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INVOLVEMENT OF PROTEASE IN L-GLUTAMINE CONTROL OF NUCLEIC ACID AND POLYPHOSPHATE METABOLISM IN CELLS TRANSFORMED BY 9,10-DIMETHYL-1,2-BENZANTHRACENE, SV40 AND H-ras ONCOGENE

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SUMMARY: Mammalian cells transformed with either 9,10-dimethyl-1,2-benzanthracene, SV40 or H-ras oncogene dramatically changed their ability to synthesize DNA and RNA and metabolize polyphosphate when L-glutamine was withdrawn from the growth medium or when heat shocked (growth at 42°C). Untransformed, DNA and RNA synthesis decreased by 50-80% when glutamine was withdrawn, but polyphosphate accumulated whether or not glutamine was supplied. Heat shock did not alter this response. Transformed isogenic cells responded differently; at 37°C, they decreased their synthesis of DNA and RNA if starved for glutamine, whereas at 42°C, synthesis was optimal without glutamine. Transformed cells accumulated polyphosphate at 37°C when starved for glutamine, but at 42°C, no polyphosphate accumulated. This apparent non-dependence on glutamine by transformed cells when heat shocked was found to be due to the production of glutamine from serum proteins through induction of a protease(s).

Mammalian cells in culture may be transformed in different ways: spontaneously (1), through infection with tumour-causing viruses (2), by treatment with chemical carcinogens (3), upon transfection with oncogenes (4) and by radiation (5). That the cells are indeed transformed is demonstrable by *in vivo* assay in which the cells are injected into syngeneic immunosuppressed hosts resulting in tumour production (6,7). This method is expensive, time consuming, and susceptible to errors because factors such as choice of host, method of cell preparation, site and mode of injection and the type of transformed cell used affect the outcome (8,9). More commonly, *in vitro* transformation assays relying on parameters such as cell morphology and cytology, growth characteristics, certain physical and biochemical properties and genetic markers are used. But none of these is an invariant indicator of transformation (10-15).

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A puzzling feature of transformed cells is that they express considerably higher levels of proteolytic enzyme activity than untransformed cells (16-18). The precise role of the proteases is not fully understood although it is thought that they contribute to some of the distinguishing characteristics of transformation (19), and they have well known functions in nutrition. This communication presents data relating transformation, proteases and nutrition in a novel way.

In 1979, we reported that transformed and untransformed cells of human, mouse, hamster and monkey metabolized polyphosphate differently when starved of L-glutamine. Transformed cells accumulated polyphosphate only when glutamine was withdrawn from the growth medium whereas untransformed cells accumulated it whether or not glutamine was present (20). That study, however, was done with a melange of genetically related and unrelated transformed and untransformed cells. We have now used different cell lines that are in isogenic pairs of transformed and untransformed states. Transformation was achieved with 9,10-dimethyl-1,2-benzanthracene, SV40 and H-ras oncogene. We show that not only the metabolism of polyphosphate, but the synthesis of DNA and RNA was altered when glutamine was removed from the growth medium of transformed cells. Furthermore, when transformed cells were heat shocked, they displayed glutamine independency in the synthesis of nucleic acids and metabolism of polyphosphate. Remarkably, inactivation of protease(s) induced by heat shock caused the cells to become glutamine dependent once again. We suggest that (a) altered polyphosphate metabolism may be exploited as an invariant diagnostic parameter of transformation and (b) proteases of transformed cells play a crucial role in changing the nutritonal milieu of the cell by their action on serum proteins.

MATERIALS & METHODS

Cells. WI-38 (a human lung diploid cell line), its SV40 transformed counterpart WI-38VA13, and On Ser (human HPRT$^-$ cell line), were obtained from the American Type Culture Collection, MD. On Ser was chemically transformed by us with 9,10-dimethyl-1,2-benzanthracene (5 µg/mL) according to the procedure of Chen and Heidelberger (3) and tested for transformation by in vitro methods. This transformed human cell has been cultured continuously for over 4 years. Mouse myoblast 10T$_{\frac{1}{2}}$ and its H-ras oncogene transformed counterpart, ciras-2, were supplied by Dr. J. Borsa, Atomic Energy Research Station, Pinawa, Manitoba.

Polyphosphate analysis. Labelling of cells with ^{32}P-orthophosphate, identification and quantitation of acid soluble nucleotides and polyphosphate by one and two-dimensional chromatography on polyethyleneimine (PEI) cellulose plastic-backed thin layer sheets followed by autoradiography have been described (21,22).

DNA and RNA analysis. Cells were labelled with either ^{3}H-thymidine or ^{3}H-uracil, DNA and RNA isolated and radioactivity determined as per Goh, Wright and LéJohn (23).

Media. Alpha-minimal essential medium (GIBCO, Canada) was used for all growth studies. Alpha-minimal essential medium lacking L-glutamine was prepared by us. Dialysed foetal calf serum (GIBCO, Canada) was routinely used as supplement.

Determination of glutamine. Glutamine was quantitated by a coupled glutaminase-glutamic dehydrogenase assay (24).

Chemicals. Radioisotopes were purchased from radiochemical companies, Amersham and New England Nuclear. Biochemicals and enzymes were obtained from Sigma Chemical Co. All other reagents and chemicals were from commercial sources.

RESULTS

Polyphosphate metabolism at 37°C and 42°C. All cells used grew optimally at 37°C but were thermally stressed at 42°C although they survived and resumed normal growth if returned to 37°C after 4h. Prolonged incubation (12h) at 42°C was lethal. Untransformed cells maintained their individual levels of the polyphosphate whether or not they were glutamine-deficient or thermally stressed (Table 1). By contrast, in transformed cells at 37°C, glutamine withdrawal caused an almost non-detectable polyphosphate to accumulate to levels that were even higher than those of untransformed cells; but at 42°C, no polyphosphate accumulated. Heat shock, therefore, conditioned transformed cells into behaving as if they were not starving for glutamine. It should be mentioned that while these cells, like most mammalian cells, possess an active glutamine synthetase, glutamine is still essential for their growth.

DNA and RNA synthesis at 37°C and 42°C. Previous work of Goh and LéJohn (22) had shown that there is an inverse relationship between nucleic acid and polyphosphate synthesis in transformed cells. The current results support that conclusion and show (table 2) that at 37°C, DNA and RNA synthesis was affected by glutamine withdrawal in both transformed and untransformed cells. DNA was labelled twice as effectively and RNA thrice as effectively in untrans-

Table 1. ^{32}P-labelled polyphosphate in isogenic transformed and untransformed mammalian cell lines grown at different temperatures in the presence and absence of L-glutamine (gln)

Cell line	Polyphosphate (% of control*) 37°C -gln	37°C +gln	42°C -gln	42°C +gln
Untransformed				
On Ser	76.5	73.5	74.6	79.4
WI-38	58.8	55.1	63.1	57.1
$10T_{1/2}$	61.7	54.9	60.7	58.0
Transformed				
On Ser-DMBA**	116.1	2.1	2.0	2.2
WI-38 VA13	107.1	4.4	3.3	3.1
$10T_{1/2}$ ciras-2	88.4	2.9	3.1	2.6
On Ser-DMBA + TLCK†	96.8	4.8	94.8	2.6

Cells were plated at a density of 0.5 x 10^6/mL in 35 mm plates and grown for at least 24h before use. Cells were labelled with ^{32}P-orthophosphate (250 µCi/mL) for 4h under the appropriate experimental condition before extraction of acid soluble nucleotides and polyphosphate and analysis as referred to in "Materials and Methods".

*Control (100%) is the amount of polyphosphate accumulated in CHO-WT cells for 4 h at 37°C in the absence of glutamine. CHO-WT is the first mammalian cell line in which the polyphosphate was identified (20,22, 23).

**9,10-dimethyl-1,2-benzanthracene

†Nα-p-tosyl-L-lysine chloromethylketone (100 µg/mL).

formed and transformed cells when glutamine was supplied than in its absence (see columns 2,4,6 and 8 of table 2). On the other hand, at 42°C, transformed cells synthesized DNA and RNA optimally and independently of glutamine (see columns 5 and 9 of table 2) while untransformed ones maintained their reliance on glutamine for optimal DNA and RNA synthesis (columns 3 and 7, table 2). Therefore, like polyphosphate metabolism, thermal stress altered nucleic acid metabolism in transformed cells in a way that caused them to become independent of glutamine for optimal synthesis of DNA and RNA.

Influence of protease inhibitors on nucleic acid and polyphosphate metabolism. While several hypotheses can be proposed to explain the phenomenon, we favoured and experimentally tested one; that is, the 'apparent' non-requirement for glutamine was due to protease degradation of serum proteins thereby providing the cells with much needed glutamine. First, protease inhibitors

Table 2. DNA and RNA synthesis by On Ser and 9,10-dimethyl-1,2-benzanthracene On Ser (On Ser-DMBA) cells in the presence and absence of L-glutamine (gln) and in the presence and absence of protease inhibitors, Nα-p-tosyl-L-lysine-chloromethylketone (TLCK) and Nα-p-tosyl-L-arginine methylester (TAME) at 37° and 42°C

	^{3}H-thymidine incorp. (% of control)				^{3}H-uracil incorp. (% of control)			
	On Ser		On Ser-DMBA		On Ser		On Ser-DMBA	
Addition	37°C	42°C	37°C	42°C	37°C	42°C	37°C	42°C
- gln	50	43	64	121	35	33	17	74
+ gln (2 mM)	100*	89	119	132	100*	118	48	73
- gln + TLCK	47	42	55	53	30		13	15
+ gln + TLCK	82	90	109	100	78		71	62
- gln + TAME	49	46	54	53		36	17	13
+ gln + TAME	95	77	113	114		80	65	59

Cells were plated at a density of 0.4 x 10^6/mL in 60 mm plates (On Ser) and at a density of 0.6 x 10^6/mL (On Ser-DMBA) and incubated at 37°C for 48h. Cell densities at the end of this period were 0.75 x 10^6/mL (On Ser) and 1.5 x 10^6/mL (On Ser-DMBA) and subconfluent. The cells were labelled with ^{3}H-thymidine and ^{3}H-uracil for 4h under the specified conditions. DNA and RNA were recovered as described (23) and radioactivity incorporated determined on a per cell basis. TLCK and TAME at 100 µg/mL.

*Control (100%) is the incorporation into DNA or RNA of On Ser in complete medium for 4h at 37°C.

Nα-p-tosyl-L-lysine chloromethylketone (TLCK) and Nα-p-tosyl-arginine methylester (TAME) were supplied to transformed cells that were heat shocked in glutamine-free medium. Normally, these cells do not accumulate polyphosphate and synthesize nucleic acids optimally. But in the presence of TLCK, they accumulated polyphosphate (table 1) and nucleic acid synthesis became dependent on exogenous supply of glutamine (table 2). Second, as shown in table 3, upon shifting the temperature from 37°C to 42°C, significant amounts of glutamine accumulated in the growth medium (initially glutamine-free) and cells of transformed but not untransformed cultures. Protease inhibitor added prior to the temperature shift prevented this (not shown). The source of glutamine produced remains to be precisely determined. Third, analysis of the medium for proteolytic activity, using TAME as substrate, revealed that medium from transformed cells incubated at 42°C had twice as much protease activity as cells incubated at 37°C. No protease activity was detected in the medium of untransformed cells.

Table 3. Accumulation of L-glutamine in the medium and cells of transformed On Ser cultures when thermally stressed

Growth condition	L-Glutamine (mM) detected in medium	cell extract
37°C, - glutamine (4h)	0.06	0.0
42°C, - glutamine (4h)	0.38	0.39

Samples were prepared as follows. Growth medium (10 mL) in Brockway flask was decanted and collected for medium analysis. The cells were washed with saline, covered with 5 mL 10 mM Tris·Cl, pH 8 and scraped from the flask. They were then homogenized by hand at 0°C, debris removed by centrifugation and the supernatant used as cell extract.

Glutamine was determined as glutamate by the coupled assay procedure of Lund (24). An aliquot of each sample was incubated for 15 min with protease inhibitor Nα-p-tosyl-L-lysine chloromethylketone (100 µg/mL) and then for 1h with glutaminase (*Escherichia coli*) at 37°C. Glutamate was then quantitated with glutamate dehydrogenase (bovine liver). Glutamine was undetected in the medium and cells of untransformed On Ser cultures incubated under the same conditions. Corrections made for L-glutamate present in the growth medium.

DISCUSSION

These results indicate that the pattern of polyphosphate metabolism in transformed and untransformed cells in response to nutritional and thermal stress may be a valuable indicator of transformation. Regardless of the mode of transformation (chemical, viral or oncogenic as shown here; spontaneous or X-irradiation (unpublished data)), mammalian cells from diverse species, tissues and organs exhibited the same response; which is that no polyphosphate accumulated when the cells were starved of glutamine at 37° and 42°C, and even when glutamine was provided at 42°C. Untransformed cells, on the other hand, accumulated polyphosphate at both temperatures whether or not glutamine was present. The change in response was established as soon as the cells were transformed and it persisted with their immortalization.

The effect of glutamine on polyphosphate metabolism is specific, for other amino acids, notably glutamate and isoleucine, are ineffective (23, and unpublished). This unique role of glutamine may be explained on the basis of its central role in carbon and respiratory metabolism of transformed cells (25-30). But the inability of glutamate to substitute for glutamine suggests that the answer may not reside entirely on the fact that glutamine is a

respiratory fuel. Glutamine and the polyphosphate may have a relationship through their involvement in nucleic acid metabolism, especially de novo synthesis of nucleotides. Indeed, we have shown through mutant studies that the polyphosphate has a probable role in purine and pyrimidine biosynthesis (22) and that it inhibits enzymes of nucleic acid biosynthesis (20,21).

The remarkable feature of heat shock on nucleic acid synthesis and polyphosphate metabolism in transformed cells is a new phenomenon that is most simply explained by protease activation. It is an established fact that transformed cells secrete abundant amounts of protease compared to untransformed cells (16-18). Because protease inhibitors reversed the effects of thermal stress which prevented polyphosphate from accumulating in transformed cells (table 1) and caused nucleic acid synthesis to become independent of glutamine (table 2), proteases clearly have a central role in the phenomenon. Thermally stressed transformed cells appear capable of generating glutamine (table 3). If glutamine is serving as a respiratory fuel inter alia, a reasonable conjecture is that glutamine is derived from proteins in the medium since it would be unproductive for the cell to derive glutamine from its own proteins. Thermal stress, therefore, may promote activation of protease(s) produced by transformed cells not by untransformed ones, and the protease(s) may aid in the recovery of amino acids essential for growth from serum proteins. By changing the chemical milieu in this manner, a transformed cell can exert profound effect either on its own fate or that of its normal neighbours. We note that this response of transformed cells to thermal stress may be important in understanding heat therapy in the clinical management of tumours.

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