mass of 132 kDa on SDS/PAGE contains a protein component which is both necessary and sufficient for Na^+/amino acid co-transport in bovine kidney BBMV. Whether this protein is in itself sufficient to account for the absolute Na^+ specificity of transport observed in intact BBMV remains to be determined. The exact composition of the 132 kDa protein band remains to be elucidated, and it has not yet been convincingly shown that only one polypeptide is present. The carrier protein can, however, be identified as that fraction of the peanut-lectin-binding protein which reacts with the monclonal antibody FD19, which will react with only one specific epitope.

Final proof that a particular protein is in fact the major renal brush-border membrane neutral amino acid carrier will require demonstration of expression of Na^+-dependent amino acid transport activity in *Xenopus* oocytes or in some other suitable cell system after injection of the specific mRNA coding for the protein.

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