Glucose catabolism in cancer cells: regulation of the Type II hexokinase promoter by glucose and cyclic AMP

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Abstract The increased glucose consumption of many tumor cells depends to a large extent on the overexpression of hexokinase Type II. In a previous study we isolated and sequenced the hepatoma Type II hexokinase promoter and showed that it is activated by glucose in the highly glycolytic AS-30D hepatoma cell line under study, but not activated in control hepatocytes [Mathupala, S.P., Rempel, A. and Pedersen, P.L. (1995) J. Biol. Chem. 270, 16918–16925]. Here we report that the promoter of the hexokinase Type II gene is maximally activated by glucose at concentrations above 5 mM. Moreover, the data strongly suggest that glucose can act alone without requirement for metabolism. Also, glucose-mediated promoter activation is markedly potentiated by cAMP. This response may serve as a strategy for cancer cells to maintain the hexokinase transcription rate high to ensure an efficient glucose utilization even under conditions where carbohydrates are limiting.

Key words: Cancer; Type II hexokinase; Glucose catabolism; Cyclic AMP; Protein kinase A; Gene regulation

1. Introduction

The capacity to perform a high rate of glycolysis even under aerobic conditions is among the most characteristic biochemical phenotypes for animal and human cancers [1-3]. Hexokinase, the first enzyme of the glycolytic pathway, plays a pivotal role in glucose metabolism in transformed cells. Its activity, mRNA levels, and transcription rate, are strikingly increased in tumor cells relative to normal cells [4-7]. The hexokinase reaction which phosphorylates glucose is essential for further metabolism of glucose and provides the cell, not only with energy, but also with precursors for biosynthesis of lipids and nucleic acids. As high hexokinase levels are mandatory for the efficient utilization of glucose in many tumor cells, it is of interest to assess the effect of glucose itself on hexokinase gene expression.

There are four hexokinase isozymes in mammalian tissues (HK I–IV). In most of the tumors studied to date it is hexokinase Type II which is found to be markedly overexpressed in highly glycolytic tumors [4-6,8]. In adipose tissue it has been demonstrated that hexokinase Type II activity can be increased in response to glucose [9,10]. Further we have shown recently that a 4.3 kbp region of the proximal promoter of the tumor hexokinase Type II gene confers glucose responsiveness to a heterologous reporter gene in transient transfection assays in hepatoma cells but not in normal hepatocytes [4]. Elucidation of the glucose signaling pathway in tumor cells might help explain the molecular basis of hexokinase overexpression.

In the present paper we first inquired whether glucose itself or a specific glycolytic intermediate is responsible for the glucose responsiveness of the tumor hexokinase Type II promoter. For these studies we used the highly glycolytic, rapidly growing rat hepatoma cell line AS-30D. This cell line has been characterized in this laboratory with respect to its high glycolytic rate and the role of hexokinase in this process [4,11]. Transient transfection of hepatoma cells with the tumor hexokinase Type II promoter fused to a luciferase reporter were used to determine the effect of glucose, glucose analogs, and other substrates on promoter activity. In a second set of studies, we inquired whether an interaction exists between glucose and the cAMP/PKA (protein kinase A) signaling pathway. Our results from the two sets of studies suggest that glucose can act alone on the hexokinase Type II promoter, and that the effect of glucose can be potentiated by activating the cAMP/PKA signal transduction pathway.

2. Material and methods

2.1. Materials

RPMI 1640 tissue culture medium, glucose analogs, forskolin and IBMX were purchased from Sigma. pGL-2 luciferase vector and pSV-β-galactosidase control vector were from Promega. H89 was purchased from Calbiochem. Chemiluminescence was measured using a TD-20e luminometer (Turner Designs/Promega). Electroporation was carried out in a Cell-Porator electroporator (Life Technologies, Inc.). AS-30D hepatoma cells were propagated, harvested, and purified as described [4].

2.2. Plasmids and electroporation

Electroporation was carried out as described by Mathupala et al. [4]. Briefly 10 μg of the hexokinase promoter–luciferase construct and 2.5 μg of a SV40 promoter β-galactosidase control vector were transfected in AS-30D cells by electroporation. The cells were incubated for 24 h either in basal medium (glucose-free RPMI 1640 supplemented with 1 mM pyruvate) or in basal medium containing the various agents as indicated. For experiments in which enzyme inhibitors (mannoheptulose, glucosamine, or H89) were included the cells were first pretreated with the inhibitor for 2 h before challenging with the inducing agent. Cell extracts were assayed for luciferase and β-galactosidase activity as we have previously described [4]. For each experiment the luciferase activity was normalized to the β-galactosidase activity derived from the cotransfected control plasmid pSV-β-galactosidase.

2.3. Enzyme assays

The activities of luciferase and β-galactosidase were assayed essentially as described by the manufacturer using 20 μl and 100 μl cell extracts respectively. Hexokinase activity was determined spectrophotometrically in a glucose 6-phosphate dehydrogenase coupled assay [12]. Hexokinase activity is expressed as milliunits (mU) defined as the formation of one nmol of NADPH per min.

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Abbreviations: 2DOG, 2-deoxyglucose; dbtcAMP, dibutyryl cAMP; IBMX, 3-isobutyl-1-methylxanthine; 3-OMetGlc, 3-O-methylglucose; PKA, protein kinase A

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3. Results and discussion

We first investigated the effect of glucose concentration on the tumor hexokinase Type II promoter in transient transfection experiments using the promoter–luciferase–reporter construct. Glucose induced promoter activity in a concentration-dependent manner (Fig. 1). The sugar did not stimulate promoter activity significantly below concentrations of 5 mM as compared to control samples grown in media without glucose. Maximal activation was achieved at a glucose concentration of 25 mM resulting in a 2–3-fold increase in promoter activity.

In order to address the question of whether glucose alone or a downstream glycolytic intermediate is responsible for activating the tumor hexokinase Type II promoter, experiments were first carried out with 5 different glucose analogs (Fig. 2). These included 3-0-methylglucose (3-OMetGlc), which cannot be phosphorylated, 2-deoxyglucose (2-DOG) which is phosphorylated by hexokinase to 2-DOG-6-P but not further metabolized, fructose, which can either be phosphorylated by hexokinase or bypass the hexokinase step upon entering the glycolytic pathway, and galactose which bypasses the hexokinase step. The data show that only glucose and fructose markedly activate the tumor hexokinase Type II promoter. The simplest explanation of these results is that both glucose and fructose are activators of the promoter as no glycolytic intermediate, i.e. neither 2-DOG-6-P derived from 2-DOG nor further downstream metabolites provided by galactose, appreciably activates the promoter. Nevertheless, the experiment is not conclusive as 3-OMetGlc which is not phosphorylated failed to activate the promoter and 2-DOG, because of its toxicity to hepatoma cells at the high concentrations (25 mM) used for the other sugars, had to be used at a low concentration. [Toxicity of 2-DOG to cells at high concentrations resulting in cell death has been observed by others [13] and is poorly understood.]

For the above reasons, we examined also the action of

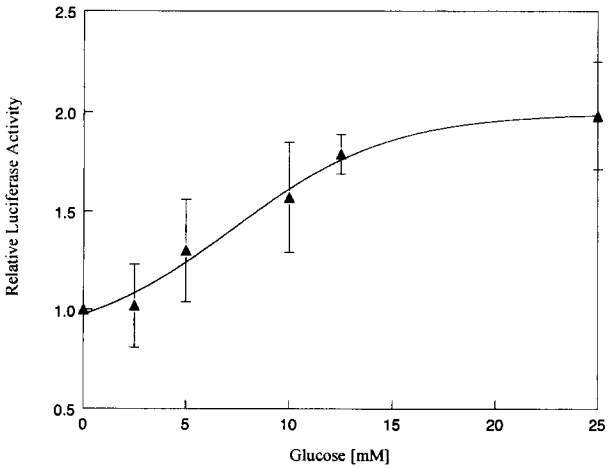

Fig. 1. Dose-dependent transcriptional activation of the tumor hexokinase Type II promotor by glucose. Glucose responsiveness of the tumor hexokinase Type II promotor was measured in transiently transfected AS-30D cells using the promoter–luciferase reporter construct. The cells were exposed to medium containing the indicated concentration of glucose. Luciferase activity in cell extracts was assayed 24 h posttransfection. Luciferase activity is expressed as relative units. The activity at 0 mM glucose was arbitrarily set at 1. Data points represent the mean ± S.E.M. of at least 5 independent experiments.

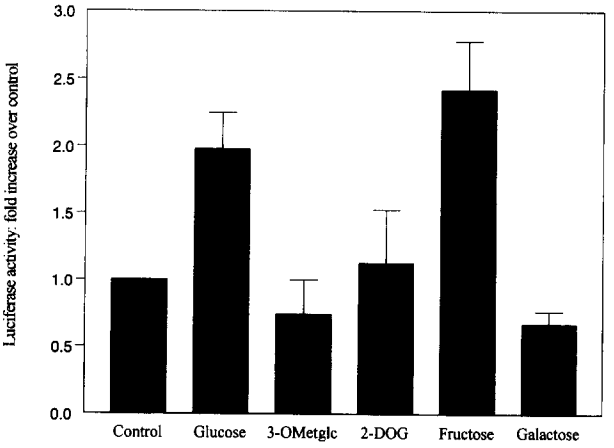

Fig. 2. Effect of various sugars and glucose analogs on the tumor hexokinase Type II promotor activity. AS-30D hepatoma cells were transfected with the promoter–luciferase construct and grown either in basal medium or in basal medium with the additions as indicated. For each compound a concentration of 25 mM was used, except for 2-DOG which was added to a final concentration of 0.08 mM. Luciferase activity was assayed 24 h posttransfection. Luciferase activity in control samples incubated in basal medium was arbitrarily set at 1. Each data point represents the mean ± S.E.M. of at least 4 independent experiments.

hexokinase inhibitors alone or in combination with glucose, on promoter activity. Should metabolites downstream from glucose in the glycolytic pathway be the activating signals, inhibition of hexokinase activity would be expected to prevent or at least diminish glucose-mediated promoter activation. We chose two different inhibitors of hexokinase, mannoheptulose and glucosamine. Fig. 3 illustrates the inhibitory potential of these two compounds on total hexokinase activity in AS-30D hepatoma cells. At a concentration of 20 mM, hexokinase activity was inhibited by 84% and 74% by mannoheptulose and glucosamine, respectively. When used in our transfection system, glucosamine exhibited no inhibitory effect while mannoheptulose imparted only a slight inhibitory effect on promoter activity (Fig. 4). However, in the presence of glucose the observed promoter activation (3.31 for mannoheptulose, 2.2 for glucosamine) was not only higher than with the corresponding glucose concentration alone, but also higher than the maximal activation achieved by glucose alone (Fig. 1). The additional activation of glucosamine is hard to visualize in Fig. 4, but for this experiment a glucose concentration of only 2.5 mM was used, which alone showed almost no effect on promoter activity (Fig. 1) compared to a 2.2-fold increase in the presence of glucosamine. This suggests that elevated intracellular glucose concentrations which accumulate due to the inhibition of hexokinase cause an increase in promoter activity. To further test this possibility, we used the glucose analog 2-DOG in combination with glucose. Due to the inhibitory effect of 2-DOG-6-P on hexokinase activity, the simultaneous addition of glucose and 2-DOG would be expected to result in promoter activation. Consistent with this view, under these conditions 2-DOG was able to activate the promoter in a concentration-dependent manner (Fig. 4). Taken together these findings point to glucose itself as an activator of the hexokinase Type II promoter. Although the failure of 3-OMetGlc to activate the promoter (see above) seems inconsistent with glucose being the stimulating agent, a low

uptake of this glucose analog, or the structural difference between glucose and 3-O-methylglucose per se, could have prevented this analog from activating the promoter. In addition to glucose, fructose might be another potent activator of the hexokinase Type II promoter, although additional experiments will be necessary to distinguish among fructose and intermediates thereof.

[It should be noted that in the set of experiments described above, in contrast to those described earlier (Fig. 2), 2-DOG could be used at relatively high concentrations without toxicity to the cells. This is most likely due to the presence of glucose in the above experiments.]

Consistent with our finding that glucose promotes transcriptional regulation of hexokinase Type II in tumor cells, are data reported by Bernstein [14] investigating hexokinase Type II activity in fat pads using an in vitro assay. Glucose and fructose but not galactose showed the capacity to increase hexokinase II activity 1.5 to 2-fold. 2-DOG caused a decrease, and 3-OMetGlc a slight increase. However, based on the observation that glucose 6-phosphate itself is an inducer of hexokinase activity, Bernstein concluded that glucose 6-phosphate mediates the glucose effect on hexokinase activity. This would be paradoxical because high intracellular glucose 6-phosphate concentrations favor down regulation of glucose phosphorylation. The known inhibition of hexokinase activity by glucose 6-phosphate [9] is consistent with this concept. Induction of hexokinase expression by glucose and fructose, the two main carbohydrates in the blood stream, seems to be more physiological. Nevertheless, our studies do not exclude the possibility that, in addition to glucose, glycolytic or post-glycolytic intermediates might function as regulators of the tumor hexokinase Type II promoter.

Following the above set of studies, we turned our attention to a study of the effects of cAMP and cAMP plus glucose on the activity of the tumor hexokinase Type II promoter. Previously we reported the activation of the tumor hexokinase Type II promoter by dibutyryl cAMP (dbtcAMP) [4], a membrane-permeable cAMP analog. In order to further evaluate the specificity of promoter activation by this cAMP analog and the possible involvement of the PKA signal transduction

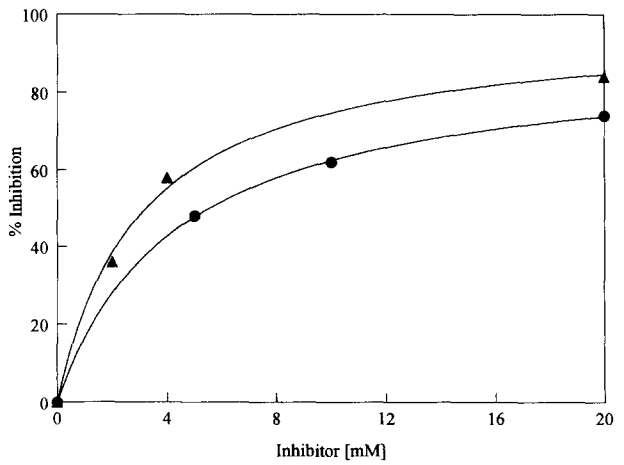

Fig. 3. Inhibition of tumor hexokinase activity by mannoheptulose (▲) and glucosamine (●). Aliquots of AS-30D hepatoma cell homogenates were incubated 10 min at 25°C with the indicated concentration of inhibitor. Hexokinase activity was assayed as described in section 2.

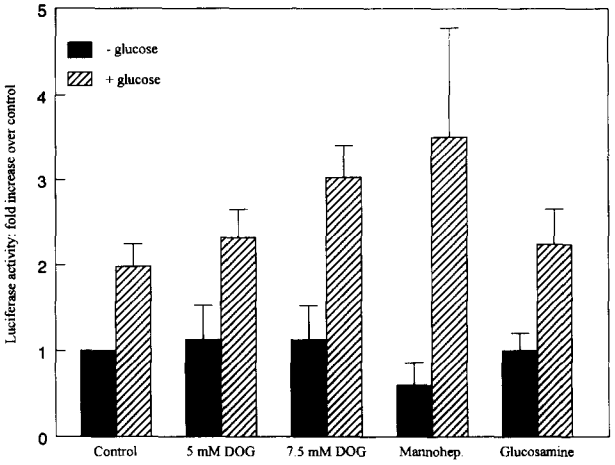

Fig. 4. Effect of hexokinase inhibitors on the glucose reponsiveness of the tumor hexokinase Type II promoter. AS-30D hepatoma cells were transfected with the promotor–luciferase construct and prior incubated for 2 h in basal medium containing the compounds as indicated. The cells were then incubated for 24 h with or without the addition of glucose. Luciferase activity was assayed in cell extracts and the activity in control samples incubated in basal medium alone was arbitrarily set at 1. (Concentrations used, from left to right: 0/25 mM glucose [Control], 12.5 mM mannoheptulose/12.5 mM glucose, 7.5 mM glucoseamine/2.5 mM glucose, 5 or 7.5 mM 2-DOG/20 or 17.5 mM glucose). Data points represent the mean ± S.E.M. of at least 4 separate experiments.

pathway in promoter regulation, we tested the effect of forskolin, an activator of adenylyl cyclase, and IBMX, an inhibitor of cyclic nucleotide phosphodiesterases. As shown in Fig. 5, incubation with either forskolin or IBMX resulted in a 2 and 1.5-fold activation, respectively, of the tumor hexokinase Type II promoter, which is in the same range as the stimulation observed with dibutyryl cAMP. Thus, cAMP generated either exogenously or endogenously increases promoter activity. To seek additional evidence that PKA mediates stimulation of promoter activity, we used H89, a selective and potent inhibitor of PKA [15]. Used alone, H89 caused a slight but consistent decrease in promoter activity. Because protein kinases exhibit a low basal activity in the absence of added cyclic nucleotides [16], this indicates that the PKA pathway contributes to the 'basal activity' of the hexokinase Type II promoter. However, H89 was not able to completely prevent the dbtcAMP-mediated activation, although it was less than with dbtcAMP alone. It might be that even at the concentrations used, H89 was not sufficiently potent to completely antagonize the activation of PKA, or that there might be a way, other than stimulating PKA, by which cAMP mediates its effect on the tumor hexokinase Type II promoter.

To obtain more insight into the glucose signaling pathway in AS-30D hepatoma cells, and as a first attempt to obtain information about its possible cross-talk with other signaling pathways, we investigated interaction of this sugar with the PKA pathway. AS-30D hepatoma cells were transfected with the promoter–reporter construct and exposed for 24 h either to glucose and dbtcAMP alone or simultaneously. As shown in Fig. 6, the increase in promoter activity (~6-fold) exhibited by the combined treatment of glucose and dbtcAMP was always more than the addition of promoter activation of both agents separately (~2-fold, each). Synergistic interactions between various signal transduction pathways are commonly involved in gene regulation providing multiple sites of regula-

tion. Protein phosphorylation by cAMP-dependent protein kinase represents the central mechanism in the signal transduction of glucagon, the hormone which is present at high concentrations when blood glucose levels are low (fasting state). The synergism of glucose and cAMP might be a strategy of the cancer cell to maintain hexokinase levels high even under conditions when glucose concentrations are low. This strategy might facilitate glucose consumption when carbohydrates are limited. More importantly, when facing a high glucose availability, again the cancer cell can utilize the available glucose efficiently without a lag period. Glucokinase which is the predominant hexokinase isozyme in normal hepatocytes is up-regulated by glucose [9]. However, cAMP decreases glucokinase expression [17]. Therefore, the synergistic activation of the tumor hexokinase Type II promoter by cAMP and glucose clearly provides the cancer cell with a growth advantage over normal cells.

Further downstream events in the glucose signaling pathway still remain an open question. A synergistic effect of glucose and cAMP has been reported for the insulin secretion of β-cells [18]. In the insulinoma cell line INS-1, glucose activates MAP kinase. This activation is synergistically enhanced by increased levels of cAMP [19]. This raises the possibility that glucose might mediate its effect on tumor hexokinase Type II promoter activity in AS-30D hepatoma cells by a similar mechanism. However, the identification of the proximate glucose signal requires further investigations.

Glucose has been implicated to play a crucial role in regulating gene expression for a variety of enzymes attesting to its importance in regulating energy metabolism. For example, the transcriptional rates of L-type pyruvate kinase and lipogenic enzymes are increased in response to glucose [20-23]. In cases studied thus far a glycolytic intermediate, most probably glucose 6-phosphate, has been suggested to mediate the glucose response. The activation of the tumor hexokinase Type II promoter by glucose itself seems to be unique. Significantly, in rapidly growing tumor cells, where hexokinase levels are many fold higher than in normal cells, and are required for the high glucose catabolic phenotype, one can hypothesize that hexokinase Type II may play a major role in determining the glucose responsiveness. Along these lines, it is important to note that we have previously shown that the tumor Type II hexokinase promoter is activated by glucose in transfected hepatoma cells (AS-30D) which express the high glycolytic phenotype, but not in control hepatocytes [4]. Thus data presented here strengthen the view that hexokinase is pivotal for tumor metabolism.

In summary, we have shown that glucose alone is an activator of the tumor hexokinase Type II promoter and that this activation can be potentiated by cAMP. This response is likely an adaptive response to allow tumor cells to better utilize glucose and hence provide them with a growth advantage over normal cells.

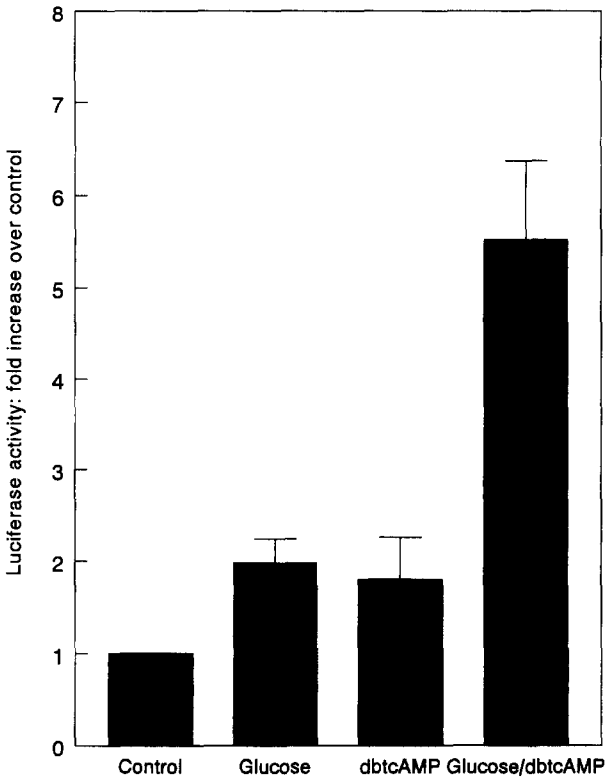

Fig. 6. Synergistic effect of glucose and cAMP on the tumor hexokinase Type II promotor activity. AS-30D cells transfected with the promotor–luciferase construct were exposed to glucose (25 mM), dbtcAMP (100 μM) or a combination thereof. Luciferase activity was determined 24 h posttransfection. The activity in control samples grown in basal medium was arbitrarily set at 1. Data points represent the mean ± S.E.M. of at least 6 independent experiments.

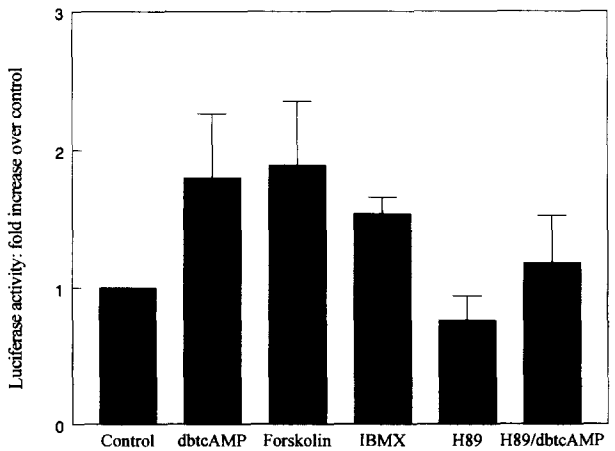

Fig. 5. Effect of exogenous and endogenous generated cAMP on the tumor hexokinase Type II promotor activity. AS-30D hepatoma cells were transfected with the promoter–luciferase construct and exposed to media containing dbtcAMP (100 μM), forskolin (20 μM), IBMX (150 μM) or H89 (30 μM). In experiments in which H89 and dbtcAMP were used together, the latter was added after a 2 h prior incubation period of cells with H89 alone. Luciferase activity in cell extracts was determined after 24 h. The luciferase activity in control samples grown in basal medium was arbitrarily set at 1. Data points represent mean ± S.E.M. of at least 4 different experiments.

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