Glucose regulates the promoter activity of aldolase A and pyruvate kinase M_2 via dephosphorylation of Sp1

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Abstract Proliferating cells and tumour cells maintain a high glycolytic rate even under aerobic conditions. FTO2B cells, a rat hepatoma cell line, show high activities of glycolytic enzymes. Within a culture period of 48 h the cell number increases 5-fold. Replacement of glucose by pyruvate in the culture medium lowers glycolytic enzyme activity and prevents proliferation. Transfection assays revealed that glucose deprivation dramatically decreases the transcriptional activities of the Sp1-dependent aldolase and pyruvate kinase promoters leading to reduced reporter gene expression. Sp1 binding activity is also inhibited by ocadaic acid, an inhibitor of protein phosphatase 1. Western blot analyses with nuclear extracts from FTO2B cells cultured in the presence or absence of glucose revealed differences in the phosphorylation state of Sp1. From these results we conclude that glucose increases the amount of the dephosphorylated form of Sp1 which has a higher DNA binding activity. As a consequence gene expression of the glycolytic enzymes is increased which is a prerequisite for cell proliferation.

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Key words: Glycolytic enzyme activity; Rat hepatoma cell; Sp1; Protein phosphatase 1

1. Introduction

One of the characteristics of rapidly growing cells is their ability to degrade glucose glycolytically at high rates. This enables tumor cells [1–3] and controlled proliferating cells [4] to produce more than 50% of their energy, and a remarkable amount of anabolic precursors for biosynthetic pathways from glycolysis. Crabtree described the inhibition of oxygen consumption by the addition of glucose resulting in enhanced aerobic glycolysis in tumour tissues [5]. This phenomenon has been demonstrated also in proliferating thymocytes [6]. Mitogen-stimulated rat thymocytes cultured in a conventional medium containing glucose induce their glycolytic enzymes 8–10-fold in the S-phase of the cell cycle and divide within a culture period of 72 h [7–9]. Deprivation of glucose from the medium prevents glycolytic enzyme induction and proliferation of thymocytes [4]. The genetic basis underlying these biochemical observations is not known.

The promoter regions of aldolase and pyruvate kinase genes contain various Sp1 binding sites [10,11], and Sp1 is known to play a critical role in the transcriptional regulation of glycolytic enzymes [12–15].

In this report we show that withdrawal of glucose from the culture medium decreases glycolytic enzyme activities and inhibits proliferation of FTO2B cells. This rat hepatoma cell line contains the isoenzymes aldolase A and pyruvate kinase M_2, which are missing a glucose response element in their promoter region. Nevertheless the promoters of aldolase and pyruvate kinase are markedly activated by high concentrations of glucose.

The aim of this paper was to elucidate the mechanism by which glucose affects the promoter activities of aldolase and pyruvate kinase. In gel shift experiments with nuclear extracts of FTO2B cells, we show that glucose treatment of the cells causes increased binding of Sp1 to its consensus sequence. The glucose-mediated increased binding of Sp1 to DNA is due to a shift to the dephosphorylated form of Sp1 in the nucleus. This is the first report showing that glucose treatment of proliferating cells decreases the amount of phosphorylated Sp1 and consequently increases its DNA binding efficiency.

2. Materials and methods

2.1. Materials

The pGL3Basic vector, the luciferase assay system and DEAE-dextran were purchased from Promega (Heidelberg, Germany); ocadaic acid, sodium salt was obtained from Calbiochem Inc. (Bad Soden/Ts.). Antibodies used in competition assays and immunoblottings were from Santa Cruz Biotechnology (Santa Cruz, USA) and [α-^{32}P]dATP was from Amersham Buchler (Braunschweig, Germany). Phosphatase, alkaline and lysis buffer were products of Boehringer (Mannheim, Germany).

2.2. Cell preparation and cell culture

FTO2B rat hepatoma cells were grown in DMEM/Ham's F-12 medium supplemented with 5% heat-inactivated fetal calf serum, 200 U ml^{-1} penicillin G and 100 U ml^{-1} streptomycin at 37°C. Cells that were used for nuclear extracts and enzyme activity measurements were treated as follows: when the cells had just reached confluence the medium was removed and the cells were washed with phosphate-buffered saline. They were incubated for 6 days in a glucose-free RPMI 1640 medium that was supplemented with 5% heat-inactivated fetal calf serum deficient in glucose (Biochrom KG, Berlin, Germany), 200 U ml^{-1} penicillin G, 100 U ml^{-1} streptomycin, 15 mM HEPES and with either 10 mM pyruvate or 24 mM glucose.

2.3. Enzyme activity measurements

Cell extracts were obtained by ultrasonication of the cells in Krebs saline buffer (KSP) for five separate periods of 6 s at 70 W with a Branson sonifier. The extracts were maintained between 0 and 4°C during sonication. The activities of glycolytic enzymes were assayed at 37°C immediately after sonication by the following methods: aldolase and hexokinase as described by Bergmeyer, Graßl and Walter [16], pyruvate kinase according to Fujii and Miwa [17], 6-phosphofructo-1-kinase according to Crane and Sols [18] and lactate dehydrogenase as described by Vassault [19].

2.4. Luciferase reporter constructs

Aldolase A and pyruvate kinase M_2 promoters were cut with the appropriate restriction enzymes and inserted 5′ upstream of the luciferase reporter gene in the commercially available luciferase basic

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vector (pGL3Basic). The aldolase promoter fragment −727 to +141 and the pyruvate kinase M_2 fragment −454 to +78 were cloned into the SstI-BglII sites of pGL3Basic.

2.5. Cell transfections and treatments

FTO2B cells were transfected by DEAE/dextran with 5 μg of the indicated plasmid construct according to the standard protocol of the technical manual of the Promega mammalian transfection system. After treatment with the DNA/DEAE-dextran mixture the cells were incubated with glucose-free RPMI 1640 medium supplemented with 10% glucose deficient fetal calf serum (Biochrom) in the presence of 24 mM glucose or 10 mM sodium pyruvate or 24 mM glucose and 100 nM ocadaic acid. Cells were harvested 48 h after transfection.

Transfected cells were harvested with a rubber policeman, washed with phosphate-buffered saline, and resuspended in 300 μl lysis buffer. After 5 min at room temperature the extracts were centrifuged for 10 min and the supernatants were used for luciferase assay or determination of protein concentration.

2.6. Nuclear extracts and electrophoretic mobility shift assays (EMSA)

Nuclear extracts from FTO2B cells were prepared according to Dignam et al. [20]. FTO2B cells were subjected to the treatment described above. Protein concentration was quantified with the Bradford reagent (BioRad Laboratories, Richmond, CA, USA). For mobility shift assays the following probes were used:

(A): A fragment of the aldolase A promoter containing two GC boxes and spanning from position +14 to −84.

(B): A fragment of the pyruvate kinase M_2 promoter containing two GC boxes and spanning from position +15 to −86.

Ten fmol ^{32}P end-labeled fragments, 1 μg double-stranded poly (dIdC) and 5 μg nuclear protein were incubated for 30 min at room temperature in binding buffer (20 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTE, 5% (v/v) glycerol, pH 8.0). The binding complex was separated by 5% polyacrylamide gel electrophoresis at 4°C in 1×TGE (25 mM Tris-HCl, 190 mM glycine, 1 mM EDTA, pH 8.3) at 35 mA.

2.7. SDS-polyacrylamide gel electrophoresis and immunoblotting

Nuclear proteins were separated by vertical SDS-polyacrylamide gel electrophoresis according to Laemmli [21] and blotted to nitrocellulose using an LKB Novablot semidry transfer apparatus according to the manufacturers instructions. Immunoblots were probed with the anti-Sp1 antibody, filters were incubated with an alkaline phosphatase conjugated goat anti-rabbit antibody and developed as described by Blake et al. [22].

3. Results and discussion

3.1. Glucose requirement for glycolytic enzyme induction and proliferation

In FTO2B cells, a rat hepatoma cell line, cultured in a glucose-free medium in which glucose is replaced by 10 mM pyruvate the relative maximal activities of the glycolytic enzymes hexokinase, 6-phosphofructo-1-kinase, pyruvate kinase and lactate dehydrogenase are significantly lower (Fig. 1). Moreover these cells do not proliferate either (Fig. 2). FTO2B cells grown in medium containing 24 mM glucose show 2–5-fold increases in glycolytic enzyme activities within 48 h of culture (Fig. 1). Thus, glycolytic enzyme induction and proliferation of FTO2B cells depend on the presence of glucose in the culture medium.

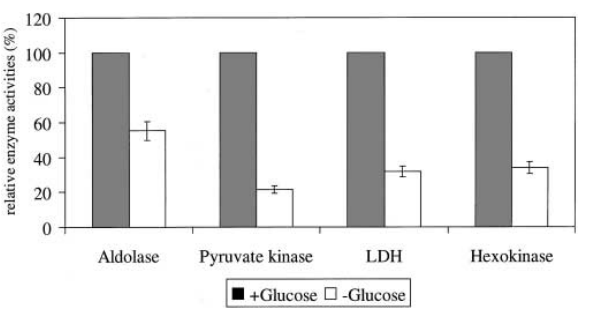

Fig. 1. Relative glycolytic enzyme activities of FTO2B cells cultured in the presence or absence of glucose. Enzyme activities were determined in extracts prepared from FTO2B cells cultured in RPMI 1640 medium containing either 0 mM glucose/10 mM pyruvate or 24 mM glucose. For details see Section 2.

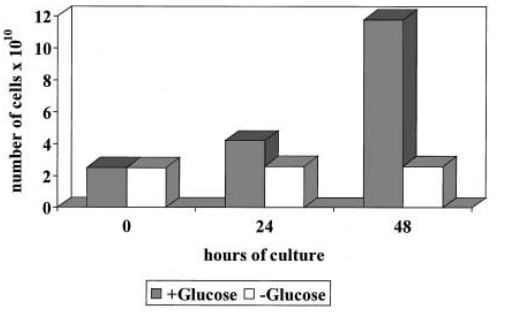

Fig. 2. Analysis of the cell number of proliferating FTO2B cells cultured in the presence or absence of glucose. Numbers of cells cultured in 10 ml of a medium containing either 0 mM glucose/10 mM pyruvate or 24 mM glucose were counted at 24 h intervals during a 48 h culture period in more than three separate experiments.

3.2. Effect of glucose on Sp1 binding

To examine the effect of glucose deprivation from the culture medium on the binding efficiency of Sp1 we performed electrophoretic mobility shift assays using promoter fragments of aldolase and pyruvate kinase containing two GC boxes each. Nuclear extracts were prepared from FTO2B cells cultured in the presence or absence of glucose. As indicated in

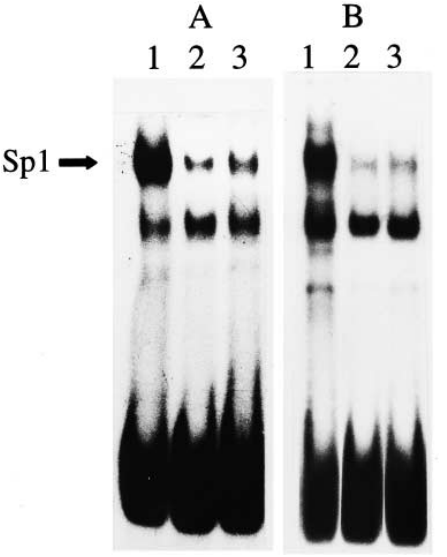

Fig. 3. Effect of glucose on Sp1 binding in vivo. Electrophoretic mobility shift assays were performed using: A: a fragment of the aldolase promoter spanning from position −84 to −200 containing two GC boxes, and B: a fragment of the pyruvate kinase promoter spanning from position +15 to −86 containing two GC boxes. The following nuclear extracts were used: lanes 1: nuclear extract from FTO2B cells cultured in the presence of 24 mM glucose; lanes 2: nuclear extract from FTO2B cells cultured in the presence of 0 mM glucose/10 mM pyruvate; lanes 3: nuclear extract from FTO2B cells cultured in the presence of 24 mM glucose incubated with anti-Sp1 antibody.

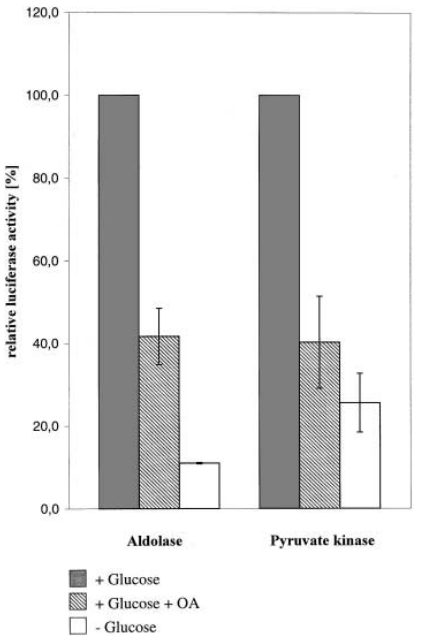

Fig. 4. Effect of glucose and ocadaic acid on the transcriptional activity of aldolase and pyruvate kinase promoter-controlled expression of a luciferase reporter gene. FTO2B cells were transfected with 5 μg of the luciferase reporter gene constructs containing the aldolase A or pyruvate kinase M_2 promoters. Luciferase activity was assayed 48 h post-transfection of the promoter-reporter constructs into FTO2B cells, which were maintained in the presence or absence of glucose or in the presence of glucose plus ocadaic acid as indicated in the figure. The luciferase activity measured in the control cells (+glucose) was set to 100%. Activities were normalized to equal amounts of protein. All values represent the mean of six independent experiments.

Fig. 3, glucose treatment leads to an increase in Sp1 binding activity at its binding sites. With both fragments one main complex was formed. Glucose deprivation from the culture medium decreased the intensity of the band indicated by arrow. The formation of the complex was efficiently inhibited in the presence of an Sp1-specific antibody. Therefore it can be deduced that the complex contains the Sp1 transcription factor.

From these results we conclude that DNA binding efficiency of Sp1 is increased in the presence of glucose. This could be caused either by a change in the absolute quantity of Sp1 molecules in the cell or by posttranslational modification of the transcription factor.

3.3. *Influence of glucose on Sp1-dependent transactivation*

To examine the functional activity of aldolase and pyruvate kinase promoters in the presence or absence of glucose, we placed (i) a fragment of the aldolase promoter spanning from position −717 to +141 or (ii) a fragment of the pyruvate kinase promoter spanning from position −454 to +78 in the pGL3-basic vector. This vector is designed to test a promoter's activity by using luciferase as a reporter gene. FTO2B cells were chosen for the transient gene expression studies to ensure the presence of signal transduction cascades and cell surface receptors characteristic of a tumor cell line. Transient expression of luciferase derived from the promoter-reporter construct was determined after transfection of FTO2B cells, by assaying luciferase activity 48 h post-transfection. As shown in Fig. 4 deprivation of glucose from the culture medium resulted in a marked decrease of transcription activity with both promoters compared to cells cultured in the presence of 24 mM glucose.

Previously it has been shown that glucose activation of Sp1 is attenuated by ocadaic acid, an inhibitor of protein phosphatase 1 (PP1) [23,24]. To determine if the Sp1 activity in FTO2B cells is modified by a phosphatase, the effect of ocadaic acid was examined. Addition of ocadaic acid to the glucose containing culture medium inhibited glucose activation of aldolase and pyruvate kinase promoters (Fig. 4).

3.4. *Western blot analysis*

In order to examine if glucose-mediated increases in the DNA-protein complexes are due to an increase in the amount of Sp1, or to the activation of existing Sp1, Western blot analysis of nuclear extracts from glucose deprivated and control cells was carried out. The amount of Sp1 did not change as a result of glucose treatment of the cells (Fig. 5A). Recently Armstrong et al. reported that Sp1 is a phosphoprotein and that phosphorylation by Casein kinase II lowers its DNA binding efficiency in liver [25]. Comparing equal quantities of nuclear extract obtained from cells cultured in a glucose containing medium and cells cultured in a glucose deprived medium we found that nuclear extracts from glucose starved

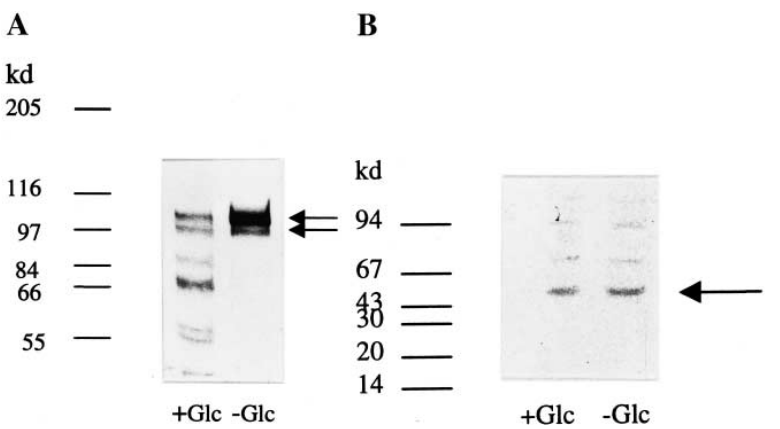

Fig. 5. A: Western blot analysis of Sp1. 25 μg of nuclear extracts prepared from control and glucose deprivated cells were run on a 7.5% SDS-polyacrylamide gel. After transfer to a nitrocellulose filter, the filter was blotted with a monoclonal antibody against Sp1. The upper arrow indicates the phosphorylated form of Sp1, the lower arrow the dephosporylated form [27]. B: Nuclear extracts, as indicated, were subjected to Western blot analysis using an antibody against the catalytic subunit of PP1 (CS1).

cells contain a higher percentage of phosphorylated Sp1 (Fig. 5A). From the immunoblot it appears that the nuclear extract from the control cells contains more proteolytic degradation products of Sp1, although both nuclear extracts were prepared in parallel and under identical conditions. This phenomenon was found in five separate preparations of nuclear extract. We assume that dephosphorylated Sp1 is more susceptible to proteolytic degradation than phosphorylated Sp1. The observed differences in the phosphorylation state of Sp1 suggest differences in the amount or activity of PP1. PP1 has been shown to dephosphorylate Sp1 in vivo [26]. In order to determine if glucose treatment had any effect on the amount of phosphatase in FTO2B cells, we performed an SDS-PAGE with nuclear extracts. As shown in Fig. 5B the blot was incubated with an antibody against the catalytic subunit of PP1 (CS1). Both nuclear extracts from the control and the glucose deprived cells cross-reacted with a specific anti-CS1 antibody to the same extent. From this result we conclude that not the amount but the activity of PP1 is reduced in glucose deprived cells.

From our results it appears that interconversion of Sp1 by phosphorylation and dephosphorylation is an effective mechanism in regulating the expression of glycolytic genes in the FTO2B tumor cell line.

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