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The relationship between sodium-dependent transport of anionic amino acids and cell proliferation

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The relationship between the transport of anionic amino acids and the proliferative status of the cell population has been studied in NIH-3T3 cells. Proliferative quiescence, verified by determinations of growth-rate quotient and incorporation of thymidine, is associated with a marked increase of the influx of L-aspartate. After 7–10 days of serum starvation, the initial influx of L-aspartate increases by 8–10-times with respect to the transport activity determined in growing cells. The operational properties of the influx of L-aspartate are similar in growing and quiescent cells; in particular, the influx of the anionic amino acid is mostly Na^+-dependent and completely suppressed by an excess of L-glutamate and D-aspartate, but not of D-glutamate. These features suggest that, in both cases, aspartate uptake occurs through system X_{AG}^-. The quiescence-related increase in aspartate transport is gradual, sensitive to the inhibition of protein synthesis and referable to the enhanced maximal capacity of transport system X_{AG}^-. Restoration of serum concentration in the culture medium of serum-starved cells causes a decrease in aspartate transport that is maximal in correspondence to late G_1/S phases. It is concluded that the X_{AG}^- system for anionic amino-acid uptake is sensitive to the proliferative status of the cell population and that, in particular, its transport activity is stimulated by the establishment of proliferative quiescence.

Introduction

Two distinct systems are devoted to transport anionic amino acids in cultured human fibroblasts [1–5]. One, named system x_C^-, is a Na^+-independent, electroneutral exchange transport mechanism that recognizes L-cystine and anionic amino acids with relatively long side chains [1–2]. The other system, named X_{AG}^- [3–5], is Na^+-dependent and transports L-aspartate and L-glutamate with comparable affinities; it is sensitive to membrane potential and characterized by a stereoselective anomaly that consists in an efficient interaction with D-aspartate but not with D-glutamate. This system appears to be widely distributed and its peculiar stereoselectivity has been recently confirmed in NIH-3T3 cells [6].

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Abbreviations: BrdU, bromodeoxyuridine; CS, calf serum; DMEM, Dulbecco's modified Eagle medium; DOC, deoxycholate; EBSS, Earle's balanced salt solution; TCA, trichloroacetic acid.

Previous studies from our group have indicated that system X_{AG}^- is selectively suppressed in NIH-3T3 cells transformed in vitro by transfection with *ras* oncogenes; in these cells transport activity for L-aspartate ranges between 3% and 12% of the value obtained in non transfected cells [7]. This alteration is specific, since no other transport system for amino acids is suppressed in *ras*-transformed cells [7]. That result prompted us to identify what distinctive feature of non-transfected and transfected cells could be involved in the transformation-associated suppression of anionic amino-acid transport. Among the features taken into consideration, we decided to study cell proliferation in detail, since the proliferative activity of *ras*-transformed cell populations is much higher than that exhibited by control cell populations. Therefore, the observed alteration in the transport of anionic amino acids could be explained by a sensitivity of transport activity of system X_{AG}^- to the proliferative status of the cell population.

The present report recounts the results of a study undertaken to assess the validity of this hypothesis. Some of these results have been reported in abstract form [8].

Materials and Methods

Materials

Calf serum (CS), growth medium and antibiotics were purchased from Gibco. L-[2,3-^{3}H]Aspartic acid (22.8 Ci/mmol), L-[2,3-^{3}H]arginine (55 Ci/mmol) and [*methyl*-^{3}H]thymidine (6.7 Ci/mmol) were from DuPont deNemours. L-[5-^{3}H]Proline (10.5 Ci/mmol) was obtained from Amersham International. Plastic cultureware was from Nunc and Falcon. Sigma was the source of all other chemicals.

Cell culture

NIH-3T3 cells (gift of R.A. Weinberg, Whitehead Institute) were routinely grown in 10-cm diameter dishes (Falcon) in Dulbecco's modified Eagle medium (DMEM) containing 10% CS. The conditions of culture were: pH 7.4, atmosphere 5% CO_2 in air, temperature 37°C. All measurements of amino-acid transport were made on subcultures resulting from 10^4 cells seeded into 2-cm^2 wells of disposable 24-well trays (Nunc) in 1 ml of growth medium.

Measurements of cell growth

Cell number. Cell number was estimated with a Coulter Counter ZM after trypsinization of the subculture. The proliferation rate was calculated from the cell number through the growth-rate quotient (Q) following the equation [9]:

$$Q = (N_n - N_{(n-1)})/\Delta t \cdot (1/N_{(n-1)}) \cdot 100 \quad (1)$$

where N_n is the cell number at the final counting, $N_{(n-1)}$ is the cell number at the penultimate counting, and Δt is the time elapsed between the two countings (in h).

Incorporation of thymidine. Incorporation of thymidine in the acid-insoluble compartment was performed supplementing growth medium with [^{3}H]thymidine (2 μCi/ml) for 60 min. At the end of this period the incubation was terminated with three washes with ice-cold $MgCl_2$ (0.1 M) followed by three washes with TCA 5%. Extraction of acid-insoluble material was performed with DOC 0.5% in 1 M NaOH for 60 min at 37°C.

Incorporation of bromodeoxyuridine (BrdU). BrdU incorporation was performed for 60 min. Cells were washed with PBS, fixed in acetone and stained with anti-BrdU peroxidase-conjugated antibody (Amersham International). Labelling index was calculated from five microscopic fields (×200, average number of cells 72).

Uptake assay

Amino-acid uptake has been measured under conditions of initial entry rates using the cluster-tray method

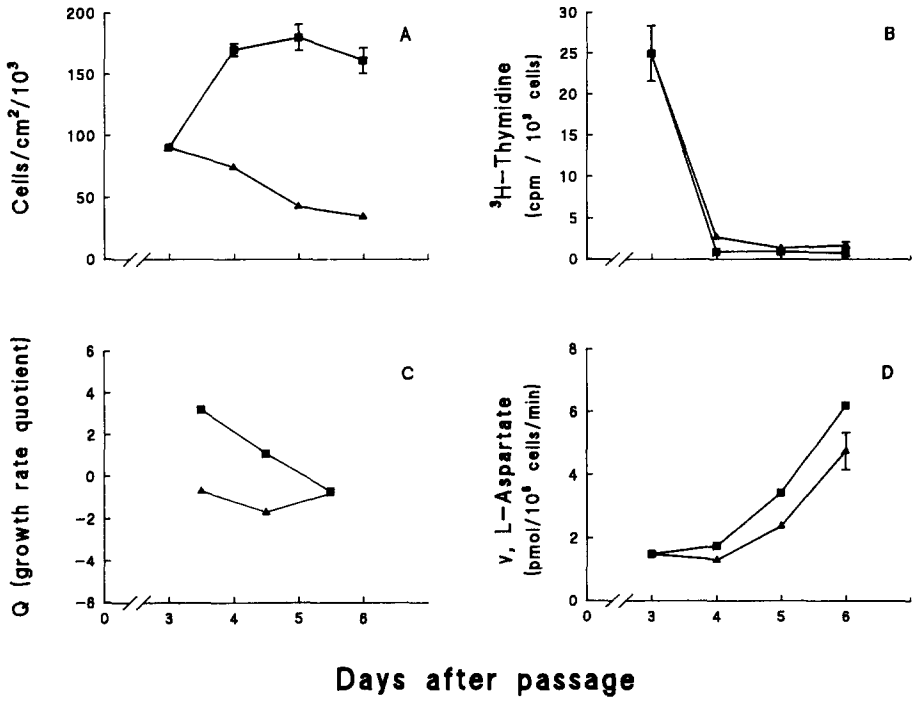

Fig. 1. Transport of L-aspartate and cell proliferation in NIH-3T3 cells. NIH-3T3 cells were seeded into 24-well multiwell dish. After three days the medium was replaced with either new complete growth medium (■) or medium supplemented with 0.5% CS (▲). The parameters determined were cell number (panel A), the incorporation of thymidine (panel B), the growth rate quotient Q (panel C) and the transport of 10 μM L-aspartate (panel D). These parameters were determined on day 3 (before change of medium) and, thereafter, at daily intervals in both conditions. See Materials and Methods for description of the methodology employed. The points are means of three independent determinations with S.D. indicated when greater than the size of the point.

for the rapid measurement of solute fluxes in adherent cells described by Gazzola et al. [10]. The activity of system X^-_{AG} has been measured by determining the initial influx of the preferential substrate L-aspartate [3]; L-proline has been employed as a probe of transport system A [11] and L-arginine of system y^+ [12]. For both types of measures, unless otherwise stated, cell monolayers were incubated for 1 min at 37°C in DMEM at pH 7.4 in the presence of the labelled substrate and, when required by the experimental design, of unlabelled inhibitors. The Na^+-independent uptake was determined in a Na^+-free saline solution in which choline replaced sodium in Earle's balanced salt solution (EBSS). Transport assay was terminated by three rapid washes of the cell monolayer with ice-cold 0.1 M $MgCl_2$ and cells were fixed in place by the addition of 0.2 ml of ethanol. Extracts were added to 2.5 ml of scintillation fluid and counted for radioactivity with a Packard 460C liquid scintillation spectrometer. Cell monolayers were then dissolved with 0.5% sodium deoxycholate in 1 M NaOH and assayed for protein content directly in the well as described previously [10].

Calculations

Amino-acid uptake was expressed as pmol/mg of cell protein per min or as fmol/10^6 cells per min. The kinetic parameters were evaluated by the analysis of initial velocity data using a BASIC program applying the Marquardt's algorithm. The equation used was:

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]} + K_d \cdot [S] \qquad (2)$$

This equation describes the transport process as the sum of a saturable system plus diffusion.

Results

Effect of proliferative quiescence on the influx of L-aspartate in NIH-3T3 cells

Proliferative quiescence of NIH-3T3 cells was obtained either by serum deprivation (incubation in media supplemented with 0.5% CS) or by maintenance of cells at confluence density. Serum starvation caused a rapid decrease in cell number that fell to less than 50% of the control (Fig. 1, panel A) and stabilized thereafter (see below, Fig. 3, panel A). Both serum deprivation and attainment of confluence density were associated with a rapid fall of the incorporation of thymidine into the acid-insoluble fraction (Fig. 1, panel B). After 2 days of treatment cell proliferation was completely stopped, as indicated by very low values of the growth-rate quotient (Fig. 1, panel C). Proliferative quiescence was accompanied by a progressive increase of the inward transport of L-aspartate (Fig. 1, panel D). The increase in aspartate transport did not occur immediately after serum deprivation or attainment of confluence density; rather, in both situations it became detectable on the second day after treatment, i.e., when thymidine incorporation was at very low values from at least 24 h. The increase in aspartate transport reached a maximum after 7–10 days of proliferative quiescence; at this time, the initial influx of aspartate was 8–10-fold increased with respect to the values measured in actively growing cells (see below, Fig. 3, panel D).

In the experiment shown in Fig. 2, panel A, the initial velocity of influx of L-aspartate was measured at various times during the third day of serum starvation in the presence or in the absence of the protein synthesis inhibitor cycloheximide. In the control cells, the influx of the amino acid progressively increased; this increase was significantly diminished in the presence of

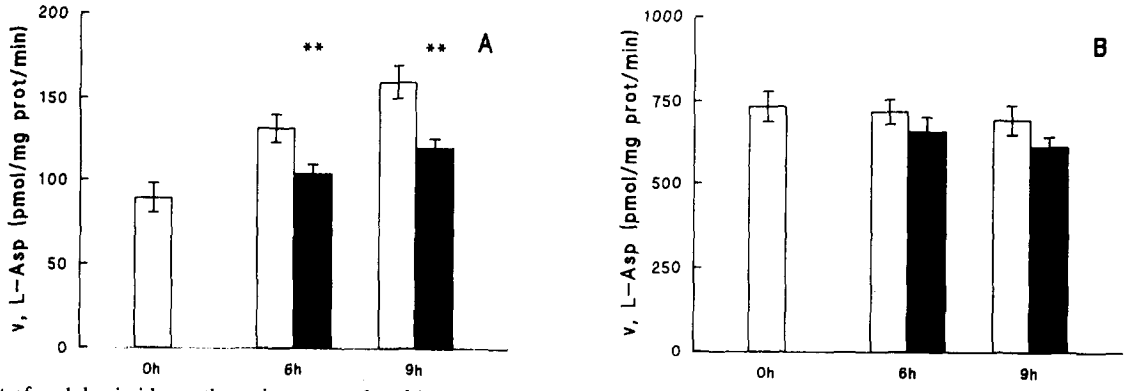

Fig. 2. Effect of cycloheximide on the quiescence-related increase in the transport of L-aspartate. NIH-3T3 cells were incubated either for 2 days (panel A) or for 9 days (panel B) in a medium supplemented with 0.5% CS. In both conditions, cycloheximide (18 μM) was added to the culture medium and the incubation was prolonged in the absence (open bars) or in the presence (filled bars) of the inhibitor. The transport of L-aspartate (10 μM, 5 μCi/ml) was determined at time 0 (before the addition of cycloheximide) or after 6 h or 9 h. The points are means of three independent determinations with S.D. indicated. The level of significance was assessed with a two-tailed *t*-test for unpaired data (** $P < 0.01$, cells incubated in the absence of cycloheximide vs. cells incubated in the presence of the inhibitor).

TABLE I

Kinetic parameters for the transport of L-aspartate in growing and quiescent NIH-3T3 cells

Transport of L-aspartate was determined in a range of extracellular concentrations of the amino acid from 2.5 to 675 μM either in actively growing cultures (incubated with medium supplemented with 10% CS) or in quiescent cultures (incubated for four days at 0.5% CS, as indicated). Parameters were obtained by non-linear regression analysis of transport data and are shown with S.E. indicated.

	K_m (μM)	V_{max} (pmol/10^6 cells per min)	K_d (min^{-1})
Growing	16.8±4.13	48.0± 8.23	0.079±0.013
Quiescent	18.8±4.14	153.7±22.2	0.084±0.028

the inhibitor of the protein synthesis. The same experiment was also performed with cells that had been incubated for 9 days at 0.5% CS (Fig. 2, panel B); in this case, no increase of transport was detected in control cells and cycloheximide did not significantly alter aspartate influx. This experiment indicates that the quiescence-related increase of L-aspartate transport is dependent upon an active protein synthesis.

Kinetic and operational features of L-aspartate transport in quiescent and growing cultures

Table I summarizes the kinetic parameters of L-aspartate transport in actively growing NIH-3T3 cells, maintained in a medium supplemented with 10% CS, and in quiescent NIH-3T3 cells, incubated for 4 days in a medium supplemented with 0.5% CS. The kinetic analysis indicates that in both cases the transport data were satisfactorily fitted by an equation (Eqn. 2, see Materials and Methods) that describes the transport process as the sum of a saturable agency and of a non saturable component, formally undistinguishable from diffusion. The kinetic parameters obtained indicate that the increase in the transport of L-aspartate observed in quiescent cells was solely due to an increase of the transport capacity (V_{max}) while neither the K_m of the saturable component nor the diffusion constant K_d were significantly different in the two cell popula-

TABLE II

Sodium dependence and sensitivity to inhibition by anionic amino acids of L-aspartate transport in growing and quiescent NIH-3T3 cells

The initial influx of L-aspartate (10 μM, 5 μCi/ml) was measured in cells maintained for 3 days at 10% CS (growing cells) and in cells incubated for 8 days at 0.5% CS (quiescent). Transport assay was performed in DMEM (control), in a modified Na^+-free Earle's balanced salt solution (Na^+-free), or in DMEM supplemented with the indicated anionic amino acids at a concentration of 1 mM. Data are means of three independent determinations with S.D. indicated.

	Growing cells		Quiescent cells	
	$V_{L\text{-Asp}}$ (pmol/mg per min)	% of control	$V_{L\text{-Asp}}$ (pmol/mg per min)	% of control
Control	111 ±26.5	–	903 ±36	–
Na^+-free	3.11± 0.52	3	5.1± 0.8	<1
+L-Aspartate	4.62± 1.12	4	19.7± 1.02	2
+D-Aspartate	15.6 ± 2.88	14	46.3± 8.09	5
+L-Glutamate	5.79± 0.53	5	21.9± 1.14	2
+D-Glutamate	58.5 ±10.2	53	393 ±56	44
+L-Cysteate	10.4 ± 1.06	9	22.8± 8.17	3
+D-Cysteate	67.3 ± 5.49	61	345 ±62.2	41
+L-Aminoadipate	39.9 ± 2.80	36	250 ±37.2	28

TABLE III

Transport of L-aspartate, L-proline and L-arginine in growing and quiescent NIH-3T3 cells

The uptake of L-aspartate (10 μM, 5 μCi/ml), L-proline (100 μM, 3 μCi/ml) and L-arginine (20 μM, 3 μCi/ml) was measured in cells maintained for 3 days at 10% CS (growing cells) and in cells incubated for 3 days at 0.5% CS (quiescent). Transport assay was performed in EBSS either in cells just washed from growth medium (unstarved cells) or in cells starved for 4 h in EBSS supplemented with 0.5% dialyzed CS (starved cells). Data are means of three independent determinations with S.D. indicated.

	Transport activity (pmol/mg protein per min)					
	Unstarved cells			Starved cells		
	L-Asp	L-Pro	L-Arg	L-Asp	L-Pro	L-Arg
Growing	106±11	1600±130	656±46	135± 7	3870±92	524±29
Quiescent	259±41	1150± 55	383± 9	383±48	1490±81	285± 6

tions. These results indicate that in both types of cells the mediated transport of aspartate is due to the operation of a single agency whose capacity markedly increases in quiescent cultures.

The experiment reported in Table II was performed in order to ascertain if the operative and recognition characteristics of L-aspartate transport change upon the establishment of proliferative quiescence. In both quiescent and growing cultures of NIH-3T3 cells the transport of the anionic amino acids is strictly Na^+-dependent, with an exceedingly small Na^+-independent component. When measured in the presence of a 100-fold excess of a panel of selected anionic amino acids, the influx of 10 μM L-aspartate was almost completely suppressed by D-aspartate, L-glutamate and L-cysteate. Other compounds enclosed in the panel of inhibitors tested, such as L-aminoadipate, D-glutamate and D-cysteate, exerted a less marked, although significant, inhibition. The relative insensitivity to the inhibition by D-glutamate, but not by D-aspartate, constitutes the characteristic stereoselective anomaly typical of a transport process mediated by system X^-_{AG} [3].

Specificity of the effect of proliferative quiescence on the transport of aspartate

In the experiment recounted in Table III the specificity of the stimulative effect of quiescence on the activity of system X^-_{AG} was evaluated. In this experiment the conditions described in Fig. 1 were adopted to study the influx of L-aspartate and, in parallel, those of L-proline and of L-arginine. These amino acids are preferential substrates, respectively, of system A and system y^+, two other transport systems widely distributed in mammalian cells (see Ref. 13 for review). In order to study the transport activities in proper conditions, uptake assays were performed in EBSS either in cells just washed from growth medium or in cells starved with a 4-h preincubation in EBSS supplemented with 0.5% dialyzed CS. This treatment was aimed to deplete intracellular amino-acid pool so as to minimize the *trans*-effects that modify amino-acid transport (*trans*-inhibition, system A [11]; *trans*-stimulation, system y^+ [12]) and also to pull the activity of system A towards very high values due to the occurrence of the adaptive regulation [14]. The results indicate that aspartate transport is significantly higher in quiescent (3 days at 0.5% CS) than in growing cultures both in unstarved and in starved cells. On the contrary, the influx of L-proline through system A was significantly decreased in quiescent cells both before and after amino-acid starvation (when proline influx was significantly enhanced for the combined effects of adaptive increase and release from *trans*-inhibition). Moreover, both in starved and in un unstarved cells

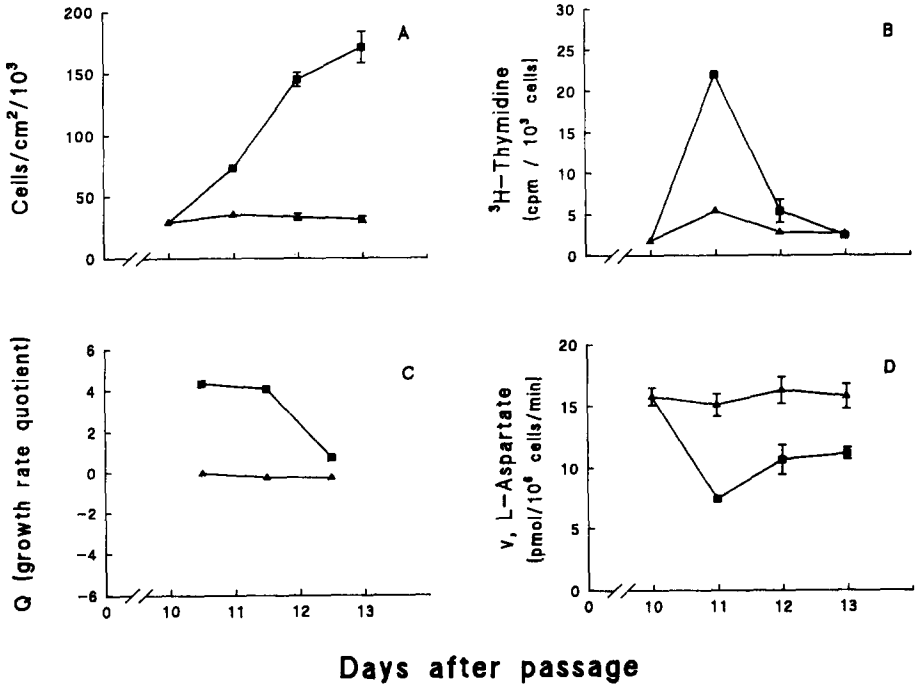

Fig. 3. Effect of restoration of serum concentration on the transport of L-aspartate in quiescent NIH-3T3 cells. NIH-3T3 cells were incubated for 7 days in the presence of 0.5% CS (medium changed on the third day). After this period the medium was substituted with fresh medium supplemented with 0.5 (▲) or 10% CS (■). Cell number (panel A), the incorporation of thymidine (panel B), the growth rate quotient Q (panel C) and the transport of 10 μM L-aspartate (panel D) were measured on day 3 (before change of medium) and thereafter at daily intervals in both conditions. See Materials and Methods for description of the methodology employed. The points are means of three independent determinations with S.D. indicated when greater than the size of the point.

L-arginine influx through system y^+ was also slower in quiescent than in growing cultures; as expected, in either condition, unstarved cells exhibited an enhanced uptake of L-arginine in respect of starved cells, due to *trans*-stimulation of system y^+ transport activity.

These results indicate that the quiescence-related stimulation of transport appears to be a specific feature of system X^-_{AG}.

Reversion of quiescence-dependent stimulation of L-aspartate transport

Fig. 3 describes the effect of serum restoration on the transport of aspartate in quiescent NIH-3T3 cells. The results indicate that the proliferative burst of NIH-3T3 cells caused by the increase of serum concentration (Fig. 3, panels A–C) was accompanied by a decrease in the transport of L-aspartate (Fig. 3, panel D). L-Aspartate influx fell at 50% of the control value 24 h after the restoration of serum in the culture medium. Subsequently, when the cell number approached the confluence density (Fig. 3, panel A, cf., Fig. 1, panel A), the proliferative activity of the culture declined (Fig. 3, panels B and C) and the transport of aspartate was partially restored (Fig. 3, panel D). Again, the behavior of L-proline transport through system A is opposite, since, in the same conditions, it almost doubles upon readdition of serum to quiescent cells (not shown).

A more in-depth analysis of the serum-induced decline in aspartate transport of quiescent NIH-3T3 cells is shown in Fig. 4. In this experiment, the transport of L-aspartate was measured before the addition of serum ($t = 0$) and after 6, 12, 15, 18 and 24 h. In parallel cultures cell number (not shown), cell proteins, and BrdU-labelling index were monitored. BrdU incorporation increased after 12 h from serum readdition and peaked at 15 h. At these same times the values of L-aspartate influx (note the expression as pmol/mg of protein per min) were significantly lower than those measured before serum readdition, although cell proteins and cell number (not shown) had not changed appreciably. This fact indicates that, in these conditions, a true decrease of transport activity was detected rather than an artifact dependent upon an increase in cell protein associated to the rescue of cell proliferation. At the longest time points considered (18–24 h) aspartate influx underwent a further decrease; at these times cell proteins and cell number (not shown) were significantly greater than the control values, suggesting that, in these conditions, de novo synthesis of carriers does not keep pace with growth of cells.

Discussion

The results recounted here demonstrate that aspartate transport is much higher in quiescent than in growing NIH-3T3 cells. The analysis of the kinetic and of the operative features of the transport indicates that this behavior is due to a modulation of the activity of transport system X^-_{AG} for anionic amino acids. This system appears to be extremely sensitive to the proliferative activity of the cell population. In particular, the activity of the system is greater in quiescent than in actively growing cells. Under this regard, the behavior of this membrane agency recalls that of several cell functions, encoded by the so-called growth-arrested specific genes [15], that have received much attention, since they appear to be involved in growth suppression [16]. The behavior of L-aspartate transport appears to be unique, since it constitutes, at our knowledge, the first example of a transport activity for nutrients depressed by cell proliferation. Conversely, it has been known since many years (and confirmed here for L-proline transport through system A and for L-arginine transport through system y^+) that several transport activities for nutrients are stimulated by the onset of cell proliferation [17–20].

The increase in transport activity for aspartate associated to the establishment of quiescence is reversible; the rescue of cell proliferation, triggered by the addi-

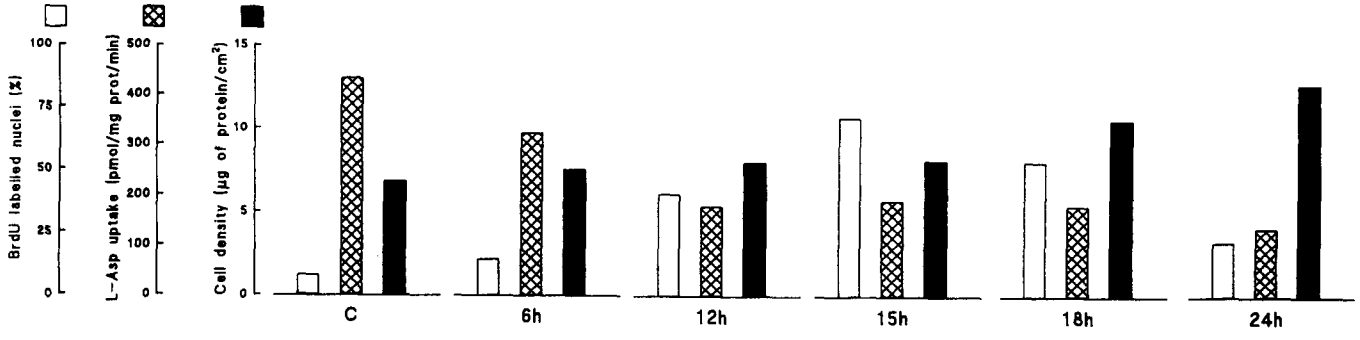

Fig. 4. Time-course of aspartate transport decrease in serum-stimulated quiescent NIH-3T3 cells. Conditions of culturing were similar to those employed in the experiment shown in Fig. 3. Cell protein (filled bars), BrdU-labelling index (open bars), influx of 10 μM L-aspartate (cross-hatched bars), and cell number (not shown) were determined at the indicated times. Data of transport and cell density are means of three independent determinations with S.D. indicated.

tion of serum to serum-starved quiescent cells, inhibits L-aspartate transport. The analysis of this decrease, shown in Fig. 4, indicates that the decline in transport activity is gradual and reaches the maximal extent in the late G_1/S phases.

The conclusion reached by this study validates the hypothesis that the suppression of system X^-_{AG} activity, observed in *ras*-transformed NIH-3T3 cells [7], could be related to the higher proliferative activity of transformed in respect of control cells. Under this regard, it is interesting to note that in proliferating NIH-3T3 cells [21], as well as in other models [22,23], the maximal expression of *ras* genes is observed in late G_1/S phases (i.e., with a temporal pattern similar to the decline of transport activity following readdition of serum to serum-starved cultures). The phenomenon observed here also helps to explain a previous observation that demonstrated a fall in aspartate transport following a long-term treatment of NIH-3T3 cells with phorbol esters [24]. It is noteworthy that, in these cells, phorbol esters stimulate cell proliferation [25]. On the other hand, in a strain of cultured human fibroblasts whose proliferation is not stimulated by phorbol esters, the activity of system X^-_{AG} is enhanced by the tumor promoters [26].

The data presented here cope with the observation, made by our group in cultured human fibroblasts several years ago [4], that transport activity of system X^-_{AG} is higher at high culture density. Probably, this phenomenon is more related to the lower proliferative activity of dense cultures than to culture density per se. It is interesting to remark, under this regard, that very similar values of aspartate transport are detected in cultures fully comparable for proliferative behavior and markedly different for cell density (see Fig. 1). Conversely, cultures with comparable cell densities, but with different proliferative activities, show very different transport values for aspartate (see Fig. 1).

The metabolic consequences of the growth-related modulation of system X^-_{AG} activity have not been ascertained. In particular it is not possible to infer from the data presented here whether there are modifications in the intracellular content of anionic amino acids related to the transport alterations. For this purpose it will be necessary to determine the intracellular concentrations of anionic amino acids and related compounds in the various experimental conditions adopted here. Moreover, a direct approach aimed to assess the influence of an excess of anionic amino acids on the DNA synthesis triggered by serum readdition has been hindered by a marked toxicity exerted by these compounds on serum-starved cells (data not shown).

The mechanism underlying the regulatory effects described does not appear to be referable to a perturbation of trans-membrane gradient of sodium since intracellular sodium concentration was 13.5 ± 1.8 mM (quiescent cells, $n = 6$) vs. 12.1 ± 1.6 mM (growing cells, $n = 6$). Moreover, the variations in the activity of system X^-_{AG} do not appear to depend upon changes in membrane potential. Indeed, the activities of two transport systems known to be extremely sensitive to membrane potential, system A [27] and system y^+ [28], are significantly lower in quiescent than in growing cultures (see Table III). On the other hand, several data suggest that changes in transport activity can be accounted for by a change in the number of active carriers on the plasma membrane; these data are (*i*) the long time required for the expression of the quiescence-related increase in transport; (*ii*) the sensitivity of the quiescence-related increase to inhibitors of protein synthesis; (*iii*) the substantial similarity between the operational features of anionic amino-acid transport in quiescent and growing cells and (*iv*) the kinetic evidence of a pure change in V_{max} without modifications of K_m. It is therefore possible that a sustained synthesis of new carriers occurs after the establishment of proliferative quiescence. This phase ceases after 8–10 days of incubation of cells at low serum; at that moment, indeed, transport activity is nearly constant (although at very high values) and not appreciably sensitive to the block in protein synthesis caused by cycloheximide. Cells maintained at low serum for several days remain sensitive to proliferative rescue induced by serum and start DNA synthesis within 12–15 h after serum readdition; at this time transport of aspartate falls by 50%: the extent and time-course of the decrease indicate that it can not be simply explained by a block in the synthesis of new carriers. It is therefore possible that, under this condition, an active mechanism of inhibition and/or degradation of carriers is triggered.

Recently, significant advances have been made in the molecular biology of anionic amino-acid transport in the central nervous system and peripheral tissues [29–31]. The operative features of the transport systems cloned resemble those of system X^-_{AG}. Therefore, it is possible that system X^-_{AG} is associated with one of the cloned transporters or, at least, belongs to the same gene superfamily. In either case those molecular devices would favour the ascertainment of the precise site of action of the regulatory mechanism described here.

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