

Differences in DNA-binding efficiency of Sp1 to aldolase and pyruvate kinase promoter correlate with altered redox states in resting and proliferating rat thymocytes

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Abstract Thymocytes induce their glycolytic enzymes as they undergo transition from the resting to the proliferating state. Corresponding increases in mRNA levels point to a transcriptional regulation. Electrophoretic mobility shift assays revealed that the DNA-binding efficiency of Sp1 is increased when nuclear extracts from proliferating compared to resting rat thymocytes were used. Here we demonstrate that hydrogen peroxide, added to nuclear extract from proliferating cells, decreases the Sp1 DNA-binding activity, whereas in nuclear extracts from resting cells dithioerythritol fully restores DNA-binding efficiency. Moreover we show that in contrast to resting thymocytes, production of reactive peroxide anions upon priming with phorbol 12-myristate 13-acetate is nearly abolished in the proliferating cells. From these results we propose that reactive oxygen intermediates affect the interaction of the Sp1 transcription factor with its consensus sequence and subsequently regulate glycolytic gene expression.

Key words: Glycolytic enzyme induction; Rat thymocytes; Reactive oxygen; Redox regulation; Sp1

1. Introduction

Primary cultures of rat thymocytes provide a useful model to study the energy metabolism as they switch from partly oxidative to completely glycolytic glucose metabolism during transition from the resting to the proliferating state [1,2].

A complete cell cycle of mitogen-activated thymocytes has been obtained [1,3,4]. Cell division is completed after 72 h of culture with the S-phase peaking between 44 h and 48 h. Induction of glycolytic enzymes has been shown to be well correlated with controlled proliferation [2]. In addition, similar increases in the mRNA levels of these enzymes have been reported [5,6], pointing to a transcriptional regulation.

The promoter regions of aldolase and pyruvate kinase are G+C rich and contain various Sp1-binding sites [7–9]. The DNA-binding affinity of Sp1 [10] and other transcription factors like AP-1, NFκB [11,12], USF [13] and the glucocorticoid receptor [14] have been shown to be affected by reactive oxygen intermediates.

The present study demonstrates that the transcriptional fac-

tor Sp1, present in nuclear extracts from both resting and proliferating thymocytes, binds to its cognate DNA sequences with a decreased efficiency in resting, compared to proliferating cells. To address the question whether this difference is caused by reactive oxygen intermediates, we studied the DNA-binding activity of Sp1 in nuclear extracts treated with oxidizing and reducing agents. In addition, chemiluminescence measurements revealed that resting, but not proliferating thymocytes can produce peroxide anions upon priming with PMA. From these experiments we suggest that changes of the redox state contribute to the regulation of glycolytic gene expression during transition of thymocytes from the resting to the proliferating state.

2. Materials and methods

2.1. Materials

Female outbred Wistar rats (6–9 weeks old) were used for all experiments. RPMI 1640 medium was purchased from Biochrom (Berlin, Germany). Percoll was obtained from Pharmacia (Freiburg, Germany). Antibodies used in competition assays and immunoblottings were from Santa Cruz Biotechnology (Santa Cruz, USA) and [α -³²P]dATP was from Amersham Buchler (Braunschweig, Germany).

2.2. Cell preparation and cell culture

Thymocytes from the thymus gland of 6–9 week old female outbred Wistar rats were prepared as described in [15] and collected in sterile PBS containing 136 mM NaCl, 4.7 mM KCl, 5 mM NaH₂PO₄·H₂O, 1.3 mM CaCl₂·2H₂O, 1.2 mM K₂HPO₄·3H₂O, 0.8 mM MgSO₄·7H₂O at pH 7.4. Large thymocytes, able to proliferate, were separated from non-dividing small thymocytes by Percoll density gradient centrifugation according to Salisbury et al. [16]. Large thymocytes (10–15% of total thymocytes) were stimulated by ConA (10 μ g ml⁻¹). The stimulated cells were cultured at a cell density of 1–2 × 10⁸ per 70 ml of RPMI 1640 medium supplemented with 2 mM glutamine and 70 μ M mercaptoethanol, 10% heat inactivated fetal calf serum and 5–10 units ml⁻¹ interleukin-2 at 37°C in a 5% CO₂ incubator. Resting cells were cultured under the same conditions without ConA and interleukin-2. The cultured cells were harvested after 44–48 h by centrifugation and washed with PBS, pH 7.4.

2.3. SDS-polyacrylamide gel electrophoresis and immunoblotting

Nuclear extracts from rat thymocytes were prepared according to Dignam et al. [17]. Protein concentration was quantified with the Bradford reagent (BioRad Laboratories, Richmond, CA, USA). Nuclear proteins were separated by vertical SDS-polyacrylamide gel electrophoresis according to Laemmli [18] and blotted to nitrocellulose using an LKB Novablot semidry transfer apparatus according to the manufacturer's instructions. Immunoblots were probed with a polyclonal antibody against Sp1. After incubation 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. [19].

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

For mobility shift assays the following probes were used:

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Abbreviations: ConA, concanavalin A; DTE, dithioerythritol; PBS, phosphate buffered saline; PMA, phorbol 12-myristate-13-acetate

- A a fragment of the aldolase A AH1 promoter spanning from position +14 to –84 containing two GC boxes [7]
 B a fragment of the pyruvate kinase M₂ promoter spanning from position +15 to –86 containing two GC boxes [8]
 C an oligonucleotide containing the consensus sequence of the transcription factor C/EBP

10 fmol ³²P end-labeled fragments, 2 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 MgCl₂/0.5 mM DTE/5% (v/v) glycerol, pH 8). 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 25 mA. Competition experiments were performed by incubating the nuclear extracts for 1 h at 4°C with the anti-Sp1 antibody prior to the addition of the ³²P-labeled probe. Where indicated, the extracts were incubated with 0.8 µl of 125 mM H₂O₂ (final concentration 5 mM) for 1 h and 0°C and treated with 0.6 µl 250 mM DTE (final concentration 7.5 mM) for 1 h and 0°C. The labeled probes were then incubated with the extracts as described above.

2.5. Measurement of reactive oxygen species

Peroxide anion (O₂²⁻) production was measured at 37°C by chemiluminescent analysis using a LKB Wallac Luminometer 1250. The incubations were carried out in a total volume of 1 ml containing 1–5×10⁷ resting or proliferating rat thymocytes (cultured for 44–48 h), 4 mM glucose and 170 µM Luminol in PBS pH 7.4 with 0.02% EDTA.

Priming of peroxide anion generation was achieved by addition of

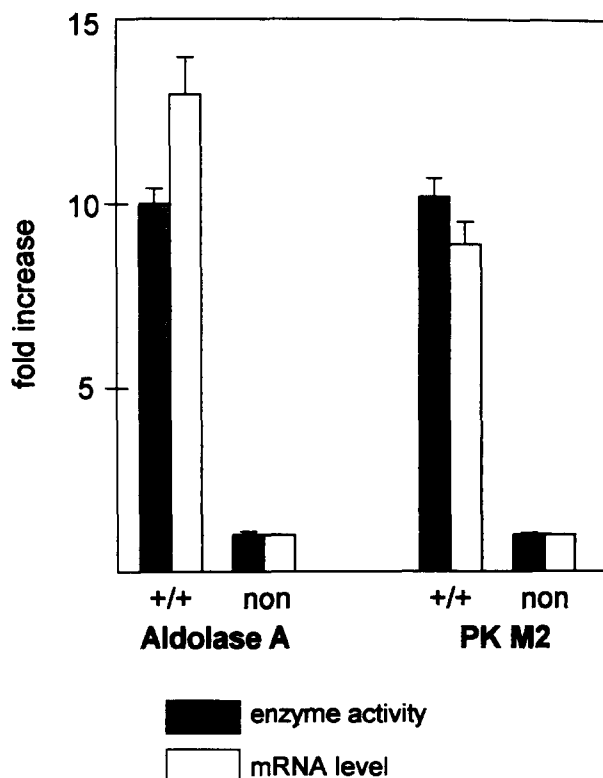


Fig. 1. Changes in cellular activities and mRNA levels of aldolase and pyruvate kinase in proliferating thymocytes (+/+) compared to resting thymocytes (non). Rat thymocytes were cultured for 44–48 h. At 44–48 h the activities and mRNA levels of aldolase and pyruvate kinase were determined. The relative mRNA levels were quantified by scanning of Northern blots and corrected for the changes in the cellular RNA content during proliferation.

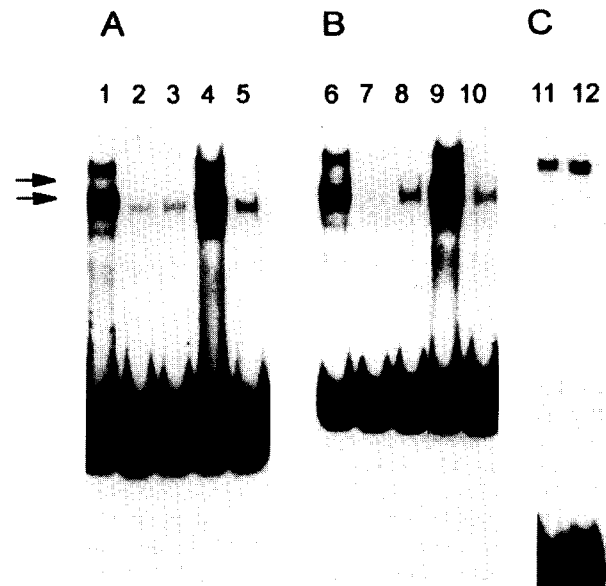


Fig. 2. Hydrogen peroxide inhibits and dithioerythritol restores the Sp1 DNA-binding activity. Electrophoretic mobility shift assays were performed using (A) a fragment of the aldolase A AH1 promoter spanning from position +14 to –84 containing two GC boxes, (B) a fragment of the pyruvate kinase M₂ promoter spanning from +15 to –86 containing two GC boxes, (C) a C/EBP oligonucleotide. The following nuclear extracts were used: lanes 1, 6 and 11: nuclear extract from proliferating thymocytes; lanes 3 and 8: nuclear extract from resting thymocytes; lanes 2, 7 and 12: nuclear extract from proliferating thymocytes incubated with 5 mM H₂O₂; lanes 4 and 9: nuclear extract from resting thymocytes incubated with 7.5 mM DTE; lanes 5 and 10: nuclear extract from proliferating thymocytes incubated with anti-Sp1 antibody.

2 µg ml⁻¹ PMA (dissolved in dimethyl sulfoxide) and 1 µg ml⁻¹ calcium ionophore A23187 (dissolved in dimethyl sulfoxide). The chemiluminescence emission of oxidized Luminol was detected at 425 nm and registered for 10–15 min with a Hitachi 2500 chromato-integrator.

3. Results and discussion

The parallel increases of both the activities [1] and the mRNA levels [5,6] of aldolase A and pyruvate kinase M₂ observed in mitogen-stimulated rat thymocytes during the S-phase of the cell cycle (44–48 h after stimulation) suggest that the induction of these enzymes is caused by enhanced transcription (Fig. 1).

The existence of various Sp1-binding sites in the activating promoter regions of aldolase and pyruvate kinase points to a role of the Sp1 transcription factor in enhanced gene expression.

To test the binding activity of Sp1 to the aldolase and pyruvate kinase promoters nuclear extracts from resting and proliferating thymocytes were prepared. ³²P-labeled fragments of the aldolase A (Fig. 2A) and pyruvate kinase M₂ promoter (Fig. 2B), each of them containing two recognition sites for Sp1, were utilized as probes in gel shift assays. Two major DNA-protein complexes were generated with both probes. The formation of the slower migrating complex was efficiently inhibited by the antibody whereas the formation of the faster migrating complex was inhibited to a lesser extent (Fig. 2A, lane 5; Fig. 2B, lane 10). These data indicate that the upper complex contains Sp1, the lower complex may include an Sp1-

related protein. The formation of two major complexes with the Sp1-binding site was also reported by others [20,21]. From the band intensities it appears that the nuclear extract from proliferating thymocytes yields more intense Sp1 bands (Fig. 2A, lane 1; Fig. 2B, lane 6) compared to the nuclear extract from the resting thymocytes (Fig. 2A, lane 3; Fig. 2B, lane 8).

Western blot analyses of the nuclear extract from proliferating (Fig. 3, lane 1) and resting thymocytes (Fig. 3, lane 2) revealed that the content of Sp1 is identical. The proportion of phosphorylated Sp1 (Fig. 3, upper arrow) and unphosphorylated Sp1 (Fig. 3, lower arrow) is the same in both nuclear extracts, suggesting that the observed differences in binding efficiency are due neither to a different amount of this transcription factor nor to its phosphorylation state [22,23].

It has been shown that oxidation of Sp1 affects its DNA-binding efficiency [10]. We therefore investigated whether the different band intensities observed in the gel shift assays are a result of different redox states of the Sp1 protein in the nuclear extracts. In order to test the sensitivity of Sp1 to oxidizing treatment, nuclear extracts from proliferating cells were incubated with hydrogen peroxide. DNA-binding ability of Sp1 to the promoter fragments from both aldolase and pyruvate kinase was markedly decreased (Fig. 2A, lane 2; Fig. 2B, lane 7). Incubation of nuclear extracts from resting cells with DTE resulted in an increase of binding intensity similar to that observed with nuclear extracts from proliferating cells (Fig. 2A, lane 4; Fig. 2B, lane 9). This result suggests that the low DNA-binding efficiency of Sp1 observed with nuclear extracts from resting cells may reflect the sensitivity of Sp1 to peroxide anions.

In a control experiment nuclear extracts from proliferating cells were incubated with the consensus sequence of the transcriptional factor C/EBP, which is known to be unaffected by the redox state [24,25]. As expected, C/EBP-DNA-binding efficiency was not changed by treatment with hydrogen peroxide (Fig. 2C).

From these results we conclude that the Sp1 protein in the

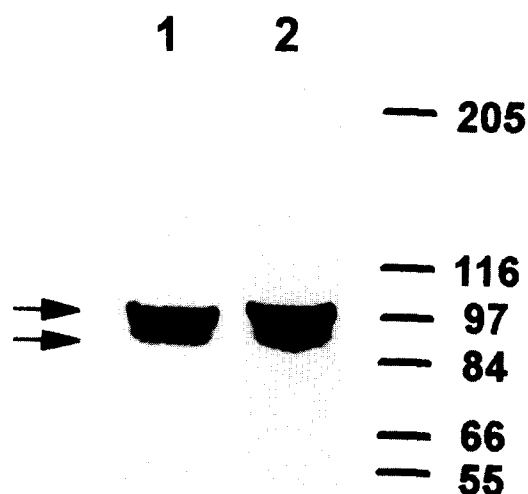


Fig. 3. Immunoblot of nuclear extracts from resting and proliferating rat thymocytes. 25 μ g of nuclear protein from proliferating (lane 1) and resting (lane 2) thymocytes were loaded on each lane. The proteins were detected with a polyclonal antibody against Sp1. The upper arrow indicates the phosphorylated form of Sp1, the lower arrow indicates the dephosphorylated one.

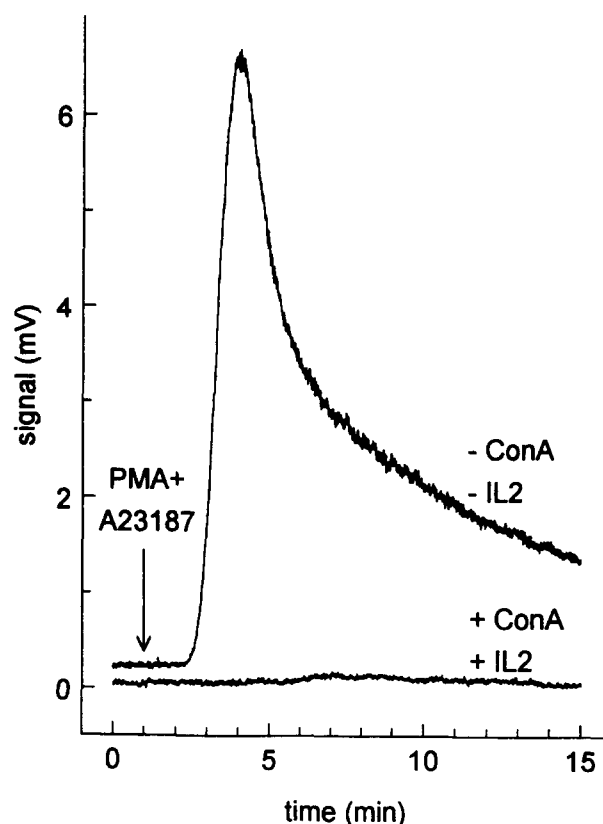


Fig. 4. PMA-initiated chemiluminescence response of non-stimulated and ConA plus IL-2-stimulated rat thymocytes. For cell culture conditions see Section 2. The incubations in a total volume of 1 ml contained 5×10^7 cells each, 4 mM glucose and 170 μ M Luminol in PBS pH 7.4 with 0.02% EDTA. Luminol chemiluminescence as a measure of peroxide anion production was initiated by the addition of 2 μ g ml⁻¹ PMA and 1 μ g ml⁻¹ A23187 calcium ionophore. Photon emission rate at 37°C from one assay vial is plotted on the vertical axis in mV and traced for 10 min by continuous recording with a chromato-integrator, model 2500 Hitachi. One representative experiment out of seven is shown.

nuclear extracts from proliferating thymocytes is predominantly present in the reduced form and therefore able to bind to the GC boxes, thus provoking enhanced gene expression during proliferation. In contrast, Sp1 in nuclear extracts from resting cells appears to be partly oxidized leading to decreased DNA-binding efficiency and subsequently reduced gene expression. These studies imply a role of reactive oxygen intermediates in the regulation of Sp1-binding activity.

Potent oxidizing agents *in vivo* such as peroxide anions are generated in a variety of oxidative metabolic reactions. Measurements of reactive oxygen species revealed striking differences between resting and proliferating thymocytes. Resting cells show a marked PMA-initiated Luminol chemiluminescence, indicative of the formation of peroxide anions, whereas stimulated cells do not produce measurable amounts of this reactive oxygen species (Fig. 4). This might be explained either by the presence of an effective scavenging system such as reduced glutathione or by the reduction of metabolic reactions producing reactive oxygen intermediates in these cells.

Taken together, these results suggest that the greatly diminished peroxide anion generation observed during the S-phase allows activation of the transcription factor Sp1 which in turn

leads to enhanced expression of aldolase A and pyruvate kinase M₂ in proliferating rat thymocytes.

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