TRANSPORT INHIBITORS RELEASED BY 3T3 MOUSE CELLS AND THEIR RELATION TO GROWTH CONTROL

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INTRODUCTION

Transport rates of certain RNA precursors are related to contact or density-dependent inhibition of division by the 3T3 cell line. Within 15 min after treatment of contact-inhibited 3T3 cells with fresh dialyzed calf serum to initiate cell division, two- to threefold increases in the rate of inorganic phosphate transport and three- to fourfold increases in the rate of uridine transport occur (1). Cells so stimulated manifest their partial release from contact inhibition by continuing to incorporate uridine at high rates for about an hour and by exhibiting periods of increased protein synthesis (several hours after serum treatment), DNA synthesis (maximum rate at 20–22 hr), and mitosis (maximum rate at 30 hr) (5, 6). Subconfluent, rapidly growing cultures of 3T3 cells accumulate uridine and phosphate at high rates and show no stimulation of transport by serum. In contrast, the rate of transport of both substrates by confluent cells is reduced and is sensitive to rapid serum stimulation. These observations suggest that control of these uptake systems is correlated with contact inhibition of this cell line. In addition, polyoma virus–transformed 3T3 (Py3T3) cells, which do not exhibit density-dependent inhibition of cell division, transport these substrates at high rates which do not decrease after confluency is reached and are not sensitive to serum stimulation (1).

A dialyzable inhibitor released by contact-inhibited 3T3 cells which reduces the serum-stimulated incorporation of uridine into RNA by these cells has been demonstrated by Yeh and Fisher (7). In light of the observation of Cunningham and Pardee (1) that this stimulated incorporation can be entirely accounted for by increased transport of uridine without any change in the actual rate of RNA synthesis, we investigated the implication that the inhibitor reported by Yeh and Fisher acted on transport by 3T3 cells. Results of this study demonstrate that confluent 3T3 cells release into the medium substances of low molecular weight which specifically inhibit transport of uridine and phosphate by 3T3 cells but not by Py3T3 cells. The transport-stimulating activity of serum cannot, however, be fully accounted for in terms of binding inhibitor; serum must, therefore, contain factors capable of increasing transport by some other mechanism.

MATERIALS AND METHODS

Transport of uridine-^{3}H and phosphate-^{32}P into the acid-soluble fraction of mycoplasma-free 3T3 and Py3T3 cells was assayed at pH 7.4 as previously described using a 20 min incubation period (1). Duplicate determinations were made for all assays,

and the average variation of the absolute values of the duplicates from their mean was found to be less than 4% of the mean. Confluent 3T3 cells used for uptake assays were grown from a density of 6×10^4 cells per 35 mm plate (approximately 13% of the density at confluency) to confluency over a 3 day period; medium from these cultures was designated as "3T3-altered medium."

RESULTS

The presence of transport inhibitor in 3T3-altered medium was demonstrated by the observation that 3T3-altered medium to which fresh serum was added to a final concentration of 10% stimulated transport by confluent 3T3 cells significantly less than did fresh 10% serum medium (Table I). This indicated that removal of transport-stimulating serum factors alone could not account for the reduced activity of medium which had supported the growth of 3T3 cells. Yeh and Fisher (7) likewise found that 3T3-"depleted" medium to which 25% serum was added stimulated uridine incorporation by no more than 37% of the maximum rate.

Dialysis of 3T3-altered medium against fresh defined medium (i.e., medium without serum) significantly increased its transport-stimulating activity toward both uridine and phosphate (Table I). The possibility that these increases in activity toward both uridine and phosphate transport resulted from replacement of some defined medium components exhausted by cells was ruled out by the observation that activity was largely restored by dialysis against buffered isotonic saline as well as against defined medium. Additional evidence indicating that removal of inhibitor of small molecular weight, rather than replacement of limiting defined medium components, accounted for these results was (*a*) transport-stimulating activity of dialysis-restored medium was significantly higher than that of fresh defined medium, and (*b*) uptake by confluent 3T3 cells continued and the normal serum effect on transport was seen when the uptake assay was carried out in buffered isotonic saline.

Lowering of specific activity of the phosphate-^{32}P by addition of unlabeled inorganic phosphate from serum or its release by cells was ruled out by performing phosphate determinations (2) on test media. Yeh and Fisher (7) concluded that significant amounts of unlabeled uridine were not released by 3T3 cells, since media which had supported their growth did not reduce the incorporation of labeled uridine by other cell lines. In addition, uridine uptake by Py3T3 cells was much less affected by 3T3-altered medium than was uptake by 3T3 cells (Table II).

TABLE I

Transport-Stimulating Activity of 3T3-Altered Media

Test medium	Transport-stimulating activity*	
	Phosphate	Uridine
3T3-altered medium‡	0	0
Fresh 10% serum medium	100	100
3T3-altered medium + 10% serum	65	33
3T3-altered medium, dialyzed§	48	60
3T3-altered medium, dialyzed + 10% serum	90	108
3T3-altered medium + bovine serum albumin (5 mg/ml)	40	5
3T3-altered medium + bovine serum albumin (5 mg/ml), dialyzed	40	68

* Transport-stimulating activity in this and other experiments is given as the per cent of the increase in uptake rate brought about by fresh 10% serum medium seen in a test medium, and is calculated as: activity =

$$\left(\frac{[\text{uptake rate in test medium}] - [\text{uptake rate in 3T3-altered medium}]}{[\text{uptake rate in 10\%-serum medium}] - [\text{uptake rate in 3T3-altered medium}]}\right) \times 100$$

‡ 3T3-altered medium was produced by growing 3T3 cells in fresh 10% serum medium from a density 13% that at confluency to confluency over a 3 day period in 35-mm tissue culture plates (2 ml medium per plate).

§ Samples were dialyzed in the cold against two changes of a 100-fold volume of Dulbecco phosphate-buffered saline, followed by two changes of a 10-fold volume of fresh defined medium. At least 12 hr were allowed for equilibration between changes. Dialysis tubing was washed several times in detergent solution, then thoroughly rinsed in tap, distilled, and double-distilled water and soaked overnight in fresh defined medium prior to use. Samples of fresh defined medium were dialyzed along with test samples and their activity was compared with that of fresh defined medium as a test for possible release of interfering substances from the tubing.

The inhibitor(s) acted specifically on uridine and phosphate transport, rather than as a general block of active uptake systems. The energy dependence of uridine and phosphate transport was demonstrated by the reduction of the uptake of these substrates to about 10% of normal rates by addition of 2 mM sodium cyanide and 10 mM sodium iodoacetate to test medium or by assaying at 0°–2°C. Under similar conditions, adenosine transport was reduced to 50% by cyanide and iodoacetate and to 15% at 0°–2°C, but it was unaffected by the inhibitors released by 3T3 cells. Adenosine transport shows no serum effect (1), nor is it affected by dialyzed 3T3-altered medium. The fact that the inhibitor of uridine transport could operate without affecting phosphate transport, as discussed below, is inconsistent with the possibility that the transport inhibitors simply reduced over-all energy production by the cells.

The effects of inhibitor and serum on phosphate and uridine transport were rapidly reversible, since uptake rates for at least the first 30–40 min were characteristic of the test medium, regardless of the order of testing.

Several experiments suggest that the transport inhibitors released by 3T3 cells and the stimulatory factors in serum exert their antagonistic effects directly on the cells, rather than on one another (Table I). Activity of exhaustively dialyzed 3T3-altered medium, from which free inhibitor was presumably removed, was still significantly increased for transport of both substrates by addition of fresh 10% serum, suggesting that serum possesses transport-stimulating activity beyond that which might be the result of simple binding of inhibitor. The possibility that dialysis failed to restore full activity to 3T3-altered medium because of binding between large serum molecules and functional inhibitor was ruled out by the observation that addition of 10% serum either before or after dialysis of 3T3-altered medium resulted in media with equally high transport-stimulating activities. The non-correlation of ability to bind inhibitor and ability to stimulate transport was also indicated by the failure of purified bovine serum albumin in fresh defined medium to affect transport rates, although it did bind inhibitor, as shown by its stimulatory activity for phosphate transport in 3T3-altered medium. By a similar test, albumin appeared unable to bind the inhibitor of uridine

TABLE II

Uptake by 3T3 and Py3T3 Cells

Cell type	Test medium	Uptake*	
		Phosphate	Uridine
		nanomoles/mg protein	*picomoles/mg protein*
Confluent 3T3‡	No medium change (3T3-altered medium)	0.92	2.8
	Fresh defined medium (medium without serum)	0.94	5.1
	Fresh 10% serum medium	2.4	10
Subconfluent 3T3§	No medium change	3.2	16
	3T3-altered medium	2.9	6.6
	Fresh 10% serum medium	4.0	16
Confluent Py3T3	No medium change	9.0	8.0
	3T3-altered medium	8.4	6.3
	Fresh 10% serum medium	10	9.2

* Uptake was measured as described in Materials and Methods by determining radioactivity incorporated into the acid-soluble fraction of cells during a 20 min incubation with uridine-^{3}H and/or phosphate-^{32}P immediately following addition of the indicated medium to the cells.

‡ Confluent 3T3 and Py3T3 cells were grown from an original density 13% that at confluency to confluency over a 3 day period.

§ Subconfluent 3T3 cells were grown to a density 10% that at confluency over a 2- or 3 day period.

transport. Dialysis experiments confirmed these conclusions: 3T3-altered medium with 5 mg/ml albumin showed no change in activity toward phosphate transport after dialysis, as might be expected if the inhibitor were bound to albumin but still partially functional. On the other hand, activity toward uridine transport was increased after dialysis to about the same level as for dialyzed 3T3-altered medium, confirming the lack of binding between this inhibitor and albumin.

TABLE III

Transport-Stimulating Activity of Medium Altered by Subconfluent 3T3 and Py3T3 Cells

Test medium	Transport-stimulating activity*	
	Phosphate	Uridine
Subconfluent-3T3–altered medium‡	24	17
Subconfluent-3T3–altered medium, dialyzed	18	72
Subconfluent-3T3–altered medium+ 10% serum	83	77
Py3T3-altered medium§	33	25
Py3T3-altered medium, dialyzed	38	60
Py3T3-altered medium + 10% serum	84	73

* Transport-stimulating activity is defined as in Table I.

‡ Subconfluent-3T3–altered medium was produced by growing 3T3 cells at a density 10% that at confluency for a 24 hr period in fresh 10% serum medium in 25-cm² tissue culture flasks (1 ml medium per flask).

§ Py3T3-altered medium was produced in a manner analogous to that for 3T3-altered medium (see Table I).

If the transport inhibitors are related to contact inhibition, one would expect them to be less effective on cells not subject to contact inhibition, such as Py3T3 cells. To test this possibility, sensitivity of Py3T3 and also of subconfluent 3T3 cells to transport inhibitor was measured. As seen in Table II, Py3T3 cells were quite insensitive to the inhibitors produced by 3T3 cells, although uridine uptake by the transformed cells was slightly reduced in 3T3-altered medium. 3T3 cells gained sensitivity to the inhibitor of phosphate transport only after growing to confluency. On the other hand, sensitivity to uridine-transport inhibitor continued throughout growth, since subconfluent 3T3 cells were equally as sensitive to inhibitor of uridine transport as were confluent cells.

The ability of subconfluent 3T3 and Py3T3 cells to reduce the transport-stimulating activity of their medium was also measured, and the results are given in Table III. Subconfluent 3T3 cells were fully able to reduce the transport-stimulating activity of fresh medium, at a rate roughly equal to that for confluent 3T3 cells. This was shown by similar reductions in activity of fresh 10% serum medium incubated 24 hr over confluent and subconfluent 3T3 cells, on the basis of a constant ratio of amount of medium

TABLE IV

Contact Inhibition and Transport Factors

Cell type	Inactivates transport-stimulating serum factors*		Affected by transport-stimulating serum factors		Releases transport inhibitor‡		Affected by transport inhibitor	
	Pi	Ur	Pi	Ur	Pi	Ur	Pi	Ur
3T3								
Confluent	+	+	+	+	+	+	+	+
Subconfluent	+	+	±§	−	−	+	±	+
Py3T3								
Confluent or subconfluent	+	+	−	−	−	+	−	−

* Inactivation of transport-stimulating serum factors was indicated by susceptibility of dialyzed test medium to further increase in activity by serum addition.

‡ Presence of inhibitor was demonstrated by reduced activity even after addition of 10% fresh serum to a test medium and by increased activity after dialysis.

§ The notation "±" indicates a slight or intermediate effect.

to cellular protein. Py3T3 cells likewise reduced the activity of the medium which had supported their growth. The criteria of degree of activity change after dialysis and after serum addition were used to distinguish between inactivation of stimulatory serum factors and release of transport inhibitor. As summarized in Table IV, noncontact-inhibited cells (subconfluent 3T3 and Py3T3 cells) released inhibitor for uridine transport only, although they inactivated stimulatory factors for both uridine and phosphate transport. It is noteworthy that Jainchill and Todaro (4) have shown that normal and virus-transformed 3T3 cells inactivate serum factors required for cell division of 3T3 cells only.

DISCUSSION

These results demonstrate the existence of a specific mechanism for decreasing uptake of certain RNA precursors when 3T3 cells grow to confluency and cell division stops. The release of specific transport inhibitors and sensitivity to them by 3T3 cells indicate that the decreases in uptake of uridine and phosphate are not simply results of a slower metabolic state of the contact inhibited cells. This conclusion is supported by the previous finding by Foster and Pardee (3) that active uptake of amino acids is only very slightly decreased after 3T3 cells grow to confluency.

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