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Aldolase A is present in smooth muscle cell nuclei

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Previously we have shown that aldolase (ALD; EC 4.1.2.13) is present in cardiomyocyte nuclei. Now, we focused our attention on ALD localization in smooth muscle cells. Immunocytochemical methods were used to study the subcellular localization of ALD. Aldolase was localized in the cytoplasm as well as in the nuclei. Within the nuclei ALD was located in the heterochromatin region. Native polyacrylamide gel electrophoresis followed by aldolase activity staining in gel was used to study the ALD isoenzyme pattern in porcine smooth muscle cells. Two ALD isoenzymes, A and C, were found in these cells but in the nuclei only the muscle isoenzyme was detected. To support the nuclear localization of ALD, measurement of aldolase activity in the smooth muscle cell nuclei isolated from porcine stomach was performed. The ALD activity in the isolated nuclei was detectable only after preincubation of the nuclear fraction with Triton X-100 and high concentration of KCl.

Keywords: aldolase, heterochromatin, isoenzymes, nucleus, smooth muscle

INTRODUCTION

D-Fructose 1,6-bisphosphate aldolase [ALD; EC 4.1.2.13] catalyzes the reversible cleavage of fructose 1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. In vertebrate tissues, three ALD isoenzymes have been observed: the muscle isoenzyme (aldolase A), the liver isoenzyme presents also in the kidney (aldolase B), and the brain isoenzyme (aldolase C) (Lebherz & Rutter, 1969; Penhoet *et al.*, 1969a). ALD exists either as a homotetramer (i.e. in mammalian skeletal muscle) or as a heterotetrameric forms, i.e. in mammalian brain where A and C hybrids have been observed (Lebherz & Rutter, 1969; Penhoet *et al.*, 1969a).

In most tissues ALD associates with actin-containing filaments (Pagliaro & Taylor, 1988; Wang *et al.*, 1997). In skeletal muscles and cardiomyocytes ALD is also localized on the M-line as well as on the Z-line (Rakus *et al.*, 2003a; Mamczur & Dzugaj, 2004; Mamczur *et al.*, 2005). In these tissues, ALD has also been found on the sarcoplasmic reticulum (Xu & Becker, 1998). However, our previously presented data indicated that ALD localization was not restricted to the cytoplasmic compartment, but in cardiomyocytes ALD was also present in the nuclear heterochromatin (Mamczur & Dzugaj, 2004). Presently, we have focused our attention on mammalian smooth muscle tissues. The primary aim of the present study was to determine whether ALD is localized in the smooth muscle cell nuclei. To study the subcellular localization of ALD, immunocytochemical methods were employed. Moreover, aldolase isoenzyme pattern in the smooth muscle tissue as well as in the isolated nuclei from this tissue were also determined.

MATERIALS AND METHODS

Mammalian tissues were obtained from a local distributor. Animals were killed in agreement

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Abbreviations: ALD, D-fructose 1,6-bisphosphate aldolase; SDS/PAGE, Sodium dodecyl sulfate/polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline.

with the rules of the Scientific Research Ethical Committee of the University of Wroclaw. Gold (5 nm)-conjugated anti-mouse immunoglobulins were purchased from ICN (USA). Phosphocellulose P-11 was purchased from Whatman (England), paraformaldehyde and glutaraldehyde were obtained from Fluka (Switzerland) and polyester wax was bought from EMS (USA). Vivaspin 0.5 ml concentrator was purchased from Vivascience (Sartorius Group). PageRuler™ Prestained Protein Ladder was obtained from Fermentas. Other reagents were bought from Sigma (USA). All reagents were of the analytical grade.

ALD purification and the activity determination. Rabbit (*Oryctolagus caniculus*) and pig (*Sus scrofa domestica*) muscle aldolases were purified according to Penhoet *et al.* (1969a). The aldolase activity was determined as described by Mamczur and Dzugaj (2004). The concentration of proteins was determined spectrophotometrically with the use of Bradford reagent (Sigma). The purity of ALD was checked by 9% SDS/PAGE (sodium dodecyl sulfate/polyacrylamide gel electrophoresis) (Laemmli, 1970). All spectrophotometric measurements were performed with an Agilent 8453 diode array spectrophotometer.

Partial purification of ALD from various porcine tissues. Porcine brain, skeletal muscle and smooth muscle from stomach were used for partial purification of ALD (Penhoet *et al.*, 1969a) for further investigation of aldolase isoforms pattern. Five grams of each tissue were homogenized in 45 ml of buffer (pH 7.5): 50 mM Tris, 0.5 mM EDTA, 0.5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM β-mercaptoethanol, 250 mM sucrose, and centrifuged at 21 000 × ***g***. The supernatants were brought to 45% ammonium sulfate saturation, centrifuged as above and the precipitates were discarded. Then, the supernatants were brought to 60% ammonium sulfate final saturation, centrifuged as above and the precipitates were dissolved in 2 ml of the homogenization buffer. The partially purified aldolases were dialyzed overnight against buffer (pH 7.5) containing: 20 mM Tris, 0.5 mM EDTA, 0.1 mM PMSF, 5 mM β-mercaptoethanol, then they were used for native polyacrylamide gel electrophoresis.

PAGE and Western blotting. SDS/PAGE was performed according to Laemmli (1970). The specificity of the immunoglobulins was checked by Western blotting (Towbin *et al.*, 1979). Native polyacrylamide gel electrophoresis was used to analyze the ALD isoform pattern from porcine tissues. Partially purified aldolase (60 mU) from porcine tissues (prepared as described above) was dissolved in 20% glycerol (final concentration) in NaOH/glycine buffer (50 mM glycine, 28 mM NaOH, pH 9.8) and used for electrophoresis in 6% polyacrylamide gel in NaOH/glycine buffer with addition of 1 mM β-mercaptoethanol. The native PAGE was performed at 40 mA for 80 min. Then, the gel was used in either Western blot analysis or activity staining of ALD. For the Western blot, the proteins separated by native PAGE were transferred on a nitrocellulose membrane in the presence of NaOH/glycine buffer (pH 9.8). Subsequently, the membrane was blocked by 5% bovine serum albumin for 3 h at 37°C. Then, it was incubated with mouse anti-aldolase immunoglobulins (overnight at 4°C in 1:300 dilution) and goat anti-mouse peroxidase-conjugate antibodies (for 30 min at room temp. in 1:10 000 dilution). After each incubation the unbound antibodies were removed by washing of the membrane with 0.1% Triton X-100 in TBS (Tris-buffered saline: 50 mM Tris, 138 mM NaCl, 2.7 mM KCl, pH 7.4). Finally, the peroxidase activity was visualized by incubation of the membrane with 3,3′-diaminobenzidine. The Western blotting experiment was performed three times.

Activity staining of ALD in polyacrylamide gel. The staining of ALD activity in the gel was performed according to Penhoet *et al.* (1966), with modifications. The developing mixture contained the following reagents: 0.025 mg/ml phenazine metasulfate, 0.42 mg/ml nitroblue tetrazolium, 0.3 mg/ml nicotinamide adenine dinucleotide, 8 mM D-fructose 1,6-bisphosphate, 0.15% sodium arsenate, 7.7 U/ml glyceraldehyde 3-phosphate dehydrogenase in 0.2 M Tris, pH 8.0, at 25°C. The experiment was performed five times.

Immunocytochemistry. The production, purification and specificity of mouse immunoglobulins against rabbit muscle ALD were described previously (Mamczur & Dzugaj, 2004). Small fragments of smooth muscle tissue dissected from porcine stomach and fragments of rat (*Rattus norvegicus*) stomach were fixed in Carnoy's fluid and embedded in polyester wax. Dewaxed tissue sections were used for immunocytochemistry as described by Mamczur and Dzugaj (2004), with modifications. Before immunostaining, the sections were treated with 1% H_2O_2 for 30 min, washed in PBS and incubated with 10% normal goat serum for 30 min at room temp. Subsequently, the sections were incubated overnight at 4°C with mouse anti-muscle aldolase immunoglobulins (in 1:300 dilution). The sections were washed in PBS with 0.1% Triton X-100 (5 min), PBS without Triton X-100 (2 × 5 min), and then incubated with goat anti-mouse biotin-conjugate IgG (Fc-specific) (for 1 h at room temp. in 1:500 dilution) and washed as above. Then, the sections were incubated with peroxidase-conjugated ExtrAvidin (20 μg/ml, 30 min at room temp.). Finally, the peroxidase activity was developed using 3,3′-diaminobenzidine. The immunogold method was performed according to Mamczur and Dzugaj

(2004). In controls, the primary antibodies were omitted or non-immune mouse serum was used as the first layer.

Isolation and purification of smooth muscle cell nuclei. Smooth muscle nuclei were isolated from a porcine stomach according to Misquitta *et al.* (1999), with modifications described by Gizak *et al.* (2005). ALD activity in isolated nuclei was determined after pre-incubation of the sample with a solution containing 1% Triton X-100 and 250 mM KCl for 15 min at room temp. To determine the number of isolated nuclei, a Thom-Zeiss cell was used.

Determination of ALD isoforms present in nuclei. The pure fraction of smooth muscle cell nuclei obtained from 177 g of porcine smooth muscles was used for aldolase partial purification followed by ALD activity staining in polyacrylamide gel. The nuclear suspension was placed in 10 ml of homogenization buffer (250 mM sucrose, 10 mM Hepes, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM PMSF, 2 mM $MgCl_2$, 1% Triton X-100, 250 mM KCl), homogenized and centrifuged at 21 000 × *g*. The supernatant was brought to 20% ammonium sulfate saturation, centrifuged as above and the precipitate was discarded. Then, the supernatant was brought to 80% ammonium sulfate saturation, centrifuged (at 21 000 × *g*) and the precipitate was dissolved in 1 ml of buffer: 20 mM Tris, 0.1 mM EDTA, 5 mM β-mercaptoethanol (pH 7.4, 4°C). The partially purified ALD was concentrated and desalted using Vivaspin 0.5 ml concentrator (Vivascience, Sartorius Group) according to the manufacturer's specification. Then, the ALD activity, protein concentration, native PAGE and ALD activity staining in gel were performed. The experiment was performed three times.

RESULTS

Immonocytochemistry

To analyze the localization of ALD in smooth muscle cells, the immunoperoxidase method as well as the immunogold method were used. Rat and porcine smooth muscles from the stomach were used to study the localization of ALD. In both species, aldolase was localized diffusely in the cytoplasm (Fig. 1A and B). There was also a high accumulation of ALD inside the nuclei (Fig. 1A and B). To support the nuclear localization of aldolase, the immunogold method was used. The results showed that ALD is localized in the heterochromatin region of smooth muscle cell nuclei (Fig. 2B). No labeling occurred in the absence of the primary antibody, nor when normal serum was used instead of the primary antibody (Fig. 1C and D, Fig. 2C and D).

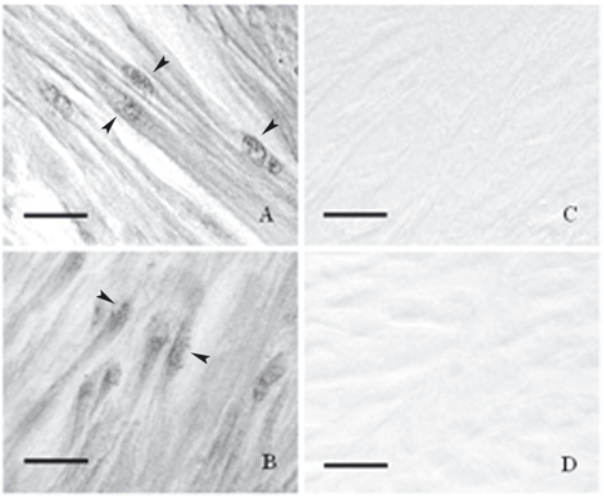

Figure 1. Immunocytochemical localization of aldolase in smooth muscle cells by light microscopy.
A. ALD localization in rat stomach smooth muscle. **B**. ALD localization in porcine stomach smooth muscle. **C**. and **D**. Control reactions for rat and porcine smooth muscle, respectively. Arrowheads indicate nuclei. Bar: 20 μm.

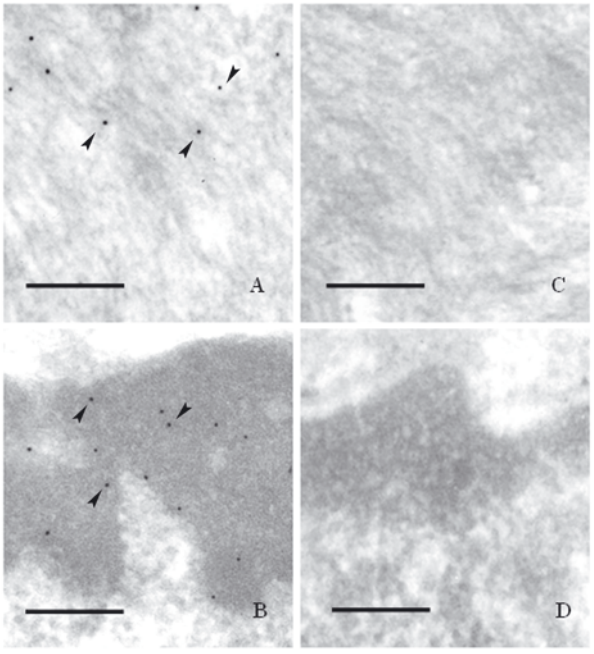

Figure 2. Localization of aldolase in porcine smooth muscle cells using immunoglod method and electron microscopy.
A. ALD localization in cytoplasm. **B**. ALD localization in heterochromatin region. **C**. and **D**. Control reactions without the first antibody for cytoplasm and heterochromatin, respectively. Arrowheads indicate aldolase localization. Bar: 125 nm.

Table 1. Aldolase activity in smooth muscle homogenate and in isolated smooth muscle cell nuclei.

The effect of mixture of Triton X-100 and KCl on aldolase activity.

Fraction	Aldolase activity (U/g tissue)		% activity
	A	B*	
Homogenate	4.13 ± 3.4	7.04 ± 4.3	100
Nuclear fraction	0 ± 0.0002	0.0026 ± 0.0008	0.037
	(U/mg protein)		
Purified aldolase	4.14 ± 0.1	4.65 ± 0.8	

*Fractions pre-incubated with solution containing 1% Triton X-100 and 250 mM KCl; ±, standard deviation of triplicate determinations

ALD activity in nuclei of smooth muscle cells

To support the nuclear localization of ALD, the enzyme activity in smooth muscle cell nuclei isolated from porcine stomach was determined. Each 1 ml of nuclear fraction contained 40–56 × 10^6 pure smooth muscle nuclei.

To ensure the destabilization of nuclear envelopes and the dissociation of ALD from nuclei, the fractions were pre-incubated with 1% Triton X-100 and 250 mM KCl for 15 min at room temp. (Mamczur & Dzugaj, 2004). There was no detectable ALD activity in the nuclear fraction which was not pre-incubated with the detergent and a high concentration of KCl (Table 1). The control reaction indicated that Triton X-100 and KCl had no effect on the ALD activity determination (Table 1). This indicated that the aldolase activity measured in the fraction was related to the enzyme which was released from the nuclei.

ALD isoform pattern in porcine smooth muscle cells

To check the aldolase isoform pattern in the pig stomach smooth muscle, native PAGE and ALD activity staining in gel were performed (Fig. 3). Purified rabbit skeletal muscle ALD was used as a control. To compare the ALD isoforms, the partially purified aldolases from porcine skeletal muscle and porcine brain were also applied. This experiment showed that in the smooth muscle, two isoforms of ALD exist: the muscle isoform (aldolase A) and the brain isoform (aldolase C) (Fig. 3B). Unexpectedly, there were no detectable ALD heterotetrameric isoforms (Fig. 3B), in contrast to ALD from the brain, where the heterotetrameric isoforms are abundant (Fig. 3D) (Lebherz & Rutter, 1969; Penhoet *et al.*, 1969a). The native PAGE also showed that there are differences between the rabbit and pig aldolase A electrophoretic mobility (Fig. 3).

To determine which ALD isoform is present inside the nuclei, partial purification of the aldolase from isolated nuclei was performed, followed by ALD activity staining in gel. This experiment showed the presence of only one aldolase isoform in

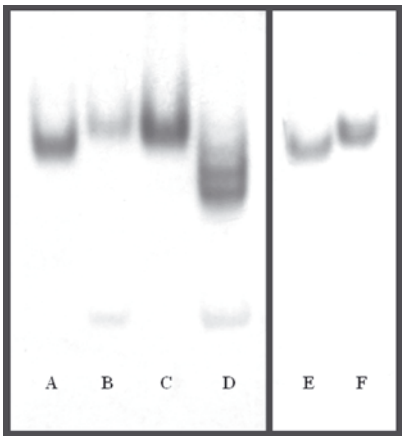

Figure 3. Activity staining of aldolase in polyacrylamide gel.
A. Purified rabbit ALD from skeletal muscle (21 U/mg protein). **B**. Partially purified smooth muscle ALD from porcine stomach (1.8 U/mg protein). Two bands are observed: upper one corresponds to muscle aldolase and lower one to aldolase C isoenzyme. **C**. Partially purified porcine skeletal muscle ALD (11.9 U/mg protein). Single band, aldolase A, is observed. **D**. Partially purified porcine brain ALD (2.6 U/mg protein). Five bands are observed: the lower one is aldolase C, and the higher one is aldolase A; next three bands correspond to heterotetrameric ALD isoenzymes. **E**. Purified rabbit skeletal muscle ALD (21 U/mg protein). **F**. Partially purified ALD from porcine smooth muscle cell nuclei (0.36 U/mg protein). Nuclear aldolase has the same mobility as porcine aldolase A.

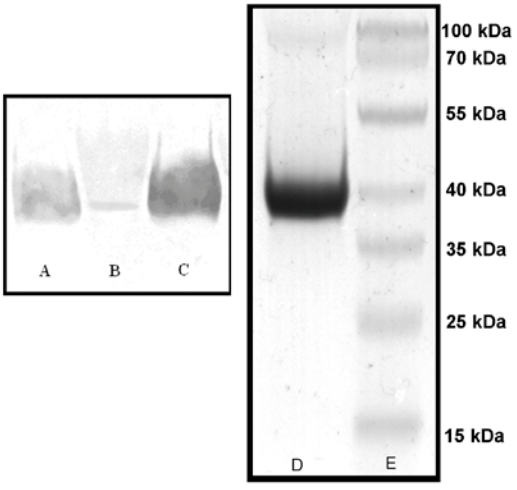

Figure 4. Western blot and SDS/PAGE.
Western blot (preceded by SDS/PAGE) on 5 μg of purified porcine skeletal muscle aldolase (A), 25 μl of porcine smooth muscle homogenate (B) and 5 μg of purified rabbit skeletal muscle aldolase (C). In the homogenate, single band corresponding to muscle aldolase is observed. SDS/PAGE of purified ALD (5 μg) from rabbit skeletal muscle (D) and protein markers (PageRuler™ Prestained Protein Ladder) (E).

Figure 5. Western blot analysis of aldolase after native PAGE.
A. Purified rabbit aldolase from skeletal muscle. **B**. Partially purified smooth muscle ALD from porcine stomach. **C**. Partially purified porcine skeletal muscle ALD. **D**. Partially purified ALD from porcine brain.

the nuclei. This ALD has the same mobility as the porcine skeletal muscle aldolase A. It strongly suggests that aldolase A is present inside the nuclei.

Western blot analysis

The specificity of the interaction between the antiserum and its antigen was checked by SDS/PAGE and Western blot analysis. The results showed a single band in the homogenate of pig smooth muscle, corresponding to purified pig and rabbit muscle aldolases (Fig. 4). We also performed a Western blot analysis preceded by native PAGE on partially purified aldolases from the porcine brain, stomach muscle and skeletal muscle (Fig. 5). Purified rabbit skeletal muscle ALD was used as a control. This experiment showed that mouse polyclonal anti-aldolase antibodies recognized almost exclusively the muscle isoform of ALD (Fig. 5).

DISCUSSION

Since, it has been reported that porcine tracheal smooth muscle contains aldolase C (Baron *et al.*, 1995), it was necessary to check which ALD isoenzymes are present in the porcine stomach smooth muscle. Our investigations revealed the presence of aldolase A and C in smooth muscle cells from the porcine stomach. We also detected aldolase A in nuclei of smooth muscles. Expression of both ALD isoenzymes in the smooth muscle has been shown previously (Adamson, 1976). It rises a question concerning the physiological role of both ALD isoenzymes in the smooth muscle. It has been postulated that aldolase A is a glycolytic enzyme involved in fructose-1,6-bisphosphate cleavage and that aldolase B is tailored for the gluconeogenic pathway catalyzing fructose-1,6-bisphosphate synthesis (Penhoet *et al.*, 1969b). The specific role of aldolase C was unknown. Since the discovery of the glyconeogenic pathway in the skeletal muscle, it has turned out that aldolase A catalyses the reaction in both directions — glycolytic and glyconeogenic, depending on which enzymatic complex it is associated with (Rakus *et al.*, 2003a; Mamczur *et al.*, 2005).

As we have previously shown, the presence of Triton X-100 and high KCl concentration in heart muscle homogenate increases of ALD activity, which means that a significant amount of aldolase is associated with subcellular structures and dissociates in the presence of the detergent and high ionic strength (Mamczur & Dzugaj, 2004). The same phenomenon was observed here, in the case of smooth muscle homogenate, indicating that, like in cardiomyocytes, ALD is associated with subcellular structures of the smooth muscle cell.

In the striated muscle, ALD associates mainly with the proteins of the I-band of the sarcomere, and is involved in the formation of the glycolytic metabolon (multi-enzyme complex) directly providing ATP to myosin ATPase (Kraft *et al.*, 2000; Sullivan *et al.*, 2003; Mamczur *et al.*, 2005). ALD is also associated with muscle fructose 1,6-bisphosphatase (FBPase) [EC 3.1.3.11] and with α-actinin (Rakus *et al.*, 2003b; Mamczur *et al.*, 2005). The aldolase–FBPase complex formation results in the desensitization of FBPase toward AMP inhibition allowing the glyconeogenesis to proceed (Rakus & Dzugaj, 2000; Rakus *et al.*, 2003b; Dzugaj, 2006). The aldolase-FBPase-α-actinin complex is probably part of the glyconeogenic metabolon located on the Z-line (Rakus *et al.*, 2003a; Mamczur *et al.*, 2005).

Hardin and Roberts (1995) have reported that in smooth muscle cells gluconeogenesis proceeds. ALD in the smooth muscle cell cytoplasm is mainly localized on actin-containing filaments, where it presumably is involved in the creation of a glycolytic metabolon (Wang *et al.*, 1997; Srere & Ovadi, 1990; Kraft *et al.*, 2000; Ovadi & Srere, 2000). The localization of the gluconeogenic metabolon in smooth muscle cells is unknown. It is feasible that aldolase A and C isoenzymes in smooth muscle cells are involved in the creation of a different metabolons — glycolytic or gluconeogenic. It is open for question whether glyconeogenesis occurs in smooth muscle cells too.

Some studies have indicated that ALD is present in the nuclei. It has been demonstrated that ALD accumulates in the nuclei of many types of tumors (Ronai *et al.*, 1992), hepatocytes and renal cells (Saez & Slebe, 2000) as well as in cardiomyocytes (Mamczur & Dzugaj, 2004). The increase of ALD accumulation in the cell's nuclei has been presented

in the Hoechst-resistant mutant cells (Satou *et al.*, 2004). Moreover, it has been shown that aldolase associates with AT-rich DNA sequences (Ronai *et al.*, 1992; Satou *et al.*, 2004). Nevertheless, its function in the nuclear compartment is still unknown. In cardiomyocytes, ALD has been localized inside the nuclei, in the heterochromatin region (Mamczur & Dzugaj, 2004). Here, we have presented evidence that ALD is also present in the nuclear heterochromatin in smooth muscle cells.

The localization of ALD in the heterochromatin region of smooth muscle cells nuclei raises a question concerning the physiological role of this cytosolic enzyme in this compartment. Several enzymes of carbohydrate metabolism are involved in nuclei in processes highly distinct from their function in the cytoplasm. Glyceraldehyde 3-phosphate dehydrogenase is engaged in DNA transcription and repair (Ronai, 1993; Sirover, 1997), lactate dehydrogenase participates in DNA reparation (Ronai, 1993), phosphoglycerate kinase may be involved in DNA synthesis as well as in the cell-cycle progression (Popanda *et al.*, 1998).

Previously, we have found that FBPase (an enzyme indispensable in either glyconeogenesis or gluconeogenesis) in muscle cells is present predominantly in the cytoplasm, but has also been found in the nuclei of cardiomyocytes (Gizak & Dzugaj, 2003) and smooth muscle cells (Gizak *et al.*, 2005). However, this enzyme was absent in the nuclei of skeletal muscle fibers (Gizak *et al.*, 2003). Recently, we have discovered that FBPase is present in the nuclei of satellite cells (Gizak *et al.*, 2006). The presence of FBPase in the nuclei of proliferating cells or cells having the capacity to proliferate, might indicate that FBPase participates in this process. We have not found aldolase in the nuclei of skeletal muscle, but previously we have shown that ALD is present in the nuclei of cardiomyocytes, and here — in the nuclei of smooth muscle cells. Like FBPase, the nuclear aldolase A might participate in cell proliferation.

The ALD function in the cytoplasm is well understood. On the other hand, its function in heterochromatin is still unknown, and further investigation is required to elucidate its functions in the nucleus.

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