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Increased Inosine 5'-Monophosphate Dehydrogenase
Gene Expression in Replicating Cells:
A Response to Growth Factors, Not to Changes in Cell Cycle Parameters

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ABSTRACT

We have analyzed levels of inosine 5'-monophosphate dehydrogenase (IMPDH; E.C. 1.1.1.205) type II mRNA levels in a human melanoma cell line, SK-MEL-131, and a Chinese hamster ovary cell line synchronously progressing through the cell cycle following treatment with aphidicolin. Following release from the aphidicolin block at the G₁-S phase boundary, the type II IMPDH gene was found to be constitutively expressed at a similar level during all stages of the cell cycle. To analyze growth regulation, as opposed to cell cycle regulation, stable SK-MEL-131 transfectants that express a type II IMPDH-promoted heterologous construct were assayed following deprivation of serum growth factors and after restimulation with fresh serum. Serum deprivation resulted in down-regulation of both steady state type II IMPDH mRNA levels and promoter activity, while restimulation with serum resulted in up-regulation of these parameters. These findings support the conclusion that the increase in IMPDH type II gene expression in replicating cells is mainly due to growth factor regulation rather than changes in cell cycle parameters and that this regulation is mediated primarily by a transcriptional mechanism. The increased level of IMPDH expression and activity found in many tumors may therefore also be due to a transcriptionally mediated response to growth factors.

Inosine 5'-monophosphate dehydrogenase (IMPDH; EC 1.1.1.205) is the enzyme that catalyzes the formation of xanthosine 5'-monophosphate from inosine 5'-monophosphate. In the purine *de novo* synthetic pathway, IMPDH is positioned at the branch point in the synthesis of adenine and guanine nucleotides and is the rate-limiting enzyme in the *de novo* synthesis of guanine nucleotides [1]. This enzyme was also implicated in tumorigenesis because IMPDH activity is often found to be elevated in tumor cells [2]. In humans, two IMPDH cDNAs have been isolated as type I and type II IMPDH [3,4]. Type II IMPDH mRNA and protein levels are higher in tumor cells than in their normal counterparts and are higher in rapidly growing cells than in comparable quiescent cells [5,6] while type I gene expression appears to be relatively constant. In addition, inhibitors of IMPDH cause the cessation of cellular multiplication and the induction of differentiation in various cell types [7-12]. Thus, up- or down-modulation of type II IMPDH gene expression and IMPDH activity is closely linked to a concurrent change in cell replication.

A fundamental question regarding the linkage of IMPDH activity and gene expression to cellular proliferation, in tumors particularly, remains unresolved: Is the increase in IMPDH gene expression in tumors due to a response to growth factors or is the increase merely a consequence of an increased percentage of S phase cells, as the end product of this enzyme is required for DNA synthesis? Many genes that are highly expressed in tumors, such as histone H3 [13] or H4 [14], are cell cycle regulated; while others such as dihydrofolate reductase (DHFR) [15] or DNA polymerase α [16] are not cell cycle regulated but instead are up-regulated by the transition from a

quiescent state (G_0 phase) to entry into the cell cycle. The present study was undertaken to elucidate the general difference between growth factor regulation and cell cycle regulation of type II IMPDH gene expression and to determine a regulatory mechanism mediating such responses.

MATERIALS AND METHODS

Cells and transfection. Cells were cultured in 10-cm tissue culture dishes (Falcon Plastics, Beckton Dickinson, Oxnard, CA) at 37°C in an atmosphere of 8% CO₂ in humidified air. RPMI-1640 culture medium supplemented with 15% fetal bovine serum (FBS), glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml) (GIBCO, Grand Island, NY) was used for the human melanoma cell line, SK-MEL-131 [11]. A modified Eagle's medium supplemented with 10% FBS, glutamine, hydroxyproline (2 µg/ml), penicillin, and streptomycin was used as the culture medium for Chinese hamster ovary (CHO) cells. Transfection was performed by electroporation with a Gene Pulser apparatus (BIO-RAD, Hercules, CA). A total of 10 µg of supercoiled pIMP-N1K(+)-CAT, a plasmid consisting of a 1.1-kb *Nco*I type II IMPDH promoter fragment (sequences 1-1093 of GenBank L33842) [17] driving expression of the bacterial chloramphenicol acetyl transferase (CAT) gene, along with 2 µg of pRSV-NEO in TE (10 mM Tris, 1 mM EDTA) buffer was mixed with 1×10^7 SK-MEL-131 cells and phosphate-buffered sucrose. The cells in this solution were electroporated with a pulse of 250 V for 35-40 ms. Following electroporation, the cells were allowed to recover for 48 h prior to selection with 0.4 mg/ml of G418 (Geneticin, GIBCO) in medium. Transfectants were picked, and clones that stably expressed the CAT mRNA

were isolated.

Cell synchronization. To arrest the cells at the G₁-S boundary, log-phase cultures were treated for 20 h with aphidicolin (Sigma, St. Louis, MO) at concentrations of 1.0 μ g/ml for SK-MEL-131 cells and 0.25 μ g/ml for CHO cells [18]. To relieve the block to proliferation, the cells were washed three times with phosphate buffered saline (PBS; 8.0 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4, 2.7 mM KCl, 137 mM NaCl), and culture medium was added. The time at which the cultures were transferred to this medium was designated as t=0.

For analysis of growth factor regulation, 1×10^6 SK-MEL-131 stable transfectants were plated per 100-mm dish in culture medium; and 4 h later, the cells were washed three times in PBS and a medium containing only 0.1% serum was added for 48 h. For restimulation experiments, serum was added to some of these dishes to a concentration of 15% for an additional 24 h.

Analysis of DNA content. The DNA content of the cells was determined by flow cytometry to confirm the synchronous progress through the cell cycle following release of the cells from the growth arrest. Trypsinized SK-MEL-131 and CHO cells, collected by centrifugation, were washed in 5 ml of PBS, fixed in 2 ml of 70% ethanol and stored at 4°C prior to analysis. Following centrifugation for 20 min at 200 x g to remove ethanol, the cells were resuspended in 200 μ l of 0.2 M citric acid, 0.5% Tween-20 and were lightly shaken for 20 min at room temperature. Cellular DNA was stained with 2 ml of 5 mM (4', 6)-diamidino-2-phenylindol in 50 mM Tris buffer (pH 7.0). Cell cycle determination was performed by using a flow cytometer (PAS II, Partec, San Diego,

CA), and data were interpreted by using the model program provided by the manufacturer. Results represent a minimum of 10,000 cells assayed for each determination.

RNA isolation and analysis. Total cellular RNA was purified by centrifugation through a 5.7 M CsCl cushion, as previously described [19]. Total RNA (10-20 μ g) was electrophoresed through a 1% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with 32 P-labeled probes. Type II IMPDH and histone H4 mRNAs were detected by using a 1.3-kb *Eco*RI fragment of a human cDNA clone [3] or a 1.7-kb *Eco*RI/*Hind*III fragment from plasmid pF0108A [20], respectively. Hybridization with an 18S rRNA oligonucleotide probe was used for the assessment of RNA quantities in each lane. Differences in steady-state mRNA levels were determined by autoradiographic densitometry, performed with a chromatogram scanner (model CS-910, Shimadzu Co., Kyoto, Japan).

RESULTS AND DISCUSSION

To achieve cell synchronization, CHO and human melanoma SK-MEL-131 cells were treated with the DNA polymerase α inhibitor, aphidicolin. Treatment of SK-MEL-131 cells with aphidicolin resulted in blockage and accumulation at the G₁/S boundary (Fig. 1). Removal of the drug resulted in the rapid progress of the cells through the cell cycle with S, G₂/M, G₁, and the next S phase optima observed at 3h, 9 h, 18 h, and 24 h, respectively. The steady-state levels of type II IMPDH mRNA did not significantly change during this full cell cycle, while the two S phase peaks were reflected in two peaks of histone H4 gene expression (Fig. 1). Although the level of synchrony

achieved and the regulation of histone H4 mRNA levels were not as pronounced, a similar consistent level of expression of the type II IMPDH mRNA and of the protein were seen in CHO cells synchronized by aphidicolin treatment (Fig. 2). These results are not specific to aphidicolin as synchronization and cell cycle progression achieved by amino acid starvation and stimulation [21] resulted in a similar consistent level of expression during the cell cycle (data not shown). Thus, this gene's expression apparently is not regulated in a cell-cycle-dependent manner.

To study growth factor regulation, as opposed to *bona fide* cell cycle regulation, we analyzed the expression of the type II IMPDH gene following deprivation of serum and after restimulation with fresh serum. Because we were also interested in a mechanism by which any such regulation was achieved and because the promoter for this gene has been cloned only from humans [17], these experiments focused on the human SK-MEL-131 cell line. SK-MEL-131 transfectants that stably express the bacterial CAT gene controlled by the type II IMPDH promoter were isolated following cotransfection of this plasmid and pRSV-NEO and selection with G418 (Geneticin). The parental SK-MEL-131 cells and these transfectant clones were then treated by serum deprivation and stimulated by the addition of serum. Northern analysis revealed that type II IMPDH steady-state mRNA levels were down-regulated in response to serum deprivation for 48 h and then were induced by restimulation with serum for an additional 24 h. Results from a representative experiment are shown in Fig. 3 (top panel). To determine whether the growth regulation of the type II IMPDH gene is controlled by changes in transcriptional initiation, the Northern blots from the

transfectant clones were stripped and hybridized with a CAT probe. Upon starvation for serum growth factors for 48 h, both IMPDH promoter activity, expressed as the level of CAT message, and steady-state IMPDH mRNA levels exhibited down-regulation (Fig. 3). Stimulation of the transfected cells by the addition of serum resulted in up-regulation of both the CAT and type II IMPDH mRNAs (Fig. 3). These results give support to the conclusion that the level of IMPDH mRNA fluctuates in response to serum stimulation by a transcriptional mechanism. This observation was seen in a number of SK-MEL-131 transfectant clones, indicating that this phenomenon was not an artifact of the integration site of the construct. In addition, this observation was also noted in several other human cell systems and derivative stable transfectants, including MB-231 mammary carcinoma cells, and HT-1080 fibrosarcoma cells (data not shown). The expression of the type II IMPDH gene therefore appears to be primarily regulated by serum factors rather than by the cell cycle, and this regulation is mediated at least in part by a transcriptional mechanism.

A similar conclusion has been reached for the regulation of several other "housekeeping" genes such as DHFR [15], thymidylate synthetase [22], and DNA polymerase α [16]. This regulation is frequently mediated by a transcriptional mechanism [23]. Among a number of TATA-less "housekeeping" genes with expression that has been shown to be growth-regulated, there are frequently multiple binding sites for the transcription factor Sp1 and Sp1 has been suggested as mediating this growth regulation [23]. Alternatively, a recent report indicated that Egr-1 regulates the activity of the thymidine kinase promoter during the transition from G_0 to G_1 [24].

These two observations may be related, however, because consensus Sp1 and Egr-1 binding sites have been shown to be able to overlap in some promoters [25]. Sp1 binds to this sequence in the absence of the Egr-1 protein; but upon stimulation of cells with serum, the Egr-1 protein is synthesized as part of the "immediate-early" cascade, and the protein competes with Sp1 and binds to this sequence [25]. The promoter for type II IMPDH [17] (GenBank L33842) contains four Sp1 binding sites with two of the sites overlapping a perfect consensus Egr-1 site. It is therefore an attractive hypothesis that the growth regulation of type II IMPDH transcription is mediated by Egr-1 in a manner similar to that seen with the growth regulation of the thymidine kinase gene.

Because IMPDH is the rate-limiting enzyme in the production of the DNA precursor guanylate, replicating cells may have great demand for IMPDH activity at S phase and consequently may up-regulate IMPDH gene expression. We chose to analyze this possibility by examining type II IMPDH gene expression as previous reports concerning the proliferative up-regulation of this enzyme have used this parameter as an end point. We have demonstrated that during cell replication the type II IMPDH gene is regulated by serum factors primarily by a transcriptional mechanism. Moreover, this regulation is for the most part independent of cell cycle regulation. To explain these data, one must distinguish between cell cycle progression from a quiescent state (G_0 - G_1 -S) and that from mitosis (M- G_1 -S). As illustrated in this report, these two transitions can be fundamentally different. Previous reports have shown differential regulation of genes depending on the cell cycle phase to which the cells

were synchronized [26,27]. A separate layer of regulation may exist for IMPDH activity, as opposed to gene expression, as some differences in total IMPDH activity (the sum of type I and type II activities) have been reported in cell populations cell cycle phase-enriched by centrifugal elutriation [28]. However, comparisons of IMPDH expression between tumors and rapidly dividing cells and their nontransformed or more slowly dividing cellular counterparts have found a difference in the steady state levels of mRNA as well as in the protein's activity [5,6]. Because we have demonstrated in this report that type II IMPDH mRNA levels are invariant throughout the cell cycle, we conclude that the basis for the increased expression and activity of type II IMPDH commonly seen in tumors and rapidly dividing cells is mainly a growth factor-related phenomenon rather than a cell cycle-dependent regulation. Clarification of the mechanisms that regulate this gene will further our understanding of the role of IMPDH in cellular regulation and may provide insights into the more general area of the control of cell growth.

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FIGURE LEGENDS

Fig. 1. Synchronization and analysis of SK-MEL-131 cells following treatment with aphidicolin. SK-MEL-131 cells were treated with aphidicolin (1.0 mg/ml) for 20 h, the drug was removed by three washes with PBS, and fresh culture medium was added. The upper graph represents the results of cell cycle analysis as determined by flow cytometry. The lower three autoradiographs are Northern hybridizations to RNA from these cells probed with a type II IMPDH cDNA probe, a histone H4 cDNA probe, and an 18S rRNA oligonucleotide probe.

Fig. 2. Synchronization and analysis of CHO cells following treatment with aphidicolin. Chinese hamster ovary cells were treated with aphidicolin (0.25 mg/ml) for 20 h, the drug was removed by three washes with PBS, and fresh culture medium was added. The top graph represents the results of cell cycle analysis as determined by flow cytometry. The central three autoradiographs are Northern hybridizations to RNA from these cells probed with a type II IMPDH cDNA probe, a histone H4 cDNA probe, and an 18S rRNA oligonucleotide probe.

Fig. 3. Northern blot analysis of type II IMPDH steady state mRNA levels and promoter activity following deprivation and restimulation with serum. SK-MEL-131 transfectant clone 1 (SK-CAT-1) cells were plated in culture medium and underwent one of the following three treatments: (1) cells were allowed to continue to grow in this medium for 48 h (c); (2) cells were washed 4 h later three times with PBS and medium was

replaced with a starvation medium containing only 0.1% serum for 48 h (-); or (3) cells that were serum starved for 48 h were replenished with 15% serum for an additional 24 h (+). Total RNA was isolated and analyzed by Northern blotting by using, sequentially, type II IMPDH and CAT cDNA fragments, and an 18S rRNA oligonucleotide probe.

Figure 3
1

Type II IMPDH

Histone H4

18S rRNA

-72 0 3 6 9 12 15 18 21 24 27

Time after release from block (h)

Figure

C - +

CAT-

IMPDH-

rRNA-