Identification of the integrin $\alpha_3\beta_1$ as a component of a partially purified A-system amino acid transporter from Ehrlich cell plasma membranes

John I. McCORMICK and Rose M. JOHNSTONE

Department of Biochemistry, McGill University, 3655 Drummond St., Montréal, Quebec, Canada H3G 1Y6

We have previously reported [McCormick and Johnstone (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7877–7881] the partial purification of the Na⁺-dependent A-system amino acid transporter from Ehrlich cell plasma membranes and have suggested that a 120–130 kDa peptide, a major component of the purified fraction [octyl glucoside (OG) extract], is involved in Na⁺-dependent amino acid transport. In the present study, N-terminal sequence analysis of the 120–130 kDa peptide revealed a sequence similar to that of the α_3 subunit of the integrin $\alpha_3\beta_1$. The presence of $\alpha_3\beta_1$ was confirmed by Western blots of the OG extract probed with anti- α_3 or - β_1 antibodies. Western blots also showed that an antibody originally raised against the 120–130 kDa peptide cross-reacts with both the α_3 and β_1 integrin subunits. Co-purification of $\alpha_3\beta_1$ and Na⁺-dependent transport activity suggested that the two activities might be associated. Evidence that α_3 plays a role in transport is shown by the fact that an antibody against human α_3 , but not β_1 , removed transport activity (~25% loss) from cholate-solubilized Ehrlich membranes. Further purification of

OG extracts using concanavalin A and wheat-germ lectin columns resulted in the separation of transport activity from the bulk (but not all) of $\alpha_3\beta_1$ integrin without loss of the transport activity. These results indicate that the integrin itself is not essential for amino acid transport. Reconstitution of a purified $\alpha_3\beta_1$ -depleted protein fraction showed high levels of Na⁺-dependent, α -methylaminoisobutyric-acid-inhibitable amino acid transport in proteoliposomes, whereas reconstituted integrin alone showed little transport activity. However, in the integrin-depleted fractions, high amino acid uptake occurred in K⁺ which compromised the accurate measurement of the Na⁺-dependent component of uptake. The data suggest that α_3 may be associated with the A-system transporter and may modulate the activity of this carrier. Moreover, transfection of K₅₆₂ and RD cells with human α_3 and α_2 cDNA showed that the former but not the latter increased A-system transport, thus providing more direct evidence that α_3 may modulate A-system transport activity.

INTRODUCTION

The uptake of amino acids into mammalian cells is accomplished by a diverse array of transport systems that have been characterized by their ion-dependence, substrate specificity, hormonal regulation and tissue localization [1–4]. Many of these transporters are Na⁺-dependent, and in non-neuronal non-epithelial cells the A and ASC systems are the major Na⁺-dependent carriers [1]. In epithelial cells Na⁺-dependent neutral amino acid transport may be accomplished by a different system showing broad substrate specificity [3,5]. In neuronal cells, where specific amino acids also serve as neurotransmitters, the carriers have defined substrate specificities [4,6] and, in addition to Na⁺-dependence, may have Cl[−] [4] or K⁺ [7,8] requirements, although these additional ion requirements are not necessarily restricted to neuronal cells. The complexity and variety of amino acid transport systems, which had been suggested for many years by transport studies with intact cells or membrane vesicles, has now been confirmed by the cloning of multiple amino acid carriers. Cloning strategies have been particularly successful with the Na⁺-dependent neuronal amino acid carriers, and cDNAs have been isolated for γ -aminobutyric acid (GABA) [9], glycine [10], proline [11] and glutamate [7,12,13] transporters. Although the GABA, glycine and proline carriers show extensive sequence identity [4], the glutamate carriers are structurally unrelated and appear to belong to a separate and distinct family [14], which may include a recently cloned [15,16] transporter with some, but not all, of the properties characteristic of the ASC system.

In an effort to identify the A-system carrier, we developed a reconstitution assay [17] which was used to partially purify the

transporter from solubilized Ehrlich cell plasma membranes [18]. A 120–130 kDa peptide was identified as the major component in a purified membrane fraction and evidence suggested that a component of this fraction was involved in Na⁺-coupled amino acid transport. The 120–130 kDa peptide was enriched during purification [18], an antibody against this peptide removed transport activity from solubilized membranes [18] and, in irradiated plasma membrane vesicles, loss of transport activity and destruction of the peptide occurred at identical rates [19].

More recently we have shown [20] that, in insulin-treated Ehrlich cells, A-system transport may be increased in an RNA- and protein-synthesis-independent manner. Plasma membranes prepared from these cells show an increased content of the peptide(s) recognized by the 120–130 kDa peptide antibody which is proportional to the magnitude of the increased transport.

These observations provide additional evidence for the involvement of a component of the 120–130 kDa peptide in amino acid transport and prompted the further characterization of the peptide described in the present study.

MATERIALS AND METHODS

Antibodies

A rabbit polyclonal antibody against a C-terminal peptide from the chicken β_1 subunit [21] was obtained from Dr. R. Hynes (Massachusetts Institute of Technology, Cambridge, MA, U.S.A.). Anti-(human α_3) and -(human β_1) sera, and K₅₆₂ and Rhabdomyosarcoma (RD) cells expressing either the α_3 (KA3 and RA3 cells) or α_2 (KA2 and RA2 cells) integrin subunits, were obtained from Dr. M. Hemler, Dana-Farber Cancer Institute,

Boston, MA, U.S.A. The preparation of the antibody against the 120–130 kDa peptide has been described [18]. Concanavalin A (Con A)–Sepharose and wheat-germ-lectin–agarose were obtained from Pharmacia and Sigma respectively. [14 C]-Aminoisobutyric acid ([14 C]AIB) (specific radioactivity 55 mCi/mmol) was obtained from ICN.

Purification of Na⁺-dependent amino acid transport activity

Plasma membranes were prepared from Ehrlich ascites cells by procedures already described [22–24] and stored at -70°C in 15% DMSO. Membrane solubilization and preliminary purification were carried out as described [18] with some minor changes. In brief, washed plasma membranes were solubilized by suspension (3–4 mg of protein/ml) in 2.5% cholic acid, 4 M urea, 100 mM NaCl, 0.1 mM EDTA, 5 mM Tris/HCl, pH 7.4, and 10 μM PMSF and incubation for 30 min at 4°C . Following centrifugation (120000 g , 60 min) the supernatant (10 ml) was applied to a Sepharose 6B column (2.5 $\times$ 50 cm) equilibrated in 5 mM Tris/HCl, pH 7.4, 100 mM KCl, 0.25% cholic acid, 50 μM dithiothreitol and 0.05% aroclorin. Fractions containing the highest level of transport activity from two Sepharose 6B columns were combined and centrifuged at 105000 g for 30 min. The resulting pellet was suspended (~ 450 μg of protein/ml) in 10 mM Tris/HCl, pH 7.4, 1% (w/v) *n*-octyl β -D-glucopyranoside (OG), 0.1% aroclorin and 10 μM PMSF, incubated at 4°C for 15 min and centrifuged at 105000 g for 30 min. The supernatant from this centrifugation (OG extract) contains high levels of transport activity [18] and is the starting material for the continuing purification described here.

Con A and wheat-germ lectin column chromatography

The OG extract (~ 1100 μg of protein) was applied to a Con A column (1 cm $\times$ 4 cm) equilibrated in 20 mM Tris/HCl, pH 7.4, 0.5 M NaCl, 1 mM MnCl_2 , 1 mM CaCl_2 , 1% CHAPS, 0.1% aroclorin and 10 μM PMSF (Buffer A) at a flow rate of 10 ml/h. Following loading, the column was washed with 5 column volumes of Buffer A (30 ml/h) and bound proteins were eluted with 0.5 M α -methyl-D-mannopyranoside in Buffer A. For maximum elution of retained proteins, 1 column volume of the 0.5 M α -methyl-D-mannopyranoside solution was applied, the column outlet was closed for 15 min and elution was continued (30 ml/h). Fractions (~ 1.5 ml) were collected and assayed for transport activity using the reconstitution assay described below. Fractions containing transport activity were pooled and loaded (10 ml/h) on to a wheat-germ-agarose column (1 cm $\times$ 2 cm) equilibrated in Buffer A. The column was washed with Buffer A until the A_{280} of the eluent was zero. The bound proteins were eluted with 0.3 M *N*-acetylglucosamine (NAG) in Buffer A. The resin was incubated for 15 min with the NAG solution before elution. All column work was carried out at 4°C .

Reconstitution of Na⁺-dependent amino acid transport

Reconstitution was accomplished using procedures already described [18,25]. Aroclorin (soy bean phospholipid) was normally used as the untreated powder and was prepared by suspension (40 mg/ml) in 100 mM KCl, 5 mM Tris/HCl, pH 7.4, 0.1 mM MgCl_2 and 0.1 mM CaCl_2 (K^+ buffer) and bath sonication to clarity under nitrogen. In some experiments crude aroclorin was washed in chloroform/methanol (1:4, v/v) before use [26]. For reconstitution, aliquots (250–500 μl) of fractions generated during purification were diluted to 2 ml in K^+ buffer, mixed with 250 μl (10 mg) of the sonicated aroclorin suspension and applied to a Sephadex G50 column (1.5 cm $\times$ 28 cm) equilibrated in K^+

buffer. The turbid void volume fractions were pooled, frozen in ethanol/solid CO_2 and thawed at 22°C , and proteoliposomes were collected by centrifugation at 40000 g for 20 min at 4°C . The proteoliposomes were suspended in small volumes of K^+ buffer. In some experiments the turbid void volume fractions were stored overnight at -80°C before proteoliposome recovery. Reconstitution from solubilized plasma membranes was accomplished as previously described [17,25,26]. [14 C]AIB transport in the proteoliposomes was measured in the presence of K^+ or Na^+ [17,26], usually over a 10 min time course. Uptake in Na^+ was corrected for uptake in K^+ , and initial-velocity values were determined from plots of Na^+ -dependent AIB uptake against time.

The small quantity of protein used in some of the reconstitutions precluded measurement of both transport activity and protein concentration in proteoliposomes from single experiments. Therefore separate experiments were carried out to assess the variability of protein reincorporation into proteoliposomes from the different fractions used for reconstitution. Protein concentration was determined by a filter binding assay [27]. Protein incorporation into proteoliposomes from lectin flow-through fractions was $50 \pm 1\%$ (difference from the mean), identical to the value determined previously for reconstitution from solubilized membranes [17]. Incorporation decreased to 40% (four determinations) for reconstitutions from the OG extract. Reconstitution of $29 \pm 3\%$ (difference from the mean) of the protein was obtained with the NAG eluate from the wheat-germ column. Uptake values from the reconstitutions are therefore expressed in terms of the proteoliposome protein content, using these percentage incorporation values.

Immunodepletion of transport activity with anti-integrin antibodies

Ehrlich cell plasma membranes were solubilized (2 mg of protein/ml) in cholate/urea as described above and dialysed overnight against 100 vol. of K^+ buffer containing 1% cholic acid and 5 μM PMSF. In initial experiments, 1 ml aliquots of solubilized membranes were incubated at 4°C for 4 h with 20 μl of rabbit anti-(human α_3 or β_1) immune sera or a rabbit non-immune serum. To remove immune complexes, 500 μl (0.3 mg) of a Protein A–Sepharose suspension in K^+ buffer, 1% cholic acid and 5 μM PMSF was added and incubation continued for 15 h at 4°C . The Protein A–Sepharose was pelleted by brief centrifugation, and the level of Na^+ -dependent amino acid transport activity remaining in the supernatants was determined by the reconstitution assay. In other experiments, Protein A–Sepharose and the immune sera were incubated before addition of solubilized membranes. Here, 30 μl aliquots of anti- α_3 and anti- β_1 immune sera or a non-immune serum were diluted to a final volume of 500 μl in 20 mM Tris/HCl, pH 7.4, and incubated for 4 h at 4°C with 500 μl (0.15 mg) of a Protein A–Sepharose suspension in the same Tris buffer. The Protein A–Sepharose/IgG complex was pelleted by brief centrifugation, washed twice in 20 mM Tris/HCl, pH 7.4 (1 ml), and once in 1 ml of K^+ buffer, 1% cholic acid and 5 μM PMSF before resuspension in 1.2 ml of solubilized membranes. Following incubation for 15 h at 4°C , the Protein A–Sepharose was removed by centrifugation and the supernatants were removed and reconstituted.

The Protein A–Sepharose pellets were washed twice in K^+ buffer, 1% cholate and 5 μM PMSF (1 ml), once in 1 ml of 20 mM Tris/HCl, pH 7.4, and finally suspended in SDS/ β -mercaptoethanol in preparation for SDS/PAGE (see below).

SDS/PAGE and Western blot analysis

Gradient polyacrylamide gels (5–15%) were run using the

procedure of Laemmli [28]. Samples were boiled for 5 min or incubated at 70 °C for 15 min in 2% (w/v) SDS, 0.062 M Tris/HCl, pH 6.8, and 10% (w/v) glycerol in the presence or absence of 10% (w/v) β -mercaptoethanol. Because of the dilute protein solutions generated during purification, samples for SDS gels were either precipitated with trichloroacetic acid in the presence of deoxycholate [29,30] or dialysed against water, lyophilized and suspended in the SDS/ β -mercaptoethanol buffer. Following electrophoresis, gels were stained with Coomassie Blue or commercial silver stain kits (Silver Stain Plus; Bio-Rad).

For Western blotting, proteins were electrophoretically transferred to nitrocellulose or poly(vinylidene difluoride) (PVDF) membranes after gel electrophoresis [31] and probed with immune rabbit sera. Antibody binding was detected by goat anti-rabbit IgG conjugated to alkaline phosphatase with the appropriate colour-developing reagents (Bio-Rad). In some experiments a commercial chemiluminescent assay kit (Amersham) was used to develop blots.

$[^{14}\text{C}]\text{AIB}$ uptake in transfected cell lines

All cell lines used in this study were a gift from Dr. Martin Hemler, Harvard Medical School, Boston, MA, U.S.A. K_{562} and RD cells, which do not express functional integrin $\alpha_3\beta_1$ [32], were stably transfected with human α_3 cDNA (referred to as KA3 and RA3 cells for the K_{562} and RD transfectants respectively) or α_2 cDNA (KA2 and RA2 cells respectively) as described by Weitzman et al. [32] and monoclonal antibodies were used to confirm the expression of α_2 or α_3 on the cell surface. Cells were grown in RPMI 1640 containing 10% (v/v) fetal bovine serum. For uptake measurements in K_{562} -derived cell lines, cells were washed, suspended in choline Ringer and incubated for 15 min at 37 °C to deplete amino acid pools and remove traces of serum. Cells were again washed and suspended in choline Ringer, and transport rates were determined by dilution (8×10^6 cells/ml) into choline or Na^+ Ringer containing 0.125 mM $[^{14}\text{C}]\text{AIB}$ at 37 °C. Transport was terminated by centrifugation through mineral oil overlaid with cold buffered isotonic saline at 4 °C and counting radioactivity in the cell pellet.

RD-derived cell lines were subcultured into 24-well tissue culture dishes, and the degree of AIB uptake was determined when the cells were 70–80% confluent. Cell layers in individual wells were washed and incubated at 37 °C for 15 min in 2 ml of choline Ringer. The solution was removed, the cells were washed in choline Ringer and 0.25 ml of choline or Na^+ Ringer containing 0.05 mM $[^{14}\text{C}]\text{AIB}$ was added. Following a 2 min incubation the solution was removed, the cell layer was washed twice with 2 ml of Na^+ Ringer (4 °C) and 0.3 ml of 5% (w/v) trichloroacetic acid was added. Radioactivity was determined by counting a 0.25 ml aliquot after a 30 min incubation at 22 °C. In wells not used for uptake measurements, cells were detached by incubation in 10 mM PBS containing 2 mM EDTA and counted with a haemocytometer. Transport and cell number measurements were done in triplicate for each plate.

RESULTS

Identification of integrin $\alpha_3\beta_1$ as a component of the 120–130 kDa peptide fraction

We have reported that during purification of plasma membrane proteins and reconstitution into proteoliposomes, a peptide of ~120–130 kDa is enriched in parallel with increased Na^+ -dependent AIB transport. Preabsorption of a membrane extract using an antibody against this 120–130 kDa fraction resulted in the partial loss of Na^+ -dependent transport, suggesting that the

		1	10
120–130 kDa peptide	:	FNLDVRFLLVVKEAVNPG	
α_3 integrin	:	FNLDTRFLVVKEAGNPG	

Figure 1 N-terminal amino acid sequence of the 120–130 kDa peptide and the α_3 subunit of human VLA-3 [44]

Figure 2 Western blots of the OG extract

Aliquots of an OG extract (~10 μg of protein) were boiled for 5 min with SDS in the presence (R) or absence (NR) of β -mercaptoethanol and samples were applied to 5–15% polyacrylamide gels following addition of 20 mM iodoacetamide. Gels were either stained with Coomassie Blue (left gel), or transferred to nitrocellulose and probed with the anti-(120–130 kDa peptide) antibody serum (1:1000 dilution) or with human anti- α_3 or - β_1 sera at dilutions of 1:500. Colour development is described in the Materials and methods section. Left-hand lanes in each gel contain molecular mass markers.

peptide was a component of the transporter [18]. To characterize this peptide further, partial amino acid sequencing of the enriched 120–130 kDa peptide was carried out followed by a search through available databases. Strong sequence identity of the first 17 N-terminal amino acids with the α_3 subunit of the integrin $\alpha_3\beta_1$ was found. In two separate analyses from different samples prepared on separate occasions from different populations of membranes, only a single amino acid sequence was obtained (Figure 1).

Human anti-integrin antibodies were used to confirm the presence of α_3 in the 120–130 kDa peptide by Western blots of the OG extract after SDS/PAGE under reducing or non-reducing conditions. Under reducing conditions, Coomassie Blue-stained gels of the OG extract showed one major band of molecular mass 119–127 kDa (Figure 2). This staining pattern changed under non-reducing conditions, with the appearance of protein bands at 141 kDa and 125 kDa and the disappearance of a minor band at ~35 kDa (Figure 2). Western blots of the OG extract probed with an anti-(human α_3) antibody recognized the 141 kDa band under non-reducing conditions, confirming the presence of the α_3 subunit in the OG extract. The molecular mass changes in the presence and absence of mercaptoethanol are in agreement with the α_3 structure [33,34].

To determine if the β subunit (β_1) normally associated with α_3 was present in the OG extract, Western blots were also probed with a polyclonal antibody against human β_1 . Under non-reducing conditions a peptide (125 kDa) cross-reacted with the anti- β_1 antibody, confirming that the OG extract contains both the α_3 and the β_1 subunits of the integrin VLA-3 (Figure 2). Both antibodies reacted poorly (if at all) with the OG extract under reducing conditions. Clearly the reduction of the extensive disulphide bond network present in both α_3 and β_1 integrin subunits changes the epitopes recognized by the antibodies and dramatically lowers reactivity. The polyclonal antibody, orig-

Figure 3 Con A column chromatography of the OG extract

An OG extract (9 ml; 2673 μg of protein) was loaded on to a Con A column (1 cm $\times$ 4 cm) as described in the Materials and methods section. At fraction 6, the column was washed with the equilibration buffer; at fraction 20, 0.5 M α -methyl-D-mannopyranoside was applied to the column. Aliquots (500 μl) of the fractions (1.5 ml) were reconstituted (see the Materials and methods section) and assayed for Na^+ -dependent amino acid transport activity (○). The arrow indicates the initial activity of the OG extract (0.9 nmol/min per mg of protein).

inally raised against the 120–130 kDa peptide [18] enriched in the OG extract, reacted with both the 141 kDa and 125 kDa peptides under non-reducing conditions (Figure 2), suggesting that this antibody cross-reacted with the α_3 and β_1 subunits.

There is considerable evidence to suggest that a component of the 120–130 kDa peptide is involved in Na^+ -dependent amino acid transport [18–20]. The above results raised the possibility that transport activity might be due to the integrin $\alpha_3\beta_1$. Alternatively, the transporter might be a component of the 120–130 kDa peptide migrating together with the integrin, the two proteins having failed to separate on SDS gels. We therefore

considered it of some importance to distinguish between these possibilities, since the measurement of transport activity indicated substantial enrichment of transport in the OG extract compared with the earlier steps (see Table 1 in [18]).

The first possibility to be considered was that the integrin is responsible for transport activity. Since both the transporter and integrins are known to be glycoproteins [35–37], different lectin binding columns were used to assess whether transport activity could be separated from the integrin. The results in Figure 3 show that the majority of the transport activity in the OG extract was recovered in the flow-through of a Con A column and was not bound to Con A. The majority of the integrin was retained by the Con A column (see Figures 6 and 7 below). In the experiment shown, the maximum reconstituted transport activity in the flow-through fractions was approx. 2-fold higher than in the OG extract (Figures 3 and 4). Most of the protein loaded on to the column was released with buffer. Glycoproteins retained on the Con A column were eluted with α -methylglucoside and showed no Na^+ -dependent transport activity after reconstitution (Figure 3; see also Figure 7). Further enrichment of Na^+ -dependent transport activity was achieved by application of the Con A flow-through fractions to a wheat-germ column. The data in Figure 4 show that the transporter bound to the wheat-germ column and was eluted by NAG (compare Figures 4c and 4d), resulting in a large increase in the rate of AIB uptake compared with that of the OG extract (Figure 4a) and the Con A flow-through fraction (Figure 4b).

Table 1 summarizes the degree of purification of the Na^+ -dependent amino acid transporter using the lectin columns (four separate experiments). Each experiment shows an increase in Na^+ -dependent AIB transport on further purification of the OG extract. However, the increase in transport activity was variable, ranging from 16-fold (Expt. 1) to 2.5-fold (Expt. 3), with an average increase of 8-fold. This variability was due to the different activities in the initial OG extracts, which ranged from 1.0 to 7.1 nmol of AIB/min per mg of protein. In contrast, the activity of the final NAG-eluted fraction in the different experiments was similar, with an average value of 18.7 ± 3.3 nmol AIB/min per mg of protein. Since the initial OG extract

Figure 4 [^{14}C]AIB transport activity in purified fractions

An OG extract (1280 μg of protein) was loaded on to a Con A column as described for Figure 3 and the flow-through fractions were pooled (539 μg of protein; 42% of column load). An aliquot of the Con A column flow-through (466 μg of protein) was applied to a wheat-germ column (see the Materials and methods section). Following column washing, retained proteins (93 μg of protein; 20% of column load) were eluted with 0.3 M NAG. The flow-through fractions contained 233 μg of protein (50% of load), and the total protein recovery from the wheat-germ column was 70%. Aliquots of 250 μl of the OG extract (40 μg of protein) (a), and 500 μl aliquots of the Con A flow-through (24.5 μg of protein) (b), the wheat-germ column flow-through (12 μg of protein) (c) and the NAG-eluted wheat-germ column fraction (6.7 μg of protein) (d) were reconstituted, and [^{14}C]AIB (0.1 mM) uptake was measured in K^+ (●) and Na^+ (○). Uptake is expressed in terms of the protein content of the liposomes, based on the average percentage protein reconstituted in each fraction (see the Materials and methods section).

Table 1 Purification of the OG extract

The OG extract was purified as described in the Materials and methods section and the legend to Figure 4. Data from four separate experiments are shown. Activity is measured in nmol of AIB/min per mg of protein. 'Units' are defined as: activity (nmol of AIB/min per mg of protein) $\times$ total protein (mg). ND, not done.

Fraction	Expt. 1		Expt. 2		Expt. 3		Expt. 4	
	Activity	Units	Activity	Units	Activity	Units	Activity	Units
OG extract	1.0	1.7 (100%)	4.5	5.8 (100%)	7.1	5.8 (100%)	2.1	2.5 (100%)
Con A column flow-through	1.5	1.3* (79%)	7.4	4.0† (69%)	5.0	2.8‡ (49%)	ND	ND
Wheat-germ column flow-through	0	0	0	0	0	0	ND	ND
NAG-eluted fraction from wheat-germ column	16.6	1.1 (76%)	23.6	2.2 (45%)	17.5	0.3 (6%)	17.0	1.0 (40%)

* Only 1.1 units were applied to the wheat-germ column.

† Only 3.4 units were applied to the wheat-germ column.

‡ Only 2.4 units were applied to the wheat-germ column.

Figure 5 Amino acid transport in the NAG-eluted protein fraction from a wheat-germ column

Amino acid transport activity was purified as described in the legend to Figure 4, and 500 μ l aliquots of the NAG-eluted fraction (6.7 μ g of protein) were reconstituted. AIB uptake was measured in K^+ (●), Na^+ (○), K^+ plus Me-AIB (■) or Na^+ plus Me-AIB (□). The final Me-AIB concentration was 5 mM.

represented a 30-fold enrichment of transport activity from solubilized membranes, the additional steps described here result in significant purification (e.g. 500-fold in Expt. 1, Table 1) of the A-system transporter from Ehrlich cell plasma membranes. Purification of the A-system carrier is indicated by the Na^+ -dependence of AIB transport and by inhibition of transport by methylaminoisobutyric acid (Me-AIB) (Figure 5) in proteoliposomes prepared from the NAG eluate. However, the purified transporter also shows an increase in the degree of AIB uptake in K^+ . This is clearly shown in Figure 4, where the AIB flux in K^+ in proteoliposomes from the NAG wheat germ column eluent is dramatically higher than in the OG extract. Table 2 compares AIB fluxes at the 3 min time point, in the presence and absence of Na^+ , in purified fractions generated from three different experiments. In each experiment, uptake in K^+ increased with purification. However, a large increase in Na^+ -independent AIB uptake occurred in the NAG fraction compared with that seen in the other fractions. From these experiments AIB uptake, in the

Table 2 AIB uptake in K^+ and Na^+ by proteoliposomes generated from purified fractions

[^{14}C]AIB uptake was measured in K^+ and Na^+ , as described in the Materials and methods section. The degree of AIB uptake at the 3 min time point of a 10 min time course is shown.

Fraction	Uptake (nmol of AIB/3 min per mg of protein)					
	Expt. 1		Expt. 2		Expt. 3	
	K^+	Na^+	K^+	Na^+	K^+	Na^+
OG extract	10.9	16.1	15.5	33.5	18.1	34.8
Con A column flow-through	14.4	18.0	24.2	35.1	26.1	53.8
Wheat-germ column flow-through	22.8	27.4	38.5	34.2	52.6	41.1
NAG-eluted fraction from wheat-germ column	230.9	285.1	261.1	291.9	185.8	241.5

absence of Na^+ , was 15-fold higher in the NAG eluate (average value 226 ± 38 nmol of AIB/3 min per mg of protein) than in the OG extract (average value 14.8 ± 3.6 nmol of AIB/3 min per mg of protein). In addition, it is clear that, with purification, the Na^+ -delta (difference between uptake in Na^+ and K^+) becomes smaller when expressed as a percentage of the total AIB uptake in K^+ (Table 2), although the absolute value for Na^+ -dependence is greatest in the NAG eluate. Thus in Expt. 3 (Table 2) the Na^+ -deltas for the OG extract (16.7 nmol of AIB/3 min per mg of protein) and the NAG fraction (55.7 nmol of AIB/3 min per mg of protein) represent 93% and 30% respectively of the AIB uptake in K^+ . Therefore, although the Na^+ -delta increased with purification, the corresponding increase in AIB uptake in the absence of Na^+ tended to obscure the Na^+ -delta. In two experiments (out of six purification experiments), the Na^+ -delta was not measurable and A-system activity became apparent only when uptake was measured in the presence of Me-AIB (results not shown). The increased uptake in the wheat-germ column fraction is probably due to purification of a protein showing high amino acid uptake in both the presence and absence of Na^+ . That the uptake in the Na^+ -free medium by the reconstituted NAG eluate is still carrier-mediated is suggested by its inhibition by Me-AIB. For example, in two experiments [^{14}C]AIB (0.1 mM)

Figure 6 SDS/PAGE and Western blots of purified fractions

Samples (except for the OG extract) from the purification described in Figure 4 were dialysed against water, lyophilized and suspended in small volumes of 1% cholic acid, 0.1 mM MgCl_2 , 0.1 mM CaCl_2 and 20 mM Tris/HCl, pH 7.4. Sample buffer without β -mercaptoethanol was added, and after incubation at 65 °C for 15 min, 6 μg aliquots of OG extract (lanes 2, 6, 10 and 14), Con A column-flow-through (lanes 3, 7, 11 and 15), wheat-germ column flow-through (lanes 4, 8, 12 and 16) and the NAG-eluted fraction from the wheat-germ column (lanes 5, 9, 13 and 17) were applied to 5–15% gradient polyacrylamide gels. Following electrophoresis, gels were either silver stained (a) or transferred to nitrocellulose for Western blotting (b). Blots were probed with anti-(120–130 kDa peptide) serum (1:1000 dilution) (b, lanes 6–9), chicken anti- β_1 serum (1:1000 dilution) (b, lanes 10–13) or human anti- α_3 serum (1:500 dilution) (b, lanes 14–17). Development of blots was by chemiluminescence using a second antibody linked to horseradish peroxidase. Lane 1 contains molecular mass standards.

uptake at 10 min, in the presence of K^+ , was inhibited 16% and 36% by 4.8 mM Me-AIB. The inhibition was measurably greater in Na^+ medium (> 40%). Preliminary studies have shown that replacement of K^+ in these experiments by choline leads to the same levels of AIB uptake in proteoliposomes, indicating that the uptake is not dependent on K^+ .

Increased AIB uptake in K^+ by the NAG-eluted fraction from the wheat-germ column could be due to an increase in liposome volume during reconstitution, which could in turn increase the degree of Na^+ -independent AIB uptake [25]. However, this seems unlikely, for the following reasons. (a) If this were a non-specific phenomenon related to the reconstitution of small quantities of protein (5–10 μg) with excess phospholipid, other fractions from the purification scheme, reconstituted under these conditions, should exhibit high AIB uptake in K^+ . In fact they do not, and the observation is restricted to the NAG-eluted wheat-germ column fraction. (b) If the high uptake in K^+ , expressed in terms of protein, were due to larger intravesicular volumes, uptake expressed with respect to lipid concentration should show increases of similar magnitude. However, AIB uptake in the reconstituted NAG eluate from the wheat-germ column fraction, expressed as c.p.m./mg of phospholipid, was only 30% higher than uptake in proteoliposomes from the OG extract (results not shown), compared with the 15-fold increase measured with respect to protein (Figures 4a and 4d).

Protein composition of the purified transporter preparation

Figure 6 shows the protein composition of the OG extract, the Con A and wheat-germ column flow-through fractions and the fraction eluted from the wheat-germ column with NAG. The purification of Na^+ -dependent AIB transport activity correlates with the enrichment of ~121 and ~55 kDa peptides in the NAG eluate together with a low-molecular-mass (23 kDa) peptide (Figure 6a, lanes 2 and 5). The silver-stained gels suggest that the bulk of the $\alpha_3\beta_1$ in the OG extract is retained by the Con A column, since the Con A flow-through fraction (Figure 6a, lane 3; see also Figure 7 below) is depleted of the 125 and 141 kDa peptides, i.e. the β and α subunits respectively (see below). When the transporter-enriched fraction from the wheat-germ column was probed with antibodies, the 121 kDa peptide reacted with

both the anti- β_1 antibody (Figure 6b, lane 13) and the anti-(120–130 kDa peptide) antibody (Figure 6b, lane 9). In some purification experiments there were trace amounts of a band at 141 kDa in the NAG-eluted fraction from the wheat-germ column. This band reacted with anti- α_3 antibody, but no clear relationship between the presence of α_3 and the degree of Na^+ -dependent AIB transport has yet been determined. However, it is clear that the bulk of the $\alpha_3\beta_1$ is not required for transport, since purification of the A-system carrier is accompanied by loss of the bulk of α_3 and ~90% of the β_1 integrin subunit (compare Figure 6b, lanes 10 and 13, and lanes 14 and 17). The integrin can be recovered from the Con A column by elution with α -methylglucoside. Non-reducing SDS gels of the eluted fractions show peptides of ~136 and 117 kDa (Figure 7c) which on Western blots react intensely with anti- α_3 and - β_1 antibodies respectively (results not shown).

It is of interest to note that the ~121 kDa peptide [which reacts with the anti- β_1 and anti-(120–130 kDa peptide) antibodies] in the Con A flow-through is absent from the wheat-germ column flow-through (Figure 6b, lanes 7 and 8, and 11 and 12) but is enriched by retention on the wheat-germ column and elution by NAG (Figure 6a, lane 5 and Figure 6b, lanes 9 and 13). Thus A-system transport is enriched in the NAG eluate along with an increase in the quantity of the 121 kDa peptide. Such data imply that the 121 kDa peptide may be the transporter protein. However, since the enriched 121 kDa peptide still reacts with the polyclonal anti- β_1 antibody, as well as with the antibody to the 120–130 kDa peptide, the possibility has not been eliminated that the 120–130 kDa antibody may also react with additional unidentified proteins present in the 121 kDa peptide staining band. Since the enriched A-system transporter in the NAG eluate still contains some integrin, the possibility also remains that the two functions may be associated.

The 55 kDa peptide in the purified transporter fraction (Figure 6a, lane 5) is currently being characterized.

Reconstruction of $\alpha_3\beta_1$

Since the purification procedure led to the isolation of almost pure $\alpha_3\beta_1$ (Figure 7c), it provided an opportunity to determine if the integrin itself showed the capacity for amino acid transport.

Figure 7 Reconstitution of $\alpha_3\beta_1$ from the Con A eluate

The α -methylglucoside-eluted fraction from the Con A column (see Figure 3) was dialysed extensively against water, lyophilized and the residue suspended in 1% cholic acid, 0.1 mM MgCl_2 , 0.1 mM CaCl_2 and 20 mM Tris/HCl, pH 7.4 (68 μg of protein/ml). Aliquots were either run on non-reducing SDS gels [(c), standards in lane 1, sample (3 μg of protein) in lane 2] or (b) reconstituted using 5 μg of protein and 13.2 μmol (~ 10 mg) of partially purified asolectin. AIB (0.1 mM) uptake in proteoliposomes was measured in K^+ (●), Na^+ (○) or K^+ plus Me-AIB (■) as described in the Materials and methods section. In (a), an equivalent amount of asolectin without protein was also reconstituted and AIB uptake was measured as in (b). Proteoliposomes and liposomes were suspended in equal volumes of K^+ buffer and uptake is expressed as c.p.m./25 μl aliquot of the sample (see the Materials and methods section). Counting efficiency was $\sim 90\%$. The data shown are the average values from duplicate time courses for each condition.

Figure 8 Na^+ -dependent AIB uptake in K_{562} , KA3 and KA2 cells

K_{562} (●), KA2 (○) (expressing the α_2 integrin subunit) and KA3 (■) (expressing the α_3 integrin subunit) cells were washed in choline Ringer buffer and incubated at 37 °C for 15 min in the same buffer. Cells were washed, resuspended in choline Ringer and uptake of $[^{14}\text{C}]\text{AIB}$ (0.125 mM) was measured in choline or Na^+ Ringer. For the results shown, the total uptake in Na^+ has been corrected for uptake in choline. The cell concentration during the assay was $\sim 8 \times 10^6$ (K_{562} , KA3) or 4×10^6 (KA2) cells/ml. Uptake in Na^+ Ringer was measured in duplicate in all experiments. These data are from a single experiment, but a second experiment, done in duplicate, gave almost identical results. In eight other experiments, the average increase in Na^+ -dependent AIB transport in KA3 cells compared with K_{562} cells was 50%.

$\alpha_3\beta_1$ was reconstituted and AIB uptake in the proteoliposomes (Figure 7b) was then compared with uptake in protein-free liposomes (Figure 7a). Na^+ -dependent AIB transport in integrin-containing liposomes was not apparent. However, AIB uptake higher than that seen in protein-free liposomes was evident (Fig. 7b), and the degree of AIB uptake was proportional to the quantity of $\alpha_3\beta_1$ used in the reconstitution (results not shown).

When 10 μg of $\alpha_3\beta_1$ was used in reconstitutions, the average uptake at 10 min was 128.6 ± 9.8 nmol of AIB/mg of protein ($n = 3$), compared with 351.7 ± 55.3 nmol of AIB/mg of protein ($n = 4$) in the NAG fraction from the wheat-germ column. AIB flux in $\alpha_3\beta_1$ -containing liposomes was detectably inhibited by Me-AIB (Figure 7b). Whether the AIB uptake in proteoliposomes is due to $\alpha_3\beta_1$ itself or to a minor component of the transporter present in the α -methylglucoside-eluted fraction from the Con A column has yet to be determined. Clearly, however, no Na^+ -dependent amino acid transport activity is retained by the Con A column.

Influence of α_3 expression in K_{562} and RD cells on A-system transport

The data clearly show that $\alpha_3\beta_1$ is not responsible for the Na^+ -dependent component of amino acid transport. However, the continued co-purification of transport activity with the integrin suggested an interaction between the two activities. Direct evidence supporting this conclusion was obtained by measuring Na^+ -dependent AIB uptake in native K_{562} and RD cells and in cells transfected to express the integrin subunits α_2 and α_3 . Native K_{562} and RD cells are deficient in α_3 [32] but express endogenous β_1 . The ability to transport AIB, via the A system, in both cell lines (results not shown) argues against an obligatory role for $\alpha_3\beta_1$ in transport. However, both cell lines showed increases in AIB transport after transfection with α_3 cDNA. In K_{562} cells (Figure 8), α_3 transfection resulted in an $\sim 50\%$ increase in AIB transport over that in control cells. The level of transport was unaffected by α_2 transfection (Figure 8). Control experiments demonstrated that 3-*O*-methyl-D-glucose transport was unchanged in K_{562} and KA3 cells, suggesting a specific interaction of α_3 with the amino acid transporter (results not shown). Preliminary evidence for the specific interaction of $\alpha_3\beta_1$ with the A-system transporter has been obtained by measuring Na^+ -dependent serine transport in the presence and absence of Me-AIB to distinguish between the A and ASC systems. These experiments, with K_{562} and KA3 cells, suggest that only the

Table 3 AIB uptake in RD, RA2 and RA3 cells

AIB (0.05 mM) uptake was measured as described in the Materials and methods section. Uptake measurements and cell counts were determined in triplicate for each experiment.

Cell line	Uptake (pmol of AIB/min per 10 ³ cells)		
	Expt. 1	Expt. 2	Expt. 3
RD (control cells)	0.011 ± 0.0098 (100%)	0.009 ± 0.0016 (100%)	0.013 ± 0.004 (100%)
RA2 (α_2 integrin)	0.014 ± 0.0018 (127%)	0.012 ± 0.001 (133%)	0.028 ± 0.0047 (215%)
RA3 (α_3 integrin)	0.032 ± 0.0053 (290%)	0.022 ± 0.0047 (244%)	0.057 ± 0.011 (438%)

Table 4 Immunodepletion of Na⁺-dependent AIB transport

Aliquots (30 μ l) of non-immune or anti- α_3 and β_1 sera were diluted to 500 μ l in 20 mM Tris/HCl buffer, pH 7.4, and incubated (4 h, 4 °C) with 0.15 mg of Protein A–Sepharose. The Protein A–Sepharose/IgG complex was washed, resuspended in 1 ml of solubilized membranes (1.6 mg of protein) and incubated overnight at 4 °C. Protein A–Sepharose was removed by centrifugation, and transport activity remaining in the supernatants was determined by reconstitution.

Antiserum	Transport activity (pmol of AIB/min per mg of protein)
—	54 (100%)
Non-immune	50 (93%)
Anti- α_3	39.6 (73%)
Anti- β_1	66.8 (124%)

activity of the A system, and not the ASC system, is increased by α_3 transfection (results not shown).

Similarly, RD cells transfected with α_3 cDNA showed dramatic increases in AIB transport (Table 3), whereas cells transfected with α_2 showed only small increases in AIB uptake. Although there was day-to-day variation in the degree of AIB uptake by transfected and untransfected RD cells, it is significant that transfection with α_3 and not α_2 produced a dramatic increase in transport. Based on results from three experiments, there was a 2.08 ± 0.24 -fold increase in AIB uptake with α_3 transfection compared with α_2 transfection.

Immunoprecipitation of A-system transport with anti-integrin antibodies

That the α_3 component may influence AIB transport is also shown in Table 4. It may be seen that an antibody against the α_3 subunit of $\alpha_3\beta_1$, but not the β_1 subunit, is capable of immunodepleting transport activity. In two experiments, treatment of solubilized membranes with 20 μ l of anti- α_3 serum led to 45% and 37% decreases in Na⁺-dependent amino acid transport activity, compared with a non-immune-serum-treated control. In contrast, treatment with 20 μ l of anti- β_1 serum decreased transport activity by only 5%. Since a component of non-immune serum itself inhibits reconstituted amino acid transport (results not shown), possible interference from this component was avoided by removing IgG from serum by preincubation with Protein A–Sepharose (Table 4). In two separate experiments, anti- α_3 IgG from 30 μ l of serum decreased transport activity by 26%, whereas an identical quantity of anti- β_1 IgG had no effect on transport activity (91% and 124% of control activity). In separate immunoprecipitation experiments (results not shown),

it was observed that, unlike-anti β_1 , anti- α_3 immunoprecipitated only a small fraction of α_3 from the solubilized membranes.

DISCUSSION

In the present study the major peptide components of an OG extract from Ehrlich cell membranes, enriched in the A-system transporter, have been identified as the integrin $\alpha_3\beta_1$. Although the possibility that the co-purification of $\alpha_3\beta_1$ and Na⁺-dependent amino acid transport may be fortuitous has not been rigorously eliminated, the data suggest the more likely possibility that the integrin may be involved in the transport process defined as the A system. Purification of the OG extract resulted in a substantial, although variable, increase in transport activity and a decrease in, but not the entire elimination of, $\alpha_3\beta_1$ from the active fraction. In this further purified transport-competent extract two major components were detected, a 121 kDa peptide and a 55 kDa peptide (activity unknown). Despite the virtual elimination of the α_3 integrin and the substantial decrease in the β_1 integrin during purification, transport activity was enriched, suggesting that the integrin is not directly involved in amino acid transport. Nonetheless, since treatment of the total solubilized membrane fraction with anti- α_3 IgG did remove some of the transport activity, an association of α_3 with the amino acid transporter is implicated. Treatment of solubilized membranes with anti- β_1 antibody failed to diminish the transport activity, although anti- β_1 removed a larger fraction of β_1 than the fraction of α_3 removed by anti- α_3 . More compelling evidence for a potential role for integrins in amino acid transport came from α_3 -cDNA-transfected K₅₆₂ and RD cells. These cells showed increased A-system transport compared with wild-type cells, which do not express α_3 . In the case of α_3 -transfected RD cells the increase in uptake over untransfected cells was 3.3 ± 1.1 -fold, 2.1 times greater than that after transfection with α_2 (three experiments). The integrin-transporter association shows specificity, since α_2 cDNA transfection had much less (RD cells) or no (K₅₆₂ cells) effect. $\alpha_3\beta_1$ appears to interact only with the A system in K₅₆₂ cells, since other transporters (glucose transporter and the ASC system) were unaffected by the transfection.

A characteristic of the transporter which changed during the course of purification by lectin columns was the loss of the strict Na⁺-dependence of Me-AIB-sensitive transport. Thus AIB uptake in K⁺ in the NAG eluate from the wheat-germ column rose by 15-fold, but not all the activity in K⁺ was Me-AIB sensitive. AIB uptake in proteoliposomes prepared from the NAG eluate was more sensitive to Me-AIB in the presence of Na⁺ than in K⁺. The increased uptake in K⁺ relative to the Na⁺-dependent component may be responsible for the fact that an unambiguous Na⁺-dependent component of AIB transport in the NAG eluate was variable. Loss of the Na⁺-dependence of AIB transport has also been observed when the OG extract is purified on hydr-

oxyapatite columns (J. I. McCormick and R. M. Johnstone, unpublished work).

This is not the first example of a loss of cation-dependence during transporter purification. A similar phenomenon has also been reported for a Na⁺-dependent neutral amino acid transporter purified from renal brush border membranes [38]. This isolated brush border protein has a similar molecular size (132 kDa) to the amino acid transporter originally isolated from Ehrlich cells (120–130 kDa) [18]. Recently the 132 kDa peptide isolated from renal brush border membranes has been identified as aminopeptidase N [39]. The authors conclude [39] that the peptide does not itself transport amino acids, but suggest that it plays a role in transport, possibly that of 'channelling' amino acids to the transporter binding site. In fact, binding of amino acids to aminopeptidase N has been demonstrated [39].

There are structural and functional similarities between aminopeptidase N and the integrin $\alpha_3\beta_1$. Aminopeptidase N is an ectoenzyme [39,40] consisting of a large extracellular domain linked by a single transmembrane domain to a small intracellular N-terminal domain, and is classified as a Type II membrane glycoprotein [41]. Aminopeptidase N is an abundant protein in intestinal and renal brush border membranes [39], but it has also been reported to be a cell surface protein on human myeloid [42] and melanoma [43] cells. In the latter case [43] the protein is tightly associated with the extracellular matrix, and the target for aminopeptidase N was tentatively identified as entactin. The integrins α_3 and β_1 are Type I membrane proteins [41] with N-terminal domains external to the cell [34,44]. Integrins are a family of proteins involved in cell–cell and cell–extracellular-matrix interactions [34], and $\alpha_3\beta_1$ has been reported to bind to the extracellular matrix proteins epiligrin [32,34] and entactin [46]. Thus both aminopeptidase N and $\alpha_3\beta_1$ can interact with the same extracellular matrix protein, and both proteins are associated with Na⁺-dependent amino acid transport in different tissues. Other Type II membrane glycoproteins have been identified as possible modulators or activators of Na⁺-independent transport (System b^{0,+}) in kidney and intestine [47–49]. It appears that the activity of both Na⁺-dependent and Na⁺-independent amino acid transporters may be modified by single-transmembrane-domain polypeptides.

The integrin-depleted NAG-eluted fraction from the wheat-germ column, when reconstituted, shows characteristics of A-system transport, i.e. Na⁺-dependence and inhibition by Me-AIB. Overall, the data are consistent with the conclusion that A-system transport may be modulated by the $\alpha_3\beta_1$ integrin, and particularly by the α_3 subunit. As to the identity of the amino acid carrier in the active extract, further characterization of the peptides present in the purified fraction described here is in progress.

This work was supported by a grant (M1984) from the Medical Research Council of Canada. Our thanks are due to Dr. Martin Hemler for the anti- α_3 and - β_1 antibodies and the transfected cell lines. We also thank Dr. R. Hynes for an anti- β_1 antibody, and Marlene Gilhooly for preparing the manuscript.

REFERENCES

- Christensen, H. N. (1990) *Physiol. Rev.* **70**, 43–77
- Shotwell, M. A., Kilberg, M. S. and Oxender, D. L. (1983) *Biochim. Biophys. Acta* **737**, 267–284
- Stevens, B. R., Kaunitz, J. D. and Wright, E. M. (1984) *Annu. Rev. Physiol.* **46**, 417–433
- Amara, S. G. and Kuhar, M. J. (1993) *Annu. Rev. Neurosci.* **16**, 73–93
- Doyle, F. A. and McGivan, J. D. (1992) *Biochim. Biophys. Acta* **1104**, 55–62
- Kanner, B. I. and Schuldiner, S. (1987) *CRC Crit. Rev. Biochem.* **22**, 1–38
- Kanai, V. and Hediger, M. A. (1992) *Nature (London)* **360**, 467–471
- Brew, H. and Attwell, D. (1987) *Nature (London)* **327**, 707–709
- Guastella, J., Nelson, N., Nelson, H., et al. (1990) *Science* **249**, 1303–1306
- Smith, K. E., Borden, L. A., Hartig, P. R., Branchek, T. and Weinshank, R. L. (1992) *Neuron* **8**, 927–935
- Freneau, R. T., Jr., Caron, M. G. and Blakely, R. D. (1992) *Neuron* **8**, 915–926
- Pines, G., Danbold, N. C., Björås, M., et al. (1992) *Nature (London)* **360**, 464–467
- Storck, T., Schulte, S., Hofmann, K. and Stoffel, W. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10955–10959
- Kanai, Y., Smith, C. P. and Hediger, M. A. (1993) *Trends Neurosci.* **16**, 365–370
- Shafiqat, S., Tamarappoo, B. K., Kilberg, M. S., Puranam, R. S., McNamara, J. D., Guadaso-Ferraz, A. and Freneau, R. T., Jr. (1993) *J. Biol. Chem.* **268**, 15351–15355
- Arriza, J. L., Kavanaugh, M. P., Fairman, W. A., Wu, Y.-N., Murdoch, G. H., North, R. A. and Amara, S. G. (1993) *J. Biol. Chem.* **268**, 15329–15332
- McCormick, J. I., Tsang, D. and Johnstone, R. M. (1984) *Arch. Biochem. Biophys.* **231**, 355–365
- McCormick, J. I. and Johnstone, R. M. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7877–7881
- McCormick, J. I., Jetté, M., Potier, M., Béliveau, R. and Johnstone, R. M. (1991) *Biochemistry* **30**, 3704–3709
- Lin, G., McCormick, J. I. and Johnstone, R. M. (1994) *Arch. Biochem. Biophys.* **312**, 308–315
- Marcantonio, E. E. and Hynes, R. O. (1988) *J. Cell Biol.* **106**, 1765–1772
- Johnson, P. A. and Johnstone, R. M. (1982) *Membr. Biochem.* **4**, 189–218
- Bardin, C. and Johnstone, R. M. (1978) *J. Biol. Chem.* **253**, 1725–1732
- Colombini, M. and Johnstone, R. M. (1973) *Biochim. Biophys. Acta* **323**, 69–86
- McCormick, J. I., Silvius, J. R. and Johnstone, R. M. (1985) *J. Biol. Chem.* **260**, 5706–5714
- McCormick, J. I. and Johnstone, R. M. (1988) *J. Biol. Chem.* **263**, 8111–8119
- Kaplan, R. S. and Pedersen, P. L. (1985) *Anal. Biochem.* **150**, 97–104
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Bollag, D. M. and Edelman, S. J. (1991) in *Protein Methods*, p. 58, Wiley-Liss Inc., New York
- Peterson, G. L. (1983) *Methods Enzymol.* **91**, 95–121
- Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Weitzman, J. B., Pasqualin, R., Takada, Y. and Hemler, M. E. (1993) *J. Biol. Chem.* **268**, 8651–8657
- Hynes, R. O. (1992) *Cell* **69**, 11–25
- Hemler, M. E. (1990) *Annu. Rev. Immunol.* **8**, 365–400
- Barber, E. F., Handlogten, M. E. and Kilberg, M. S. (1983) *J. Biol. Chem.* **258**, 11851–11855
- Takada, Y., Strominger, J. L. and Hemler, M. E. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3239–3243
- Zheng, M., Fang, H. and Hakomori, S. (1994) *J. Biol. Chem.* **269**, 12325–12331
- Doyle, F. A. and McGivan, J. D. (1992) *Biochem. J.* **281**, 95–102
- Plakidou-Dymock, S., Tanner, M. J. and McGivan, J. D. (1993) *Biochem. J.* **290**, 59–65
- Hussain, M. M., Trantum-Jensen, J., Noren, O., Sjöström, H. and Christiansen, K. (1981) *Biochem. J.* **199**, 179–186
- Singer, S. J. (1992) *J. Membr. Biol.* **129**, 3–12
- Look, A. T., Ashmun, R. A., Shapiro, L. H. and Peiper, S. C. (1989) *J. Clin. Invest.* **83**, 1299–1307
- Menrad, A., Speicher, D., Wacker, J. and Herlyn, M. (1993) *Cancer Res.* **53**, 1450–1455
- Takada, Y., Murphy, E., Pil, P., Chen, C., Ginsberg, M. H. and Hemler, M. E. (1991) *J. Cell Biol.* **115**, 257–266
- Carter, W. G., Ryan, M. C. and Gahr, P. J. (1991) *Cell* **65**, 599–610
- Dedhar, S., Jewell, K., Rojiani, M. and Gray, V. (1992) *J. Biol. Chem.* **267**, 18908–18914
- Wells, R. G. and Hediger, M. A. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5596–5600
- Bertran, J., Werner, A., Moore, M. L., et al. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5601–5605
- Bertran, J., Werner, A., Chillarón, J., et al. (1993) *J. Biol. Chem.* **268**, 14842–14849