Biochimica et Biophysica Acta 904 (1987) 29–35
Elsevier

BBA 73730

Multicomponent analysis of amino acid transport System L in normal and virus-transformed fibroblasts

Stefano A. Gandolfi [c], Jeanette Anne Marie Maier [a,*], Pier Giorgio Petronini [a], Kenneth P. Wheeler [b] and Angelo F. Borghetti [a]

[a] *Istituto di Patologia Generale,* [c] *Istituto di Oftalmologia Università di Parma, Parma (Italy) and* [b] *School of Biological Sciences, University of Sussex, Brighton (U.K.)*

(Received 28 April 1987)

Key words: Amino acid transport; Kinetics; Viral transformation; (3T3 cell); (Simian virus transformed cell)

Amino acid transport System L in both normal Balb/c 3T3 cells and in those transformed with simian virus 40 (SV 3T3) was analysed kinetically under two different experimental conditions. Under 'zero-*trans*' conditions the results for both types of cell could be interpreted satisfactorily in terms of System L consisting of two components (L1 and L2) characterized by different K_m values. This conclusion is in agreement with previous reports. However, under 'infinite-*trans*' conditions, the experimental data could not be accounted for in terms of only two components; the introduction of a third component (L3) was necessary to provide a satisfactory fit. Viral transformation affects only the L1 component, either by modification or by replacement, giving it a higher 'affinity' (lower K_m) but a lower 'capacity' (lower V_{max}).

Introduction

The amino acid transport System L is widespread and transports, particularly by transmembrane exchange, neutral amino acids with branched or cyclic side chains [1,2]. Its activity is not related to the presence of Na^+ outside the cell [3] but seems to be affected by physiological concentrations of Na^+ in the cell cytoplasm [4,5]. Several observations indicate that there are differences in the nature of System L in various cells. For example, it appears to be a simple, homogeneous system in a hepatoma cell line [6] and in normal and leukemic human lymphocytes [7,8], whereas the presence of both low- and high-affinity components of System L has been proposed for rat hepatocytes [6,9] and SV 3T3 cells [10]. Petronini et al. [10], although in agreement with the hypothesis of two components, pointed out the possibility of kinetic complexity, such as negative cooperativity.

Since the two components of System L in SV 3T3 cells showed different sensitivities to experimental conditions such as cell density or the presence of Na^+ inside the cells [4,10], we have studied the effect of simian virus 40-induced transformation on the kinetic properties of System L in 3T3 cells. The activity of System L was assayed by measuring the uptake of L-phenylalanine which, in the absence of Na^+ in the '*cis*' position [11], is selectively transported by System L in both 3T3 and SV 3T3 cells [12].

* Present address: Istituto di Patologia Generale, Università di Brescia, Brescia, Italy.

Correspondence: A.F. Borghetti, Istituto di Patologia Generale, Università di Parma, I-43100 Parma, Italy.

Experimental

Cell culture

Monolayers of Balb/c 3T3 cells and those transformed by simian virus 40 (SV 3T3) were maintained in culture and propagated as previously described [10,12]. Cells were usually passaged twice a week.

Incubation and uptake assay

Measurement of amino acid uptake by cell monolayers was performed as described by Gazzola et al. [13]. The cells were always seeded at high density because both SV 3T3 cells [10] and 3T3 cells (Gandolfi, S.A. and Petronini, P.G. unpublished results) show density-dependent regulation of System L activity when reloaded with the appropriate amino acids. Depletion of the intracellular pool of amino acids ('zero-*trans*' condition) and subsequent reloading ('infinite-*trans*' condition) were achieved as described in detail previously [4,10]. Amino acid influx was assayed under conditions approaching initial rate (30 s incubation) in a Na^+-free Earle's solution, as described elsewhere [10]. Cell monolayers were dissolved in 0.5 M NaOH and samples were taken for protein determination by the method of Lowry et al. [14]. The volumes of both normal and transformed cells still attached to the substratum were calculated as previously described [10,12].

Calculations

The rate of uptake of phenylalanine is expressed as μmol/ml of intracellular water per min. The kinetic parameters of amino acid transport were calculated as described previously [12], but with the use of a wider range of substrate concentrations (34 nM–40 mM). The results relating rate of uptake to the external concentration of amino acid were corrected for the non-saturable component (which was almost identical in 3T3 and SV 3T3 cells) and then analysed by the Eadie-Hofstee method. The values of kinetic parameters giving best fit to the experimental data were derived by computer analysis. When curvilinear plots were obtained, the assumption was made that two independent Michaelis-Menten components contributed to the transport [15]. The method of parameter fitting developed by Feldman [16] was used for this analysis.

Materials

L-Phenyl[2,3-^{3}H]alanine was obtained from Amersham and the unlabelled amino acid from Sigma. The amino acid analogue 2-aminobicyclo-(2,2,1)heptane-2-carboxylic acid was purchased from Calbiochem-Behring. Media and foetal calf serum for culturing the cells were obtained from Gibco.

Results and Discussion

The kinetic parameters for phenylalanine transport were determined under 'zero-*trans*' and 'infinite-*trans*' conditions [11]. In cells depleted of amino acids ('zero-*trans*') a hyperbolic Eadie-Hofstee plot was obtained that could be resolved into two Michaelis-Menten components (L1 and L2) with different K_m values. This solution was able to fit all the experimental data satisfactorily for both 3T3 and SV 3T3 cells (Fig. 1). Note that both the V_{max} and the K_m values of the 'high-affinity' component (L1) in transformed cells were an order of magnitude lower than in normal cells (Table I). This variation cannot be ascribed to differing extents of exchange occurring in the two types of cells because, after the 60 min depletion period, the intracellular concentrations of relevant amino acids, such as phenylalanine, leucine and methionine, were virtually identical in 3T3 and SV 3T3 cells (data not shown).

TABLE I

KINETIC PARAMETERS OF PHENYLALANINE INFLUX UNDER 'ZERO-*TRANS*' CONDITIONS RESOLVED INTO TWO COMPONENTS

The units are μmol/ml intracellular water per min for V_{max} and mM for K_m. The data from experiment A are plotted in Fig. 1.

Expt.	Component	3T3 cells		SV 3T3 cells	
		V_{max}	K_m	V_{max}	K_m
A	L1	0.4	0.02	0.04	0.001
	L2	0.5	1.3	1.3	2.3
B	L1	0.5	0.02	0.06	0.001
	L2	2.5	3.0	2.0	1.6

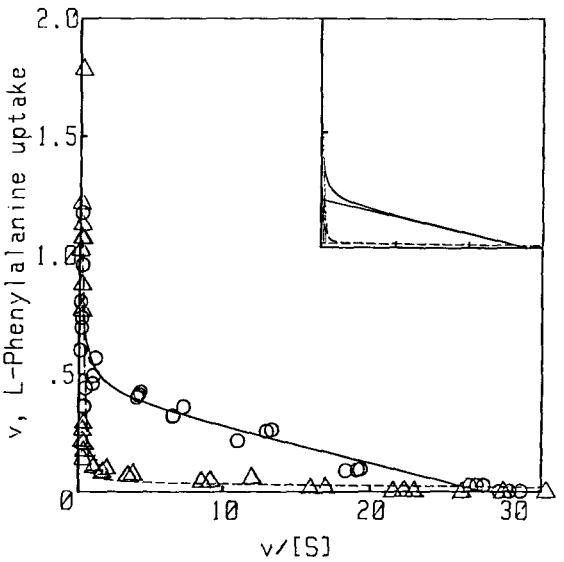

Fig. 1. Kinetic analysis of phenylalanine influx under 'zero-*trans*' conditions. 3T3 (○) or SV 3T3 (△) cells were incubated for 60 min to deplete them of endogenous amino acids. Then phenylalanine influx into the cells was measured and corrected for the non-saturable component as described. Extracellular concentrations of phenylalanine ranging from 34 nM to 40 mM were tested. The main graph shows the data analysed according to the Eadie-Hofstee method, whilst the inset shows how each curve can be resolved into two linear plots, each equivalent to a simple Michaelis-Menten component. (Solid lines represent 3T3 cells and broken lines SV 3T3 cells.)

These findings are unusual because most of the reported changes in amino acid transport induced by transformation have involved a changes in 'capacity' (V_{max}) rather than 'affinity' (K_m). However, Boerner and Saier [17] found that chemical transformation of MDCK cells changed the kinetics of System A such that non-linear Eadie-Hofstee plots resulted for the transformed cells. They concluded that transformation had produced either a new, kinetically distinct, form of System A, or something that was modifying the existing system. Segel and co-workers [7,8], whilst emphasizing the dramatic decrease in V_{max} of System L in lymphocytes present in chronic lymphocytic leukaemia, compared with normal lymphocytes, also noted an apparent increase in K_m. However, its significance was not clearly established. Hence, as far as we are aware, the results reported here give the first evidence of a change in K_m of an amino acid transport system induced by viral transformation of a mammalian cell.

There were no significant differences in the values of either V_{max} or K_m of the 'low-affinity' component (L2) between 3T3 and SV 3T3 cells (Table I).

Exactly parallel results were obtained in a single experiment in which leucine was used instead of phenylalanine. The data again could be resolved satisfactorily in terms of L1 and L2 components, with a similar pattern between normal and transformed cells (data not shown).

The kinetic parameters for phenylalanine transport under 'infinite-*cis*' conditions were then calculated. As shown in Fig. 2, a two-component Eadie-Hofstee plot does not properly fit all the experimental points for either 3T3 or SV 3T3 cells. Indeed, the computer analysis described too broad a range of possible curves to prove that the parameters obtained were reasonable. The apparent discrepancy between this finding and that reported previously for SV 3T3 cells (see Fig. 6 and Table III of Ref. 10) presumably can be

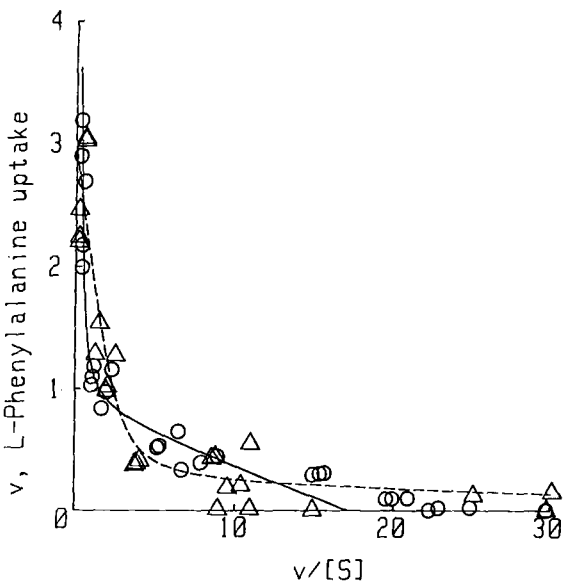

Fig. 2. Two-component kinetic analysis of phenylalanine influx under 'infinite-*trans*' conditions. 3T3 (○) or SV 3T3 (△) cells were first depleted of endogenous amino acids by incubating them for 60 min in the absence of extracellular amino acids. Then they were incubated for 15 min in Earle's solution supplemented with 1 mM unlabelled phenylalanine, giving a saturating concentration of the amino acid inside both types of cell. Phenylalanine influx and analysis of the results were then carried out exactly as described in the legend to Fig. 1.

attributed to the wider range of substrate concentrations tested here.

In drawing attention to the uncertainties of curve-fitting, Christensen [18] pointed out that analysis of a hyperbolic Eadie-Hofstee plot in terms of only two components does not always accurately describe the kinetics of a transport system. Our finding is clearly an example of such a failure and, by simple visual inspection of Fig. 2, it seems possible that analysis in terms of three Michaelis-Menten components could provide a better fit to the experimental data. We therefore, made such an analysis, using groups of experimental points that appeared to follow simple kinetics (i.e., 34 nM–50 μM, 50 μM–1 mM, 1 mM–40 m). The results, plotted in Fig. 3 and summarised in Table II, show that such analysis does provide a better and more significant fit to the experimental data. The three components obtained can be described as 'low-affinity' (L2), 'high-affinity' (L3) and 'very-high-affinity' (L1) components. The K_m of L1 is comparable to that obtained for L1 under 'zero-*trans*' conditions, although the V_{max} is greater because of the exchange process (*trans* stimulation) occurring under 'infinite-*trans*' conditions [10]. Also, the L1 component seems to be affected by viral transformation in a way similar to that shown by L1 measured under 'zero-*trans*' conditions, although the change is smaller under 'infinite-*trans*' conditions and we cannot be sure that it is significant. The other two components, L2 and L3, detected under 'infinite-*trans*' conditions have very similar kinetic parameters in 3T3 and SV 3T3 cells, so that negative cooperativity between L1 and L3 is unlikely. (It should be noted that the absolute values of these kinetic parameters do vary somewhat from experiment to experiment. We are concerned here mainly with orders of magnitude and the possible interpretations of the non-linear Eadie-Hofstee plots that are consistently obtained.) Parallel results were obtained in a single experiment with leucine instead of phenylalanine as the transported amino acid (data not shown).

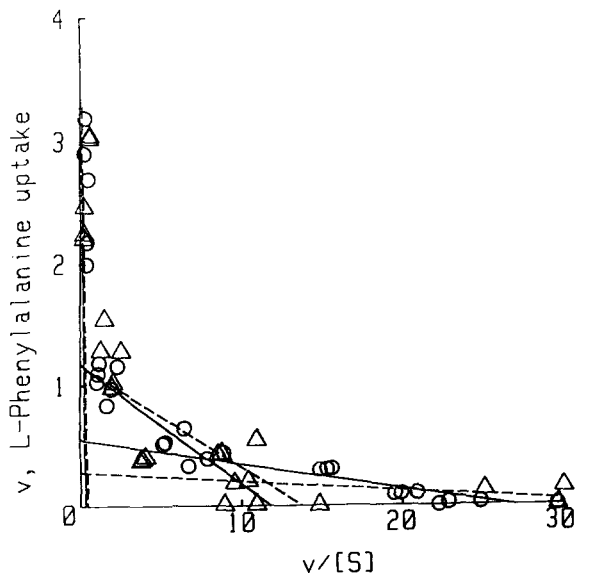

Fig. 3. Resolution of phenylalanine influx under 'infinite-*trans*' conditions into three kinetic components. The data from Fig. 2 are shown after resolution of each curve into three linear plots, each corresponding to a simple Michaelis-Menten component, as described in the text. (Symbols have the same meaning as in Figs. 1 and 2.)

TABLE II

KINETIC PARAMETERS OF PHENYLALANINE INFLUX UNDER 'INFINITE-*TRANS*' CONDITIONS RESOLVED INTO THREE COMPONENTS

V_{max} is expressed as μmol/ml intracellular water per min and K_m as mM. The data from experiment A are plotted in Fig. 2 and 3.

Expt.	Component	3T3 cells		SV 3T3 cells	
		V_{max}	K_m	V_{max}	K_m
A	L1	0.6	0.02	0.3	0.01
	L2	4.0	12	4.8	10
	L3	1.2	0.1	1.2	0.1
B	L1	0.6	0.03	0.3	0.01
	L2	4.3	14	5.8	21
	L3	1.3	0.1	1.3	0.1

The results shown in Fig. 4 support the analysis given above. The influx of phenylalanine when present externally at 5 μM (a concentration approaching the K_m of the L1 component) was higher in 3T3 than in SV 3T3 cells both before and after the 'infinite-*trans*' condition had been reached (Fig. 4a). In contrast, after reaching the 'infinite-*trans*' condition, the uptake of phenylalanine from an external concentration of 100 μM (about the K_m of L3) was not significantly different in the two types of cells (Fig. 4b). In the

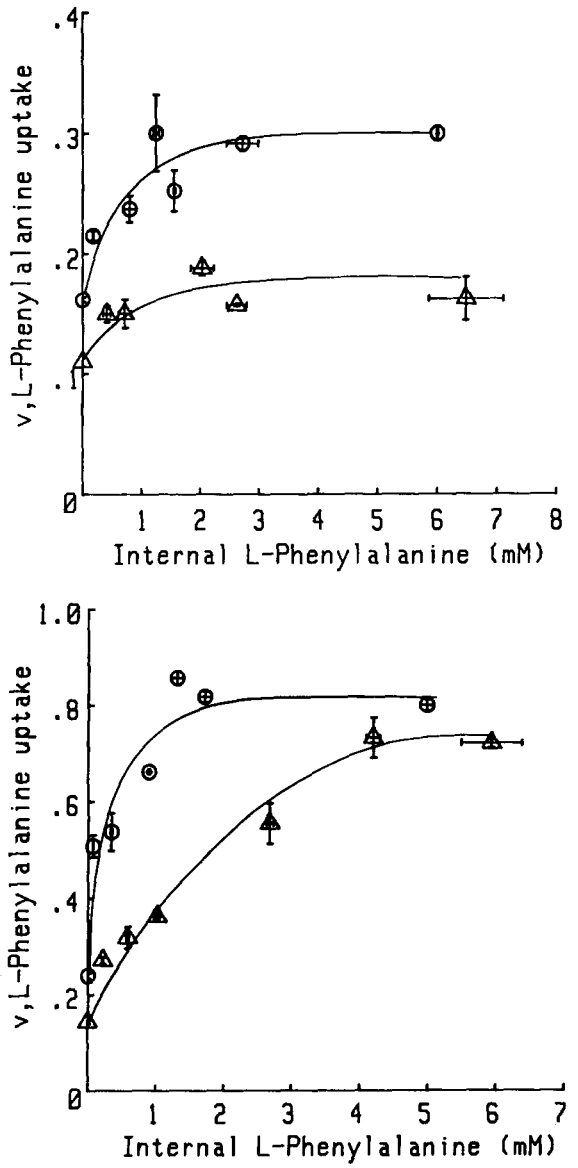

Fig. 4. Effects of intracellular and extracellular concentrations of phenylalanine on its influx into normal and transformed cells 3T3 (○) and SV 3T3 (△) cells were loaded with various different concentrations of unlabelled phenylalanine, as described previously [10], and then influx was measured with an extracellular concentration of either (a) 5 μM or (b) 100 μM labelled phenylalanine.

former situation, the L1 component predominates at all intracellular concentrations of phenylalanine tested, from 'zero-*trans*' to 'infinite-*trans*' conditions. In the latter situation, the L1 component predominates only under 'zero-*trans*' conditions because, as the '*trans*' concentration of phenylalanine increases, the L3 component becomes progressively more involved until the difference between normal and transformed cells is negligible.

The analysis given above, although suggestive, is not sufficient to prove the existence of a third component (L3) of the transport system. To gain more evidence, either for or against our interpretation, we therefore tested the inhibitory action of the substrate analogue 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid [19] on phenylalanine influx into SV 3T3 cells under conditions where only one of the proposed L1 or L3 components would be the dominating transporter. As shown in Fig. 5, the substrate analogue appeared to inhibit phenylalanine influx via the L3 component in a competitive manner, its K_i value being approximately 0.4 mM. However, inhibition of phenylalanine influx via the L1 component proved to be

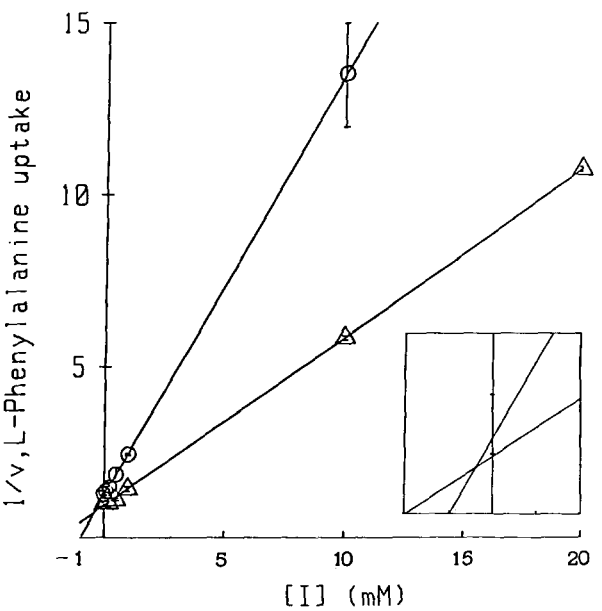

Fig. 5. Competitive inhibition by 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid of phenylalanine influx via the L3 component of System L in SV 3T3 cells. Phenylalanine influx into SV 3T3 cells under 'infinite-*trans*' conditions was measured, as described in the text, with an extracellular concentration of either 0.1 mM (○) or 0.2 mM (△) in the presence of the indicated concentrations of 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid. The results are given as a 'Dixon plot' and each point shows the mean (± S.D.) of three separate measurements. (The inset shows the same graph reproduced on a reduced scale without the points for clarity.)

non-competitive, the K_i value being about 0.3 mM (Fig. 6). Hence, both biological (transformation by SV 40) and chemical (inhibition by 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid) tests indicate that System L in 3T3 cells is heterogeneous.

Previously, we reported that System L in SV 3T3 cells slightly decreased its apparent 'affinity' for phenylalanine and leucine as the cells accumulated such amino acids. This phenomenon was restricted to the 'high-affinity' component, L1 [10]. The more detailed study described here confirms and extends that observation. The results in Table II and in Fig. 4b suggest that, as soon as the cells accumulate phenylalanine, a new component, L3, characterized by a K_m value intermediate between L1 and L2, can be detected. At present it is possible only to speculate about the possible physiological importance of these kinetically distinguishable components. L3 would constitute the main transport pathway when extracellular concentrations of amino acids are about the same as those in human blood [7,8]. With very low concentrations of external amino acids, L1 would be the dominating component and the marked decrease in its K_m under 'zero-*trans*' conditions after viral transformations of the cells (Table I) may be important. A tumour nodule has a high cell density but precarious blood supply, so that the cellular pool of amino acids may be easily depleted. This situation is closer to the experimentally induced 'zero-*trans*' condition than to the normal physiological 'infinite-*trans*' condition so that the greater 'affinity' of L1 in transformed cells could confer a selective advantage to the malignant cells in vivo. This hypothesis, which is similar to that proposed by White et al. [20] with respect to the glucose transport system, merits further study.

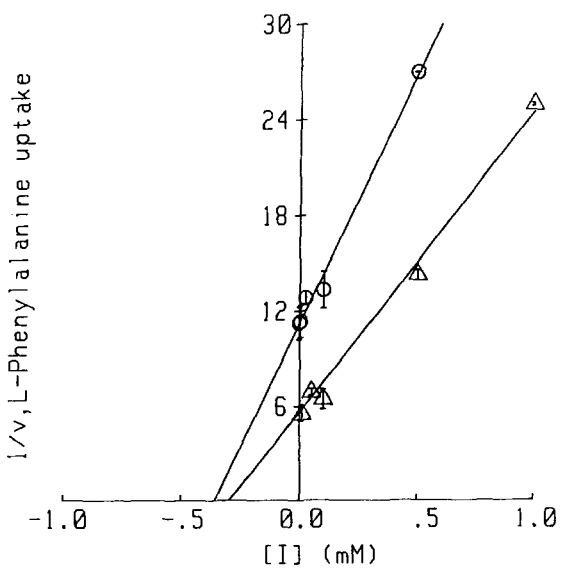

Fig. 6. Noncompetitive inhibition by 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid of phenylalanine influx via the L1 component of System L in SV 3T3 cells. The methods and analysis were exactly as described in the legend to Fig. 5, except that influx was measured with extracellular phenylalanine concentrations of 5 μM (○) and 10 μM (△).

Acknowledgements

This work has been supported by grants from the Consiglio Nazionale delle Ricerche 'Progetto finalizzato Oncologia n. 85.02047.44', 'Gruppo Nazionale Struttura e Funzione di Macromolecole Biologiche' by grants from the Ministero della Pubblica Istruzione 40% and 60%, Rome, and by the Associazione Italiana per la Ricerca sul Cancro, Milan. S.A.G. is a recipient of a fellowship from the Lega Italiana per la Lotta contro i Tumori.

References

1 Guidotti, G.G., Borghetti, A.F. and Gazzola, G.C. (1978) Biochim. Biophys. Acta 515, 329–366
2 Christensen, H.N. (1975) Biological Transport, Benjamin Inc., London
3 Oxender, D.L. and Christensen, H.N. (1963) J. Biol. Chem. 230, 3686–3699
4 Petronini, P.G., Gandolfi, S.A. and Borghetti, A.F. (1985) Biochim. Biophys. Acta 815, 361–368
5 Gandolfi, S.A., Petronini, P.G. and Borghetti, A.F. (1985) Biochem. Soc. Trans. 13, 702–703
6 Weissbach, L., Handlogten, M.E., Christensen, H.N. and Kilberg, M.S. (1982) J. Biol. Chem. 257, 12006–12011
7 Segel, G.B. and Lichtmann, M.A. (1982) J. Biol. Chem. 257, 9255–9257
8 Segel, G.B., Simon, W. and Lichtmann, M.A. (1984) J. Clin. Inv. 74, 17–24
9 Handlogten, M.E., Weissbach, L. and Kilberg, M.S. (1982) Biochem. Biophys. Res. Commun. 104, 307–313
10 Petronini, P.G., Piedimonte, G. and Borghetti, A.F. (1982) Biochim. Biophys. Acta 693, 13–21
11 Stein, W.D. (1981) in Membrane Transport (Bonting, S.L., and De Pont, J.J.H.H.M., eds.), pp. 125–158, Elsevier, Amsterdam

12 Borghetti, A.F., Piedimonte, G., Tramacere, M., Severini, A., Ghiringhelli, P. and Guidotti, G.G. (1980) J. Cell. Physiol. 105, 39–49
13 Gazzola, G.C., Dall'Asta, V., Franchi-Gazzola, R. and White, M. (1981) Anal. Biochem. 115, 368–373
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
15 Borghetti, A.B., Tramacere, M., Ghiringhelli, P., Severini, A. and Kay, J.E. (1981) Biochim. Biophys. Acta 646, 218–230
16 Feldman, H.A. (1972) Anal. Biochem. 48, 317–338
17 Boerner, P. and Saier, M.H. (1985) J. Cell. Physiol. 122, 308–315
18 Christensen, H.N. (1985) J. Membrane Biol. 84, 97–103
19 Tager, H.S. and Christensen, H.N. (1971) J. Biol. Chem. 246, 7572–7580
20 White, M.K., Bramwell, M.E. and Harris, H. (1981) Nature 294, 232–235