BLOCKS IN NUCLEOTIDE PHOSPHODIESTERASE AND ALKALINE PHOSPHATASE ACTIVITY IN TRANSFORMED MAMMALIAN CELLS

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Received 19 December 1972

1. Introduction

We have previously shown [1] that the two sugar nucleotides, UDP-*N*-acetyl-D-galactosamine and UDP-*N*-acetyl-D-glucosamine, are enzymatically hydrolyzed by normal hamster cell extracts to yield the free sugars in two steps. In the first step, the sugar nucleotides are split by a nucleotide phosphodiesterase activity to UMP and the appropriate sugar-1-phosphate, and the second enzymatic step then hydrolyzes the phosphorylated intermediate sugar to the free sugars. We have also shown that hamster cell lines transformed by Rous sarcoma virus (RSV) or Simian virus 40(SV40) were blocked in the nucleotide phosphodiesterase activity, whereas cell lines transformed by polyoma virus or after treatment with the chemical carcinogen dimethylnitrosamine (DMNA) were blocked in the second phosphohydrolase step. The present experiments were undertaken to determine the hydrolysis of GDP-mannose, GDP-fucose and UDP-glucose in normal and these transformed hamster cells, and to further characterize the phosphohydrolase activity.

Our results have shown that normal hamster cell extracts can hydrolyze in two steps these 3 sugar-nucleotides in addition to UDP-*N*-acetyl-D-galactosamine and UDP-*N*-acetyl-D-glucosamine; that the block in the nucleotide phosphodiesterase activity in RSV and SV40 transformed cell applies to all 5 sugar nucleotides; and that the phosphohydrolase that is blocked in transformed cells is an alkaline phosphatase.

Abbreviations:

SV40: Simian virus 40; RSV: Rous sarcoma virus; DMNA: Dimethylnitrosamine.

2. Materials and methods

2.1. *Cell lines*

The normal cells were secondary cultures of golden hamster embryos. The transformed cells were lines of golden hamster embryo cells transformed *in vitro* by polyoma virus, or after treatment with the chemical carcinogen DMNA [2] and lines derived from a SV40 and RSV-induced hamster tumor (Flow Laboratories, Bethesda, Md.) [1]. Cells were cultured in Eagle's medium with a 4-fold concentration of amino acids and vitamins and 10% fetal calf serum. The cells were always harvested 2–3 days after seeding 10^6 cells per 100 mm Petri dish and, unless otherwise stated, they were all in a comparable growing non-confluent phase. After removal of the medium, the cells were washed *in situ* with 0.1 M phosphate buffered saline scraped from the Petri dish with a rubber policeman, collected by centrifugation and washed with 0.1 M Tris-HCl buffer, pH 7.4. The pelleted cells were ruptured by sonic vibration and suspended in 0.083 M Tris buffer [1].

2.2. *Materials*

The sugar nucleotides UDP-([U-^{14}C] glucose), GDP-([U-^{14}C] mannose), GDP-([U-^{14}C] fucose), UDP-([U-^{14}C] galactose), and UDP-([U-^{14}C] glucoronic acid); glucose substituted with a phosphate in carbon 1, or in carbon 6 and α-D-galactose [U-^{14}C]-1-phosphate, were from the Radiochemical Centre, Amersham; α-D(+)mannose-1-phosphate, and D-mannose-1-phosphate, and D-mannose-6-phosphate, D-galactose-6-phosphate, alkaline phosphatase (from hog intestinal mucosa), phosphodiesterase (from *Crotalus adamanteus* venom, type II), 3-phosphoglyceric acid and *p*-nitro-

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phenylphosphate were obtained from Sigma Chemical Company, St. Louis. All other saccharides and reagents were products of the highest purity available.

2.3. *Assay for hydrolysis of sugar nucleotides*

The overall hydrolysis of UDP-glucose, GDP-mannose and GDP-fucose by extracts of normal and transformed hamster cells, was assayed as follows: 160 μg protein of cell extracts and 0.35 μmoles of the appropriate sugar nucleotide (70,000 cpm for GDP-mannose and GDP-fucose and 100,000 cpm for UDP-glucose) were incubated for 30 min at 37° in a total volume of 85 μl containing 2.5 μmoles Tris-HCl buffer, pH 8.6, and 0.5 μmoles $MnCl_2$. The reaction was stopped by adding 10 μl of a 1 M solution of ATP. Aliquots were chromatographed on Whatman No. 3 MM paper with ethylacetate–pyridine–glacial acetic acid–water (5:5:1:3), which will be referred to as solvent 1, in a descending system for 16 hr. Radioactive products on the paper were detected with a Packard radiochromatogram scanner (Model 7021) and the segments were counted with toluene–phosphor solution in a Packard Tri-carb scintillation spectrometer.

2.4. *Assay for alkaline phosphatase*

The reaction mixture consisted of 0.1 M Tris-HCl buffer, pH 9.4, or 0.05 M CAPS (cyclohexylaminopropane sulfonic acid) buffer, pH 10.4, 5 mM of either *p*-nitrophenylphosphate or 3-phosphoglyceric acid as substrate, 4 mM $MgCl_2$ and 200 μg of cell extract protein in a final volume of 0.1 ml. After incubation at 37° for 30 min, 0.2 ml of 10% trichloroacetic acid were added. Inorganic phosphate was determined in the supernatant as described [3], and activity was expressed as μmoles P_i/30 min per mg of protein. When *p*-nitrophenylphosphate was used as substrate, the *p*-nitrophenol liberated was estimated by measuring absorbance at 410 nm using a Zeiss Model PMQ II spectrophotometer. Alkaline phosphatase activity was determined in situ in cells growing in culture by the agar-*p*-nitrophenylphosphate technique [4].

3. Results and discussion

3.1. *Hydrolysis of GDP-mannose, GDP-fucose and UDP-glucose by normal hamster cells*

Incubation of the 3 sugar nucleotides with extracts from normal hamster cells for 10 min gave 3 radioactive peaks on the chromatogram using solvent 1 (see Materials and methods). With GDP-mannose and GDP-fucose, material from the slowest moving peak was identified by its R_f value as the non-hydrolyzed sugar nucleotide. The fastest moving peak was identified chromatographically as either free mannose or free fucose. The third peak was identified as the phosphate 1-substituted mannose or fucose by the following procedure: After 40 hr chromatography of the reaction mixture in solvent 1, the area of the third peak, which was then well separated from the slowest moving peak, was eluted with water and lyophilized. The lyophilized material was incubated for 15 min with 10 μg intestinal alkaline phosphatase in sodium-barbiturate buffer, pH 8.6 with 0.01 M $MgCl_2$. The material was then re-chromatographed in solvent 1, or in pyridine–butanol–water (6:4:3), and the radioactivity was found exclusively in the region of free mannose or fucose, thus indicating that the intermediate peak is a phosphate containing sugar. This phosphorylated sugar was identified as either mannose-1-phosphate or fucose-1-phosphate by its co-chromatography with the products of enzymatic hydrolysis of GDP-mannose or GDP-fucose by a snake venom phosphodiesterase, which splits these two sugar-nucleotides into GMP and the appropriate sugar-1-phosphate. The formation of mannose-1-phosphate rather than mannose-6-phosphate was also confirmed by co-chromatography with a commercial mannose-1-phosphate in butanol–acetic acid–water (4:2:1), which clearly separates between the 2 phosphate derivatives of mannose [5].

Incubation of UDP-glucose for 10 min with normal hamster cell extracts, and chromatography of the reaction mixture in solvent 1, also gave 3 peaks. The material from the slowest peak was re-chromatographed in ethanol–ethylmethylketone–0.5 M morpholinium tetraborate, pH 8.6, in 0.01 M EDTA [6] and identified as UDP-glucose. No UDP-galactose or UDP-glucoronic acid was detected, so that there was no epimerization or oxidation of UDP-glucose in the extracts of hamster cells under these conditions. The fastest peak was identified as free glucose, and there was no galactose formed as determined by chromatography with pyridine–butanol–water (6:4:3) on paper previously treated with 0.02 M Borax. The third peak obtained in solvent 1 was eluted from the paper and re-chromatographed for 24 hr in methylcellosolve–methylethyl-

Table 1

Sugar nucleotide hydrolysis by extracts of normal and transformed hamster cells*.

Hydrolysis products* (cpm)	Cell types				
		Transformed by:			
	Normal	Simian virus 40	Rous sarcoma virus	Polyoma virus	Dimethylnitrosamine
GMP-mannose	210	37900	38040	23100	8700
Mannose-1-phosphate	1490	820	400	16300	31600
Mannose	36400	1020	730	190	40
GMP-fucose	470	36400	37900	20400	10400
Fucose-1-phosphate	3200	1800	580	18900	28900
Fucose	35800	1730	1030	140	70
UDP-glucose	180	36310	38000	19020	6200
Glucose-1-phosphate	320	270	180	2500	3040
Glucose-6-phosphate	5240	1600	800	18200	29300
Glucose	33800	1400	1010	200	100

* The extracts were incubated for 30 min as described in Materials and methods.

ketone–3 N ammonium hydroxide (7:2:3) saturated with boric acid [7]. Two radioactive peaks were obtained and identified as glucose-1-phosphate (R_f 1.0) and glucose-6-phosphate (R_f 0.38). The interconversion of glucose-1-phosphate and glucose-6-phosphate by a phophoglucomutase is shown in table 1 and the results indicate that there was a preferential accumulation of the 6-substituted phosphate sugar.

In addition to the radioactive products that were formed upon hydrolysis of the 3 sugar nucleotides, there was also a non-radioactive peak detected on chromatograms developed with ethanol–1 M ammonium acetate, pH 7.5 (7:3, v/v). Depending on the hydrolyzed sugar-nucleotide, the non-radioactive product was characterized as uridine or guanosine. The formation of uridine and guanosine as a degradation product of UMP and GMP by enzymes such as 5′-nucleotidase [8] was blocked by adding 5×10^{-3} M of an inhibitor, *p*-chloromercuribenzoate, to the reaction mixture. The level of hydrolytic activities was the same in normal hamster cells harvested either in their growing or in confluent non-growing phase.

3.2. *Blocks in the hydrolysis of GDP-mannose, GDP-fucose and UDP-glucose in transformed cells*

Extracts from hamster cells transformed by RSV, SV40 polyoma virus or after treatment with DMNA, were tested for their ability to hydrolyze GDP-mannose, GDP-fucose and UDP-glucose. Whereas about 88–95% of the input radioactive material was converted to the free sugar by extracts of normal hamster cells, there was only 0.1–3% of such conversion by the extracts from transformed cells (table 1). The mixing of extracts from normal and transformed cells did not inhibit the ability of normal cell extracts to hydrolyze the sugar nucleotides to free sugars. The block in overall hydrolysis in transformed cells was therefore apparently not due to increased levels of inhibitors in these cells. Results obtained with different times of incubation with GDP-mannose have shown that in normal cells a nucleotide phosphodiesterase activity resulted in accumulation of the intermediate product, mannose-1-phosphate, which was then hydrolyzed by a phosphohydrolase activity to the free mannose (fig. 1). In polyoma and DMNA transformed cells, the mannose-1-phosphate accumulated due to an almost complete block in the second hydrolytic step. Similar results to those in fig. 1 were obtained with GDP-fucose

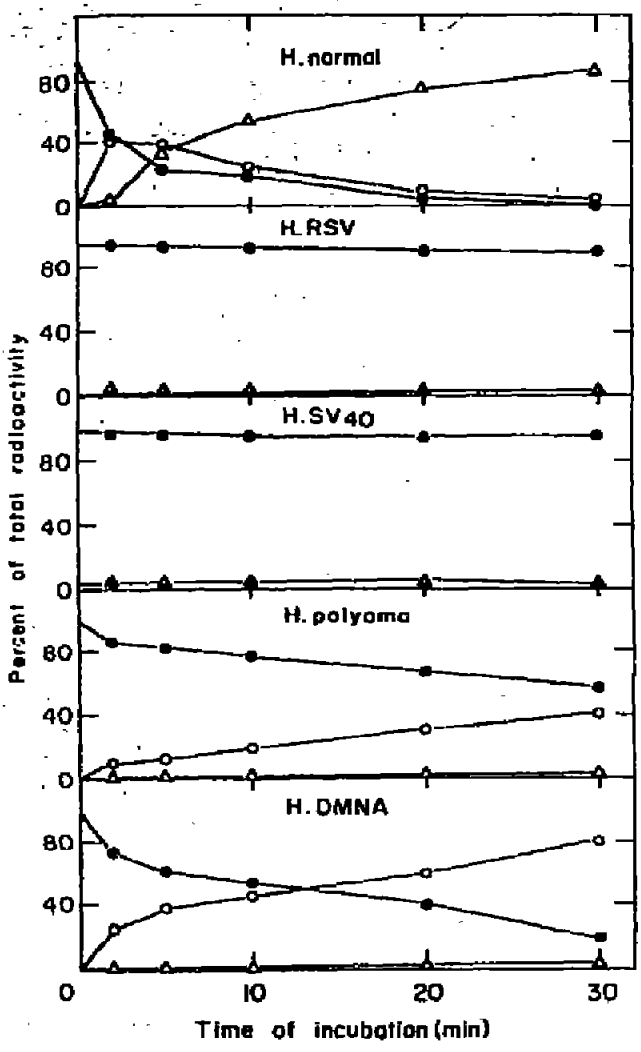

Fig. 1. Hydrolysis of GDP-mannose and the formation of mannose-1-phosphate and mannose by hamster normal and transformed cells. Incubation conditions as in Materials and methods. (●—●—●) GDP-mannose; (○—○—○) mannose-1-phosphate; (△—△—△) mannose. H = hamster.

and UDP-glucose. In order to determine whether the block in SV40 and RSV transformed cells (fig. 1) was in the first or second hydrolytic step, extracts of cells were incubated with exogenous sugar-1-phosphate. These extracts hydrolyzed the sugar-1-phosphate into free sugar, 30% and 50%, respectively, of the hydrolysis found with extracts of normal cells. The RSV and SV40 transformed lines, were therefore, presumably blocked in the nucleotide phosphodiesterase activity.

Table 2

Alkaline phosphate activity in extracts of normal and transformed hamster cells.

Cell types	(μmoles p-NP/ mg protein/ 30 min*)	(μmoles P_i/ mg protein/ 30 min**)
Normal	24.1	6.7
Transformed by:		
Simian virus 40	8.4	1.9
Rous sarcoma virus	3.2	0.8
Polyoma virus	0.09	0.02
Dimethylnitrosamine	<0.04	<0.01

* *p*-Nitrophenylphosphate as substrate.
** 3-Phosphoglyceric acid as substrate.

3.3. *Block in alkaline phosphatase activity in transformed cells*

In view of the present and previous results [1], that hydrolysis of the intermediate phosphorylated sugar compounds can be blocked in transformed cells, cell extracts were incubated with the two non-sugar phosphorous compounds, *p*-nitrophenylphosphate and 3-phosphoglyceric acid. Normal cells hydrolyzed both these substrates to free phosphor at alkaline pH, due to a phosphatase activity, and with transformed cells, there was almost a complete block in the polyoma and DMNA transformed cells, and a markedly lower than normal activity in the SV40 and RSV transformed cells (table 2). The difference in alkaline phosphatase activity was similar in growing and confluent transformed cells and was not associated with any difference in acid phosphatase activity, as measured by the method of Hubscher and West [9].

Alkaline phosphatase activity was also determined in situ on cells growing as colonies by the agar *p*-nitrophenylphosphate technique [4] at 10 days after seeding. There were no colonies with detectable alkaline phosphatase in 50,000 colonies from the DMNA transformed line, and there were 0.02% and 3% colonies with detectable enzyme in the polyoma and SV40 transformed lines, respectively. The predominantly enzyme-negative population of the 2 latter cell lines therefore contained some alkaline phosphatase positive cells. It will be of interest to determine the relationship between other cellular properties, and the presence or absence of alkaline phosphatase.

Acknowledgement

This work was supported by Contract No. 69-2014 with the Special Virus Cancer Program of the National Cancer Institute, United States Public Health Service.

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