Volume 314, number 1, 20–22 FEBS 11819 December 1992

Na^+-dependent AIB transport by neuroblastoma cells

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Received 13 October 1992

Na^+-dependent amino isobutyric acid transport by two neuroblastoma cell lines with and without amplification of the oncogene N-*myc* is studied. Surprisingly, the contribution of system A is greater in the cell line showing no N-*myc* amplification. Preliminary data support a role for essential tyrosine and cysteine residues in the active center of the carriers, mainly in system A.

Neuroblastoma; AIB transport; System A; System ASC; N-*myc* oncogene

1. INTRODUCTION

Cells need to continuously exchange matter, energy, and information to maintain the characteristic negentropy of life [1]. The flow of amino acids through the plasma membrane of eukaryotic cells is driven mainly by carrier proteins. The incorporation of amino acids against a concentration gradient is usually coupled to a favourable Na^+ gradient, which acts as the driving force [2]. The two Na^+-dependent amino acid transport systems first described and, indeed, the most conspicuous ones in very different types of eukaryotic cells are the so-called systems A and ASC [3,4]. The expression of system A is usually repressed in normal cells, but it is highly enhanced in neoplastic or proliferant cells [5].

In the present report, Na^+-dependent transport of amino acids by neuroblastoma cells is studied by using the analog amino isobutyric acid (AIB). The actual contribution of both systems A and ASC has been investigated in two neuroblastoma cell lines with different expression levels of the N-*myc* oncogene.

2. MATERIALS AND METHODS

2.1. *Materials*

Culture dishes were from Falcon (Oxnard, CA, USA). Twelve-well tissue-culture cluster dishes were from Costar (Cambridge, MA, USA). RPMI-1640 medium, fetal calf serum, and antibiotics were from Gibco (Eggenstein, Germany). 2-Amino[1-^{14}C]isobutyric acid (1.85 GBq/mmol) was from Amersham (Braunschweig, Germany). Ecolume liquid scintillation counting solution was supplied by ICN (Meckenheim, Germany). All other reagents were from Sigma (Deisenhofen, Germany).

2.2. *Neuroblastoma cell lines*

Two well-established cell lines were used: SH-EP (a homogeneous SK-N-SH-derived subline), and IMR-32, which carry 1, and 25 copies of N-*myc* oncogene, respectively. Cell culture conditions have been described elsewhere [6].

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2.3. *Transport in culture cells*

The cells used for transport experiments were seeded into twelve-well cluster dishes and they were used when confluent. The buffered medium used in the transport was 137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM HEPES adjusted to pH 7.4 with Tris. Cells were washed in this medium and transport was initiated by adding 1 ml of the above medium containing 0.1 mM AIB (with 0.2 μCi/ml of the labelled amino acid) at room temperature (20°C). The uptake was terminated by removing the transport medium and washing with 3 × 1 ml aliquots of ice-cold 137 mM NaCl, 10 mM Tris-HEPES, pH 7.4. Plates were drained and 0.3 ml 0.5% Triton X-100 added, cells were then removed with a cell scraper and resuspended in this medium. 250-μl samples were dissolved in 5 ml scintillation solution for counting and protein was determined using the method of Bradford [7]. Na^+-independent uptake was performed using 137 mM choline chloride instead of NaCl in the transport medium.

In the experiments designed to identify essential groups, cells were preincubated for 1 h in the presence of 0.17 mM 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), for 15 min in the presence of 0.5 mM *para*-chlorobenzenesulfonate (PCMBS), or for 2 min in the presence of 50 mM phenylglyoxal.

3. RESULTS AND DISCUSSION

3.1. *AIB transport in two neuroblastoma cell lines*

Pioneer work by Christensen led years ago to the proposition that abnormally strong amino acid accumulation is characteristic of neoplasia [8]. This increased accumulation could be achieved either by increased affinities and capacities of the transport systems, and/or by enhanced expression of system A, usually repressed in normal non-proliferant cells [5].

Neuroblastoma cell lines carrying different numbers of copy for the N-*myc* oncogene have been established. The N-*myc* oncogene is frequently amplified in advanced stages, but not in initial stages of human neuroblastoma [9], and N-*myc* amplification (with correspondingly enhanced N-*myc* expression) correlates with clinical outcome [10]. This fact makes neuroblastoma

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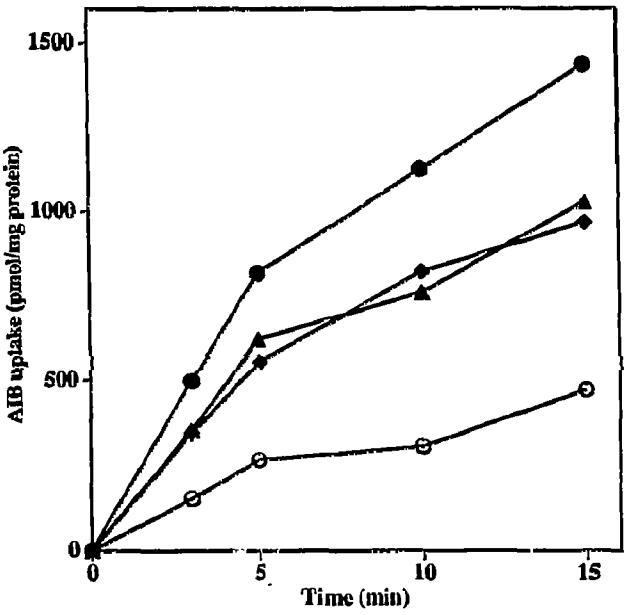

Fig. 1. Time course of AIB uptake by SH-EP cells. Transport experiments were carried out in NaCl buffer without (●) and with 5 mM threonine added (▲), as well as in choline chloride buffer (○), as described in section 2. Total Na^+-dependent AIB transport is also depicted (◆). Representative results are shown.

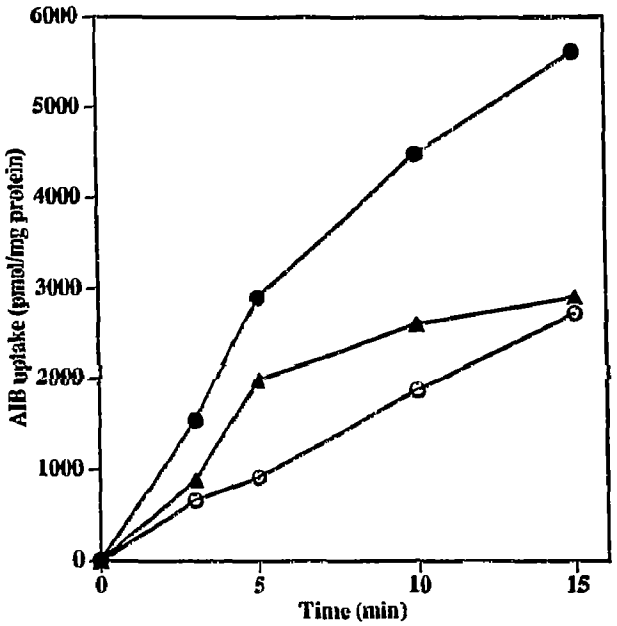

Fig. 2. Time course of AIB uptake by IMR-32 cells. Transport experiments were carried out in NaCl buffer (●), as well as in choline chloride buffer (○), as described in section 2. Total Na^+-dependent AIB transport is also depicted (▲). Representative results are shown.

an optimal model system for the study of changes in amino acid uptake related to cell proliferation.

We examined plasma membrane transport of the non metabolizable amino acid AIB in two established human neuroblastoma cell lines, which differ in their degree of N-*myc* amplification and consequent N-*myc* expression: SH-EP harbors one, and IMR-32 twenty-five copies of N-*myc*. Figs. 1 and 2 show the time course of AIB transport into SH-EP and IMR-32 cells, respectively. It is noteworthy that both Na^+-dependent and Na^+-independent AIB transports were increased in IMR-32 cells. These experimental results fit well with the expected behaviour of cells during neoplastic transformation, in accordance with the statements in the first paragraph of this section.

Table I

Inhibitory effect of some amino acids on Na^+-dependent AIB uptake by SH-EP cells

Amino acid (5 mM)	AIB uptake (%)
None	100 ± 1
Glutamine	20 ± 5
Leucine	81 ± 14
Lysine	80 ± 5
Phenylalanine	80 ± 10
Threonine	77 ± 17
Methyl-AIB	22 ± 5

Assays were carried out as described in section 2. Figures are means ± S.D. for three different determinations.

Since Na^+-dependent AIB transport by both neuroblastoma cell lines was linear with time at least for the first 5 minutes (see Figs. 1 and 2), the rest of the experimental work was carried out at this fixed time.

To examine the relative contribution of systems A and ASC to the total Na^+-dependent AIB transport by neuroblastoma cells, we used methyl-AIB, a specific inhibitor for system A [11], and threonine, which behaves as a specific inhibitor for system ASC in some cell types [12]. Tables I and II show surprising and unexpected results. The effect of methyl-AIB shows that system A accounted for almost 80% of total Na^+-dependent AIB transport in SH-EP cells (Table I); however, in IMR-32 cells, system A accounted for less than 50% of total Na^+-dependent AIB transport (Table II). Thus, our results show that, contrary to what could be expected, the contribution of system A to transport is greater in SH-EP cells, corresponding to the initial

Table II

Effects of methyl-AIB and threonine on Na^+-dependent transport of AIB by IMR-32 cells

Amino acid (5 mM)	AIB uptake (%)
None	100 ± 5
Methyl-AIB	53 ± 6
Threonine	30 ± 1
Methyl-AIB + threonine	0

Assays were carried out as described in section 2. Figures are means ± S.D. for three different determinations.

stages of neuroblastoma. No kind of gene regulation has been described for system ASC and, therefore, the actual causes of this unexpected behaviour should be investigated. On the other hand, threonine behaved as a specific inhibitor for system ASC in SH-EP cells but not in IMR-32 cells, in which threonine not only inhibited transport by system ASC but also partially inhibited transport by system A (Table II).

3.2. *Further preliminary characterization of total Na^+-dependent AIB transport by SH-EP cells*

Table I shows the amino acid specificity of Na^+-dependent AIB transport by SH-EP cells. As expected, the greatest inhibition was produced by glutamine, a well-characterized substrate for Na^+-dependent transport systems. The slight inhibitions by leucine and phenylalanine were also expected, since both amino acids are mainly transported via system L [5]. It is noteworthy that there was a slight but significant inhibition by the cationic amino acid lysine.

In order to detect specific amino acid residues in the active centers of carrier proteins, some specific compounds are available. For instance, the presence of essential sulfhydryl groups can be detected by using PCMBS [13], essential arginine residues can be detected with phenylglyoxal [14], and essential tyrosine residues can be detected with NBD-Cl [15]. We decided to use these three inhibitors in a series of preliminary experiments. The pretreatment with phenylglyoxal seemed to permeabilize SH-EP cells, as shown by high increases in recovery of radioactivity in transport assays carried out without exogenously added sodium ions (results not shown). NBD-Cl seemed to be toxic for SH-EP cells, since pretreatment with NBD-Cl produced a 36% decrease in final total protein; normalization of data with respect to protein content seemed to indicate a greater sensitivity to NBD-Cl of system A as compared to system ASC (results not shown). Finally, a short-term pretreatment with PCMBS seemed to be inocuous to SH-EP cells and did not alter the levels of Na^+-independent AIB transport, but it inhibited transport by system A (82% inhibition) and, to a lesser extent, transport by system ASC (65% inhibition).

Acknowledgements: M.A.M. is a recipient of an Alexander-von-Humboldt Fellowship. Thanks are due to Dr. Lothar Schweigerer for the use of the facilities in his lab.

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