An Altered Rate of Uridine Transport in Membrane Vesicles Isolated from Growing and Quiescent Mouse 3T3 Fibroblast Cells

(whole cell transport/mediated uptake/intravesicular transport products)

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ABSTRACT Balb/c 3T3 and simian virus-40-transformed Balb/c 3T3 (SV-3T3) cells were examined for the ability to transport nucleosides. Confluent (quiescent) 3T3 cells transported uridine at a rate 3-4 fold lower than did subconfluent cells. Adenosine uptake was independent of cell population density. Both adenosine and uridine were transported at the same rate by confluent and subconfluent SV-3T3 cells. A membrane vesicle population (plasma membrane and endoplasmic reticulum) was isolated from 3T3 and SV-3T3 cells by nitrogen cavitation. With membrane vesicles derived from SV-3T3 cells it was determined that uptake occurred by a mediated process. Uptake of adenosine and uridine by membrane vesicles isolated from 3T3 and SV-3T3 cells demonstrated the same pattern as found in whole cells; that is, membrane vesicles from confluent 3T3 cells transported uridine at a rate 3-fold lower than did membrane vesicles from subconfluent 3T3 cells. Much of the adenosine taken up was converted to inosine, hypoxanthine, and ribose 1-phosphate, whereas uridine transport resulted only in the accumulation of uridine. Results obtained with membrane vesicles indicate that the lowered rate of uridine transport by confluent 3T3 cells seems due to an alteration in the membrane itself or a component(s) thereof, rather than to changes in subsequent cellular metabolic processes.

It had been observed by Cunningham and Pardee (1) that when 3T3 cells grow to confluence and become quiescent (such cells are often termed “contact-inhibited” or “density-inhibited”), they demonstrated a decreased rate of uridine uptake as compared to subconfluent, actively growing cells. The kinetics of adenosine transport were identical in both growth states. In addition, there were no alterations in the rates of amino acid uptake (2) by confluent cells, and ribonucleic acid synthesis proceeded at 70-80% of the rate observed in actively growing cells (3). Thus, the decrease in uridine uptake by confluent cells is not due simply to a lower metabolic state. Polyoma-transformed 3T3 cells did not demonstrate the “density-dependent” change in uridine uptake.

It was further postulated by Cunningham and Pardee (1) that the change in uridine uptake was due to specific membrane alterations. However, whole cells were used for the transport determinations and, therefore, other factors could not be ruled out that might affect uridine transport subsequent to the interaction of uridine and the membrane. For example, an increase in the activity of an intracellular kinase could deplete the pool levels of uridine and lead to a heightened transport rate (4). Furthermore, Pariser and Cunningham (5) reported that conditioned medium was capable of specifically decreasing the rate of uptake of uridine by subconfluent, actively growing 3T3 cells. In order to test whether the decreased rate of uridine transport by confluent, quiescent 3T3 cells is indeed due to a membrane alteration, we isolated membrane vesicles from Balb/c 3T3 cells by the nitrogen cavitation method (6, 7). These selectively permeable membrane vesicles are a mixed vesicle population containing membrane vesicles derived from the plasma membrane (PM) and endoplasmic reticulum (ER). Adenosine and uridine transport in these vesicles was then examined by a modification of methods routinely used in this laboratory for the study of transport by bacterial vesicles (8-11).

MATERIALS AND METHODS

Growth of Cells. Balb/c 3T3 cells were obtained from Dr. Harvey Ozer; the original stock was provided by Aaronson and Todaro (12). Simian virus 40 (SV-40)-transformed Balb/c 3T3 cells (SV-3T3) were also obtained from Dr. Ozer. Cells were grown in 75 cm² flasks or in 720 cm² roller bottles at 37°C in a 10% CO₂ atmosphere using Dulbecco modified Eagle’s medium (DME) supplemented with 2 mM glutamine and 10% (final concentration) fetal calf serum (Grand Island Biol. Co.). Cultures were refed every three days with medium of pH 7.5-7.6; at harvesting the pH was not less than 7.3-7.4, as determined with a pH meter.

Harvesting of Cells. Subconfluent 3T3 and SV-3T3 cells were harvested at a density of 2-3 × 10⁵ cells/cm², confluent 3T3 cells at 8-11 × 10⁵ cells/cm², and confluent SV-3T3 cells at 4-6 × 10⁶ cells/cm². Monolayers were washed twice with warm DME; harvesting was by scraping with a rubber “police man.” The cells were collected by centrifugation at 800 × g at 2°C for 5 min and resuspended in ice-cold DME buffered with 5 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid Heps (pH 7.5). Cell counts were obtained with a hemocytometer; the final cell concentration was adjusted to 10⁶ cells/ml DME.

Whole Cell Transport Assay. 10⁵ cells in 100 μl of buffered DME were preincubated at 37°C for 15 min; the reaction was at 37°C. The reaction was started with [U-¹⁴C]uridine (538 Ci/mol) or [U-¹⁴C]adenosine (495 Ci/mol) at a final concentration of 40 μM. At various times 10 volumes of warm (37°C) DME were added. The cells were collected on 0.45 μm nitrocellulose filters and washed twice with 1 ml of cold DME. The total elapsed time from addition of cold DME to removal of filter from the suction apparatus is less than 30 sec. The

Abbreviations: PM, plasma membrane; ER, endoplasmic reticulum; DME, Dulbecco’s modified Eagle’s medium.
filters were dried and their radioactivity was measured in a gas flow counter at 16% efficiency. The amount of radioactivity taken up was 3-15 fold greater than in control samples (radioactivity added after dilution).

Preparation of Membrane Vesicles. A mixed vesicle population (containing membranes derived from PM and ER) was obtained using a nitrogen cavitation procedure (6, 7). The protocol was modified for 3T3 and SV-3T3 cells as described by Hochstadt et al. (13). The modification involved centrifuging "mitochondrial" material through a 16% dextran-110 "cushion" to free it of plasma membrane vesicles; the latter could be pooled with the mixed vesicle population and collected by centrifugation. A mixed membrane vesicle population could be stored at -60°C in 5 mM Tris-HCl (pH 7.5) plus 0.25 M sucrose at a concentration of 3-5 mg membrane vesicle protein per ml.

Marker-Enzyme Analysis. The mixed vesicle population was assayed for content of PM, ER, and mitochondria as described by Hochstadt et al. (13). The PM was monitored by 5'-nucleotidase, the ER by NADH dehydrogenase, and the mitochondria by succinate dehydrogenase. In addition, the total cell homogenate was assayed for these enzymes.

Membrane Vesicle Transport Assay. The reaction mixture (100 μl), containing 200 μg membrane vesicle protein, was made 50 mM in potassium phosphate buffer (pH 7.5) and 0.1 M in sucrose. The vesicles were pre-incubated at 37°C for 10 min. The reaction was started with [U-14C]adenosine or [U-14C]uridine at the indicated concentrations. At various times reaction mixtures were diluted with 10 volumes of warm 0.8 M NaCl, collected on 0.3 μm nitrocellulose filters, washed twice with 1 ml of warm 0.8 M NaCl, dried, and placed in a gas flow counter for radioactivity measurement. The use of a hypertonic wash solution was found, empirically, to result in low background values, minimum "leakage" of intravesicular transport products, and reproducible results. A similar method had been used originally by Kaback (14) and Hochstadt-Oer and Stadtman (8).

**TABLE 1. Marker-enzyme analysis of membrane vesicle populations**

<table>
<thead>
<tr>
<th>Cell source</th>
<th>5'-Nucleotidase %*</th>
<th>NADH dehydrogenase %*</th>
<th>Succinate dehydrogenase %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subconfluent 3T3</td>
<td>0.65</td>
<td>68</td>
<td>3.5</td>
</tr>
<tr>
<td>Confluent 3T3</td>
<td>0.90</td>
<td>59</td>
<td>5.2</td>
</tr>
<tr>
<td>Subconfluent SV-3T3</td>
<td>2.6</td>
<td>72</td>
<td>12</td>
</tr>
<tr>
<td>Confluent SV-3T3</td>
<td>1.5</td>
<td>85</td>
<td>8.5</td>
</tr>
</tbody>
</table>

5'-nucleotidase was used as a marker of the plasma membrane; NADH dehydrogenase, of the endoplasmic reticulum; and succinate dehydrogenase, of the mitochondria. All specific activity values are expressed as nmol/min per mg vesicle protein.

* Relative to total homogenate.

Chromatographic Analysis of Vesicle Contents. After a transport assay the nitrocellulose filters can be eluted with boiling water in order to remove intravesicular radioactivity. The eluate is lyophilized, resuspended in water, and spotted on thin-layer chromatography sheets (containing fluorescent indicator) as described previously (10). Development is in propionic acid:butanol:water, 62:125:87.4 (v:v:v). Spots that absorb ultraviolet light (unlabeled carrier is prespotted with sample) are assayed for co-chromatography of radioactivity by liquid scintillation counting as previously described (13).

Protein Analysis. Protein was assayed by the method of Lowry et al. (15), with bovine serum albumin as a standard.

**RESULTS**

Whole Cell Transport. Uptake of adenosine and uridine by 3T3 and SV-3T3 cells is shown in Fig. 1. The data in panels A and B indicate that uridine transport occurs at a 3-4 fold greater rate in subconfluent, actively growing 3T3 cells as compared to confluent, quiescent 3T3 cells. In contrast, adenosine uptake is not influenced by cell population density. Panels C and D show that both uridine and adenosine uptake occur at comparable rates in SV-3T3 whether the cells are subconfluent or confluent. In addition, less than 15% of the total radioactivity taken up is converted into acid-insoluble material (data not shown).

Marker-Enzyme Analysis. The validity of using 5'-nucleotidase, NADH dehydrogenase, and succinate dehydrogenase as markers for the PM, ER, and mitochondria, respectively, has been presented by Hochstadt et al. (13). Table 1 indicates that the ratio of PM to ER, based on specific activity values, is approximately the same in each mixed vesicle population derived from a specific cell type. In addition, mitochondrial contamination of these membrane vesicle populations is less than 1%, based on specific activity values (13).

Membrane Vesicle Transport. Membrane vesicle uptake follows Michaelis-Menten kinetics. Fig. 2 demonstrates the
concentration dependence of adenosine and uridine uptake by membrane vesicles from SV-3T3 cells. Uridine transport has a \( K_m \) of 8–12 \( \mu M \) and a \( V_{max} \) of 7–9 pmol/mg vesicle protein per min; adenosine transport has a \( K_m \) of 14–18 \( \mu M \) and a \( V_{max} \) of 20–25 pmol/mg vesicle protein per min. As seen in Fig. 3, membrane vesicles isolated from subconfluent 3T3 cells transported uridine at a 3-fold greater rate than did membrane vesicles from confluent 3T3 cells; adenosine uptake was approximately the same in both preparations. Furthermore, Fig. 3 also indicates that the kinetics of adenosine and uridine transport by membrane vesicles from subconfluent and confluent SV-3T3 cells appear to be identical.

**Chromatographic Analysis of Transport Products.** The data in Table 2 indicate that uridine is not metabolized as a result of transport into vesicles; greater than 80% of the uridine uptake products remain as uridine. Adenosine, however, undergoes metabolism during or after transport, involving conversion to inosine, hypoxanthine, and ribose 1-phosphate. The presence of 1 mM ribose 1-phosphate in the adenosine transport reaction mixture did not affect the final level of transport or the qualitative distribution of uptake products (data not shown). Qualitatively, adenosine and uridine transport products are the same with membrane vesicles isolated from each cell line.

**DISCUSSION**

We first measured whole cell uptake of adenosine and uridine in both confluent and subconfluent 3T3 and SV-3T3 cells. The data in Fig. 1 indicate that confluent, quiescent 3T3 cells transport uridine at a lower rate than actively growing 3T3 cells. This confirms, using harvested and resuspended cells, the observation of Cunningham and Pardee (1), who used a cell monolayer. Also, Cunningham and Pardee used Swiss 3T3 and polyoma-transformed cells whereas we used Balb/c 3T3 and SV-40-transformed cells.

In order to determine whether a change in a membrane component(s) or a change in a cytoplasmic factor is the basis of the altered uptake of uridine, we isolated membrane vesicles from subconfluent and confluent 3T3 cells, as well as from SV-3T3 cells. The membrane vesicle population contained plasma membrane and endoplasmic reticulum; there was less than 1% contamination by mitochondria as determined by succinate dehydrogenase activity (Table 1). Phase contrast microscopy was used during the membrane isolation procedure to determine that there was no contamination by whole cells or nuclei.

Initially, experiments with membrane vesicles from SV-3T3 cells were performed to verify that transport by membrane vesicles is a saturable process, occurring by a mediated uptake mechanism. The data in Fig. 2 confirm this. Concentrations of adenosine and uridine higher than 100 \( \mu M \) (Quinlan and Hochstadt, unpublished results) result in passive diffusion being the predominant uptake mechanism. Therefore, adenosine and uridine were used at concentrations below 50 \( \mu M \), at which passive diffusion rates are still minimal. We have not determined yet whether uptake of nucleosides can occur by an active, concentrative transport mechanism. The use of various metabolic inhibitors to try to answer this ques-
The results we have presented demonstrate that the decreased rate of uridine transport observed in "contact-inhibited" Balb/c 3T3 cells is due to an alteration in the membrane itself, since uridine transport by membrane vesicles isolated from confluent and subconfluent cells mirrored the transport patterns observed in whole cells. These results underscore the advantage and necessity of using purified membrane vesicles to study cell surface functions. The results obtained using membrane vesicles are strengthened further by the fact that the ratio of plasma membrane to endoplasmic reticulum was approximately the same for subconfluent and confluent cells within a particular cell type. Also, the constancy of the adenosine transport results throughout our studies serves as an internal control precluding alternative explanations for the results observed (for example, greater fragility of membrane associations in confluent, quiescent cells). In addition, an altered metabolic state alone cannot be used to explain the differences in uridine transport kinetics between actively growing and quiescent 3T3 cells, since there are no differences in the transport rates for adenosine (Fig. 1), amino acids (3), and uracil and inosine (Quinlan and Hochstadt, unpublished results).

We thus conclude that differences in uptake observed as a function of cell growth, at least in some cases, may be directly attributable to a regulatory mechanism operating at the level of the cell membrane. The possibility that growth control may in part be governed by membrane transport processes (16) is a viable hypothesis well worth continued investigation. In addition, we believe this study has clearly indicated that phenomena related to or indicative of cell growth control observable in intact cells are demonstrable in a cell-free system.

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