ON THE EXTRACELLULAR SYNTHESIS OF ADENOSINE TRIPHOSPHATE BY MAMMALIAN CELLS

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1. Introduction

Previous reports from another laboratory indicate that two glycolytic enzymes, glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12) and phosphoglycerate kinase (EC 2.7.2.3), are ecto-enzymes, i.e., they are located to some extent on the *outer* periphery of various mammalian (as well as yeast) cell membranes [1–6]. The evidence that supports this conclusion has been derived from the ability of 'intact' cells to synthesize extracellular ATP from the exogenous substrates for the two enzymes (glyceraldehyde-3-phosphate, NAD^+, orthophosphate, and ADP). The actual cells examined included Ehrlich mouse ascites tumor cells, HeLa cells, and several human cells (embryonic lung and adult skin fibroblasts, sarcoma cells, glia-like cells, glioma cells and erythrocytes). Since no function for this arrangement of the enzymes has been suggested or established, we decided to study the effects of these substrates on various properties of mammalian cell cultures. However, our unsuccessful attempts to reproduce these earlier results using a cell line (BALB/c 3T3 mouse fibroblast) that differs from the cells previously examined prompts us to communicate the following.

2. Materials and methods

Cell lines employed in these studies were the BALB/c mouse fibroblast (3T3) cells [7] and 3T3 cells non-productively transformed with the Kirsten sarcoma virus, KiSV (KiSV-3T3) [8]. The cells were isolated for study in the ATP-synthesizing medium by using 0.02% EDTA in physiological saline, according to the procedure of Ågren et al. [1], or 0.25% trypsin in Eagle's minimal essential (MEM) (Flow Laboratories, Rockville, Md., USA). In both instances the freed cells were immediately transferred into ten volumes of the same medium containing 1.5% Dextran T-40 (Pharmacia), centrifuged 10 min at 270 *g* and washed with the same solution at 4°C. The trypsin-treated cells were washed only once; the EDTA-treated cells, three times. The packed cells from EDTA or trypsin treatment were suspended in the same physiological saline or MEM solution with Dextran T-40 and counted, giving in all cases approximately 2×10^6 cells/ml.

The complete medium for ATP synthesis was prepared by adding to 0.45 ml of the cell suspension: 1) Tris–acetic acid (15 μmoles, pH 7.5) containing $MgCl_2$ (1.2 μmoles); 2) glutathione (250 nmoles, pH 7.5); 3) [^{32}P]orthophosphate (10 μCi, New England Nuclear, Boston, Mass., USA, carrier-free in 0.02 M HCl); 4) D,L-glyceraldehyde-3-phosphate (300 nmoles, pH 7.5), and 5) a solution of NAD^+ (230 nmoles) and ADP (385 nmoles) adjusted to pH 7.5. The final incubation volume in all experiments was 0.50 ml.

An incomplete medium lacking glyceraldehyde-3-phosphate, NAD^+ and ADP was used to determine possible enzyme leakage from the cells. Following

warming at 37°C for 15 min the cell suspension was centrifuged for about 5 min at 1000 rpm (270 *g*). The supernatant was split into two portions: to one, only buffer containing glyceraldehyde phosphate (300 nmoles) was added; to the other, all of the missing substrates were added to give the correct final concentration.

As an additional control, solutions lacking cells but containing rabbit muscle glyceraldehyde phosphate dehydrogenase (25 μg; Sigma Chem. Corp., St. Louis, Mo., USA) and yeast phosphoglycerate kinase; (25 μg; Boehringer Mannheim Corp., New York, N.Y., USA) were used. Either the solution contained all the substrates and enzymes for ATP synthesis from glyceraldehyde phosphate and orthophosphate or only the two enzymes were omitted.

After the last addition (NAD^+-ADP solution) the samples (cell suspension, cell-wash supernatant and cell-free solution) were incubated at 37°C. At 5 and 60 min 100 μl aliquots were withdrawn and added to 20 μl of a 60% trichloroacetic acid solution and the mixture was centrifuged at 10 000 rpm for 10 min. Ten microliters of clear supernatant, corresponding to about 140 000 cpm, were placed under a stream of warm air on a cellulose strip impregnated with polyethyleneimine (3.5 × 10 cm; Brinkmann Instruments, Inc., Westbury, N.Y., USA). The strips were eluted first with 4M formic acid (7 min) and then eluted further, without any intermediate drying of the strip, using a solution of 4 M formic acid and 1.0 M ammonium formate (23 min). The positions of ATP, ADP and orthophosphate on the dried strips were detected by their ultraviolet fluorescence or by spraying with the Hanes-Isherwood reagent. Authentic samples routinely appeared at R_f values of 0.05, 0.35, and 0.55 for ATP, ADP and P_i, respectively. Sections of the plastic strip containing radioactivity were cut off and counted in a scintillation vial containing 5 ml of Aquasol (New England Nuclear).

3. Results and discussion

In table 1 are the results using normal and virally-transformed 3T3 cells. Cells in suspension appear to synthesize ATP as shown by the increase in radioactive phosphorous incorporated into ATP during the period of incubation (60 min). The extent of radioisotopic labeling in ATP is about the same for the cell suspension and the solutions containing the purified dehydrogenase and kinase. No labeled ATP was detected in media that had not been exposed to the cells or lacked both dehydrogenase and kinase. In a separate experiment no labeled ATP was produced in a solution containing the dehydrogenase and its substrates but lacking ADP and the kinase. This result indicates that 1,3-diphosphoglycerate, another possible ^{32}P-containing species, is chromatographically separated from the nucleotides.

The observation of ATP synthesis by the cell suspension has previously been made by Ågren et al. [1]. However, at variance with their results are our data (table 1) that indicate the cell-free supernatants from washing the cells do contain the two glycolytic enzymes necessary for ATP synthesis by means of the substrate-level oxidative phosphorylation of glyceraldehyde phosphate. These results pertain regardless of the method for removing the cells from the culture flask, namely treatment with either EDTA or trypsin. Furthermore, attempts to treat the cells under milder conditions, such as fewer centrifugations or use of culture media, failed to produce different results that would agree with the earlier reports [1–6].

Thus, we must conclude that these manipulations, similar (if not identical) to those described by Ågren et al. [1], lead to significant damage to the mouse fibroblast cultured cells, as manifested by leakage of glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase into the medium. Although the extent of cell damage appears to be slight, adequate amounts of the two enzymes are released for the synthesis of radioisotopically labeled ATP in the absence of cells. We are uncertain as to the reason for these discrepant results. However, our additional observation of a potent cytotoxic effect by glyceraldehyde-3-phosphate on various mouse 3T3 cell lines (Fenselau and Long, manuscript in preparation) and the uncertainties concerning the function of the two enzymes were they located on the outside of the plasma membrane (where the necessary substrates are unlikely to be found) only raise more doubts about these earlier observations.

Our conclusion questioning the identification of glyceraldehyde phosphate dehydrogenase as an ecto-enzyme is reinforced by recent reports on the proteins of the erythrocyte membrane. Based on earlier obser-

Table 1
Total ^{32}P-ATP synthesis by mammalian cell suspensions and cell-free solutions via the substrate-level oxidative phosphorylation of glyceraldehyde phosphate

Incubation Time (at 37°C)	^{32}P-ATP Content, in cpm			
	EDTA Treatment		Trypsin Treatment	
	5 min	60 min	5 min	60 min
3T3 Cell Line				
Cell Suspension	693	4630	–	–
Cell-wash Supernatant, Complete system	755	4800	–	–
Cell-wash Supernatant, NAD^+ and ADP absent	179	170	–	–
KiSV-3T3 Cell Line				
Cell Suspension	1530	5360	1160	2330
Cell-wash Supernatant, Complete system	1580	5590	1460	2600
Cell-wash Supernatant, NAD^+ and ADP absent	535	314	45	105
Cell-free Solution				
GAPDH and PGK present	4010	5960	1350	2380
GAPDH and PGK absent	328	521	230	274

The 3T3 and Kirsten sarcoma virus-transformed 3T3 (KiSV-3T3) cells were isolated and were washed or suspended in physiological saline or MEM solution containing 1.5% Dextran T-40 (see Materials and methods for details). The complete system refers to the presence in the medium of all substrates for glyceraldehyde phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK). These two purified enzymes were also used in a complete, cell-free system. ^{32}P-ATP was separated on polyethyleneimine–cellulose chromatographic strips and counted as described in Materials and methods.

vations [5,6], the dehydrogenase would be expected to be selectively labeled by red cell membrane-impermeable reagents. However, by means of these techniques only a few proteins have been identified on the exterior surface of the erythrocyte membrane, none of which appears to be the dehydrogenase (as judged by size and/or presence of bound carbohydrate [9–13]. In fact the dehydrogenase has been shown to be loosely associated with the *inner* surface of this membrane [14–17]. This evidence, taken *in toto*, suggests that additional studies on the cell lines originally investigated are clearly warranted.

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