Histol Histopathol (2003) 18: 135-142

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Immunohistochemical localization of human fructose–1,6–bisphosphatase in subcellular structures of myocytes

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Summary. The localization of fructose-1,6-bisphosphatase (FBPase) in human skeletal muscle was determined immunohistochemically using polyclonal antibodies. Light microscopy analysis, confirmed with the use of confocal microscopy, indicated that the enzyme is localized on both sides of the Z line of myocytes. The immunohistochemical investigation was confirmed by a co-sedimentation experiment which revealed that muscle FBPase binds strongly to α-actinin - a major structural protein of the Z line. This is the first report on localization of FBPase in skeletal muscle tissue.

Key words: Fructose-1,6-bisphosphatase, Confocal microscopy, Muscle, Immunohistochemistry

Introduction

Fructose-1,6-bisphosphatase (FBPase) [EC 3.1.3.11] is regarded as a key enzyme of gluconeogenesis. Liver and muscle isozymes have been found in mammalian tissues (Tejwani, 1983; Al-Robaiy and Eschrich, 1999). FBPase catalyzes the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate in the presence of such divalent cations like magnesium, manganese, zinc or cobalt. Monovalent cations are needed for the enzyme to achieve its maximum activity (Benkovic and DeMaine, 1982; Tejwani, 1983). The enzyme is inhibited competitively by fructose-2,6-bisphosphate and allosterically by AMP (Pilkis et al., 1981; Van Schaftingen and Hers, 1981). Muscle isozyme is more sensitive to inhibition by AMP than the liver isozyme. Liver isozyme, also found in kidney, intestine and monocytes is recognized as a regulatory enzyme of gluconeogenesis (Mizunuma and Tashima, 1978; Tejwani, 1983). It has been suggested that, besides liver and kidney, lung is also a gluconeogenic organ (Latres et al., 1992). Recently we have found liver FBPase isozyme in lung tissue (Skalecki et al., 1999) and presented immunohistochemical evidence that in human lung FBPase is located in pneumocytes II (Gizak et al., 2001). Muscle isozyme of FBPase participates in the glycogen synthesis from lactate (Hermansen and Waage, 1979; McLane and Holloszy, 1979) and is involved in glycolysis regulation *via* futile cycle (Newsholme and Start, 1976).

Although the sequences of enzymatic reactions that constitute the glycolytic and gluconeogenic pathways are well known, their regulation and spatial organization are not yet fully understood. The glycolytic enzymes have been historically considered as soluble constituents of the cell but this classical concept is seen as controversial. Many studies indicate that glycolytic enzymes do not exist in a soluble form only, they also may be (reversibly) associated with cellular structures (Clarke and Masters, 1975; Masters et al., 1987; Ovadi and Srere, 2000). This association alters regulatory properties and kinetics of the enzymes (Liou and Anderson, 1980), and interactions between metabolically sequential enzymes facilitate channeling of substrates (Ovadi and Srere, 2000). It has also been proposed that some glycolytic enzymes may play structural roles in cytoplasm, in addition to having catalytic roles (Wang et al., 1996). Similarly, it has been suggested that some gluconeogenic enzymes also associate with cellular structures. Intracellular localization of FBPase has been studied in liver and kidney cells (Saez et al., 1996), but there are no data on the muscle isozyme.

Our paper is the first report on the distribution of FBPase in muscle tissue. Using light and confocal microscopy we have examined localization of FBPase in striated muscle cells. The data presented here indicate that human muscle FBPase is located on both sides of the Z line, in the isotropic regions of myocytes. The co-sedimentation experiment, indicating that muscle FBPase binds strongly to α-actinin, a major structural protein of the Z line, corroborated the existence of the interactions between these two proteins in skeletal

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muscle cells.

Materials and methods

Materials

Samples of intercostal muscle were removed surgically from patients of Lower Silesian Pulmonary Center. They were men, aged 49 to 60 years (mean 58 years) with histologically proven diagnosis of squamous cell lung cancer. Tumor was located in hillus and for this reason pneumonectomy was performed. The skeletal muscle samples were checked histologically to avoid contamination with cancer cells. The samples were taken in agreement with the rules of The Scientific Research Ethical Committee.

Antibody Diluent With Background Reducing Components and DAB chromogen were purchased from DAKO (USA), ammonium sulfate, Canada balsam, Coomassie Brilliant Blue R-250 and Paraffin Wax were from Fluka (Switzerland), Polyester Wax was from EMS (USA), xylene, CH_3OH, NaCl, H_2O_2 were obtained from POCh (Poland). Biotin Conjugated Mouse Monoclonal Anti-Rabbit Immunoglobulins, ExtrAvidin-Horseradish Peroxidase, FITC Conjugated Mouse Monoclonal Anti-Rabbit Immunoglobulins, Normal Mouse Serum, Anti-α-Actinin, α-Actinin and other reagents were from Sigma (USA).

All of the reagents were of the highest purity comercially available.

Antibody production and Western blot analysis

Antiserum against the human liver FBPase was raised in rabbit by intracutaneous injection of the electrophoretically pure enzyme (2 mg; isolated from human liver according to Dzugaj and Kochman, 1980) and Freund's complete adjuvant. Immunoserum was partially purified using acetone powder from human liver. Reactivity of anti-FBPase serum was estimated using double diffusion method (Clausen, 1988).

Immunodiffusion resulted in a strong reaction between the antiserum and the pure enzyme. To check specificity of the antibodies immunoblotting was performed as described by Towbin et al. (1979).

Immunohistochemistry

Light microscopy. Human muscle tissue samples were fixed in Bouin's fluid for 24 hours at room temperature. After the fixation they were dehydrated and embedded in paraffin. 5 μm sections were cut from the blocks of tissues and mounted on slides. Before immunostaining sections were deparaffinized and treated with 1% H_2O_2 for 30 minutes to inhibit endogeneous peroxidase. Then they were incubated with normal mouse serum (1:20), with rabbit polyclonal antibodies against human liver FBPase (diluted 1:500 in Antibody Diluent) or with rabbit anti-α-actinin antiserum (1:250) overnight at 4 °C and at room temperature for 1 hour, with Biotin Conjugated Monoclonal Anti-Rabbit Immunoglobulins (1:1000) for 30 min. at 37 °C and with ExtrAvidin-Horseradish Peroxidase complex (21 μg/ml) for 30 min. at 37 ºC.

Each incubation was followed by three washings with PBS, pH 7.4. Finally the peroxidase reaction was developed using 3,3'-diaminobenzidine (DAB), (Hsu et al., 1981) and examined by light microscopy.

In control reactions the primary antibody was omitted to detect nonspecific binding of the secondary antibodies; or IgG from normal rabbit serum were used as a first layer, to exclude nonspecific staining with the primary antibody.

Confocal microscopy

For confocal microscopy muscle tissue was fixed in Zamboni's fixative for 10 hours at room temperature, dehydrated and embedded in polyester wax. 10 μm sections were cut and mounted on slides. Before immunostaining, sections were dewaxed, and incubated with normal mouse serum. Then they were incubated with rabbit polyclonal antibodies against human liver FBPase overnight at 4 °C and with mouse anti-rabbit monoclonal antibodies FITC conjugated (1:50) 1 h at 37 °C. After extensive washing in PBS, the immunolabeled sections were mounted in 90% glycerol in PBS (pH 8.9 to reduce fading). In controls the primary antibody was omitted or nonimmune rabbit serum was used as a first layer.

Labeled muscles were imaged with a Zeiss LSM 510 confocal microscope, equipped with krypton/argon laser sources. Tissue was imaged with a x40 (NA 1.3) objective. Optical sections at consecutive intervals of 0.25 μm were imaged through the depth of the labeled muscles and saved as image stacks.

Purification procedures

Rabbit muscle FBPase was purified to homogeneity from fresh skeletal muscle according to the method of Rakus and Dzugaj (2000). Rabbit muscle aldolase was purified according to Penhoet et al. (1969). F-actin was purified according to the procedure of Pardee and Spudich (Pardee and Spudich, 1982). The FBPase, aldolase and actin purity was checked by 10% SDS-PAGE (Laemmli, 1970).

Binding experiment

Rabbit muscle FBPase was mixed with α-actinin in a buffer containing 20 mM TRIS, 1 mM EDTA, 150 mM KCl, , 2 mM Mg^{2+}, and 10% PEG 8000 (pH 7.5, T=25 °C). Each sample (100 μl) contained 50 μg/ml (1.36 μm monomer concentration) FBPase and various concentrations of α-actinin: 0-200 μg/ml (0-2 μM monomer concentration). Following incubation at room temperature for 60 min the samples were centrifuged at

250,000g, for 60 min., at 4 °C. The supernatant was decanted and tested for the FBPase activity. The pellet was resuspended in 200 μl of 2 mM TRIS/0.04 mM EDTA (pH 7.5, T=4 °C) and the quantity of FBPase was determined measuring enzyme activity. The control experiment was performed by centrifuging FBPase without actinin.

Binding of aldolase to α-actinin was performed as describe above for FBPase:α-actinin binding determination. Each sample (100 μl) contained 50 μg/ml (1.25 μm monomer concentration) of aldolase and various concentrations of α-actinin: 0-1000 μg/ml (0-10 μM monomer concentration) the quantity of aldolase was determined measuring enzyme activity.

Rabbit muscle FBPase was mixed with F-actin in the same buffer as in the above experiment. Each sample (250 μl) contained 100 μg/ml (2.72 μm monomer concentration) FBPase and various concentrations of F-actin (0.05-5 mg/ml). Following incubation at room temperature for 60 min. the samples were centrifuged at 100,000g for 30 min. at 4 °C. The supernatant was decanted and tested for the enzyme activity. The pellet was resuspended in 125 μl of 2 mM TRIS/0.04 mM EDTA (pH 7.5, T=4 °C) and the quantity of FBPase was determined by measuring enzyme activity. The control experiment was performed by centrifuging FBPase without actin and incubating FBPase with actin without centrifugation.

FBPase activity was assayed in 50 mM BTP, 2 mM $MgCl_2$, 150 mM KCl, 1 mM EDTA, 0.5 mM NADP, 5 units/ml glucose-6-phosphate dehydrogenase, 2 units/ml glucose-6-phosphate isomerase, 50 μm fructose-1,6-bisphosphate, pH 7.5; the assay was carried out at 37 °C (Rakus and Dzugaj, 2000). The substrate was used to start the reaction.

Aldolase activity was assayed as described Penhoet et al. (1969).

Results

Light microscopy. Localization of the FBPase in striated muscle was checked with the use of conventional light microscopy. Incubation of skeletal muscle tissue sections with anti-FBPase antibodies, biotinylated secondary antibodies, and avidin-peroxidase complex resulted in dark striation, presumably on the Z line. Incubation of serial sections of the tissue with anti-FBPase antibodies (Fig. 1A) or with antibodies against α-actinin (Fig. 1B), a major structural protein of the Z line (Djinovic-Carugo et al., 1999) revealed the same striation pattern.

In all the cases no labeling occurred in the absence of the respective primary antibodies (Fig. 1C) or when the normal sera were used instead of primary antibodies.

Additionally, Western blots were performed to check specificity of the interaction between antisera and their antigens and to exclude cross-reactivity. Results demonstrated that antiserum against liver FBPase detected muscle isozyme, but did not react with α-actinin (Fig. 2) or with other proteins from muscle crude extract (Fig. 3). Antibodies against α-actinin did not bind to FBPase (Fig. 2).

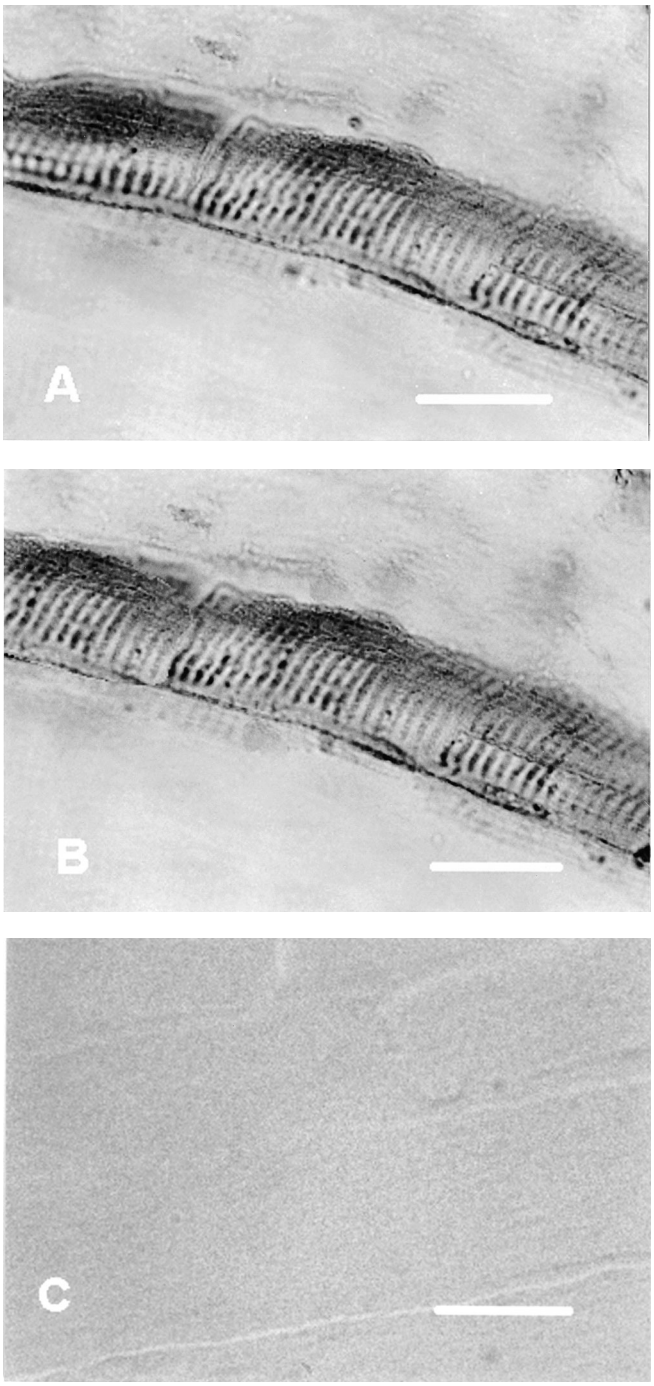

Fig. 1. Serial sections of human muscle tissue stained with: anti-FBPase antibodies **(A)**; anti-α-actinin antibodies **(B)**; and avidin-peroxidase method. **C.** Control reaction (with omission of the specific antibodies). Bar: 20 μm.

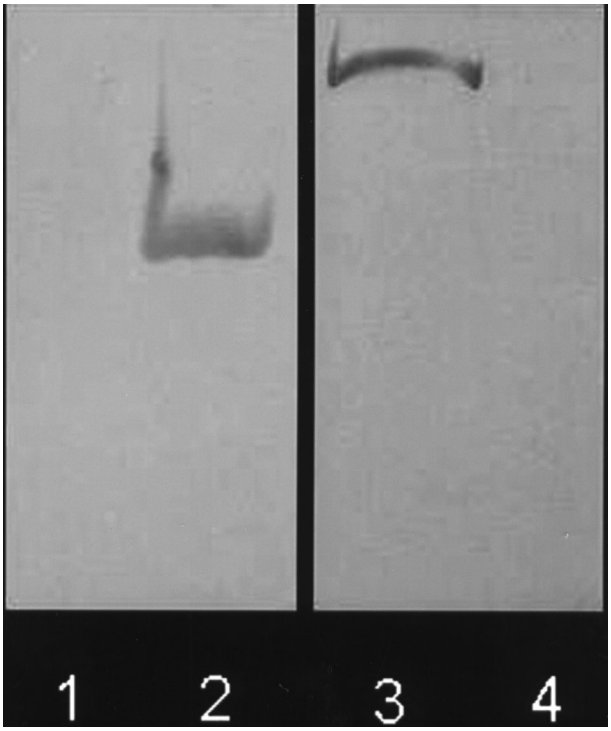

Fig. 2. Western blot analysis of purified rabbit muscle FBPase (lane 2 and 4, MW 37 kDa) and rabbit muscle α-actinin (lane 1 and 2, MW 100 kDa). Blottings were carried out using anti-liver FBPase serum (lane 1 and 2) and anti-muscle α-actinin (lane 3 and 4).

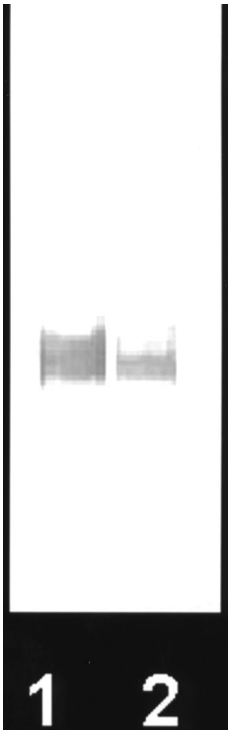

Fig. 3. Western blot analysis of crude rabbit muscle extract. Blottings were carried out using anti-liver FBPase serum. Lane 1: muscle extract: lane 2: pure FBPase.

Binding experiment

Results of the saturation of electrophoretically pure rabbit muscle FBPase (Fig. 4) with variable concentrations of α-actinin are presented in Fig. 5. Assuming that 1 molecule of FBPase binds 1 molecule of α-actinin, binding constant of FBPase and α-actinin was calculated as $K_A = 5.4x10^7$ M-1 with the use of the GraFit program (Leatherbarrow, 1992).

Interaction of FBPase with F-actin resulted in heterologous complex formation. As is shown in Fig. 6, an increasing F-actin concentration resulted in an increase in the amount of bound enzyme. The calculated FBPase:F-actin binding constant assuming 1 single-site ligand binding equation (Leatherbarrow, 1992) was $6.9x10^4$ M^{-1}.

Saturation of aldolase with α-actinin is shown in Fig. 7. The determined aldolase:α-actinin binding constant assuming that 1 monomer of α-actinin binds 1 aldolase monomer (Leatherbarrow, 1992) was $1x10^5$ M^{-1}.

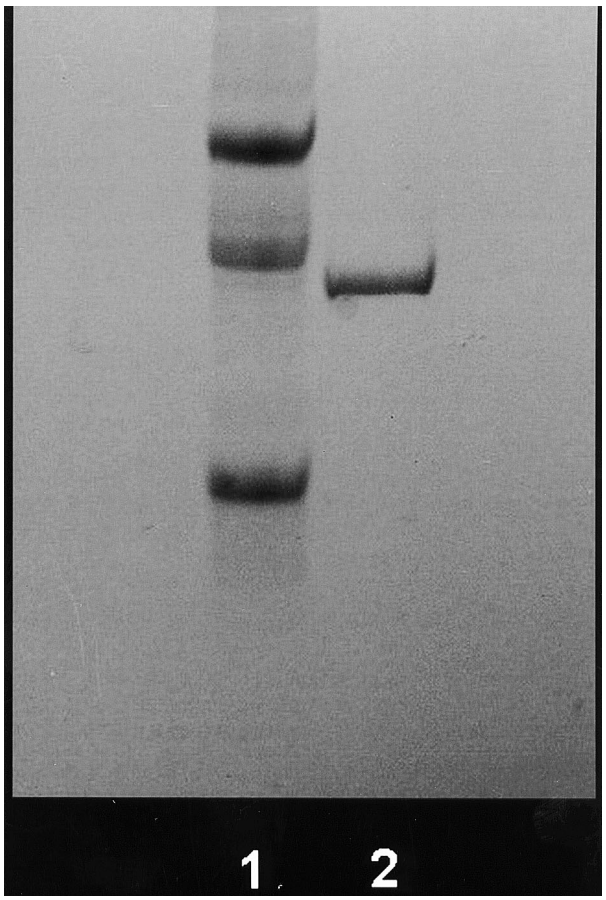

Fig. 4. SDS-PAGE (10%) of rabbit muscle FBPase. The gel was stained with Coomassie Brilliant Blue R-250. Left lane (molecular mass standards): albumin egg (MW 45 kDa), glyceraldehyde-3-phosphate dehydrogenase (MW 37 kDa), and carbonic anhydrase (MW 29 kDa); right lane: rabbit muscle FBPase.

Confocal microscopy

Analysis of the wax sections of muscle tissue with the use of FITC-conjugated secondary antibody revealed that FBPase was located on both sides of the Z line (Fig. 8A).

Staining with nonimmune serum resulted in extremely weak fluorescence, presumably due to a small amount of nonspecific adsorption. When photographed, no fluorescence was visible (Fig. 8B).

Discussion

Since the discovery of the Embden-Meyerhof-Parnas pathway in the late thirties, up to the seventies it was a common belief that the glycolytic enzymes were just soluble proteins, evenly distributed in the cytosolic compartment of the cell.

After the discovery of the mitochondrial structure and its function, a corresponding organelle for glycolysis was hoped to be found, unfortunately without succes.

Arnold and Pette (1968) have been among the first who reported that glycolytic enzymes are associated with the structural proteins of cells. By now, a number of papers describing the binding of the enzymes to the structural cell proteins are available (Minaschek et al., 1992; Volker et al., 1995). The degree of the association seems to be variable, depending on the state of the cell. For example, the distribution of muscle hexokinase II into soluble and particulate fractions depends on insulin (Vogt et al., 1998).

Basically, two kinds of experiments have been performed: an investigation of the interaction of the enzymes with the protein of the structural components of cells; and an immunocytochemical study. The experiments of the first kind revealed high affinity of the glycolytic enzymes to F-actin. The second type enabled the localization of the enzymes in the subcellular structures of cells.

The localization of the liver FBPase, the regulatory enzyme of gluconeogenesis has also been investigated. Saez et al. (1996) presented evidence on the localization of FBPase in the nuclear periphery of liver and renal cells. Our paper is the first report on subcellular localization of muscle FBPase. Immunocytochemical investigation in a light microscope revealed that FBPase is located on the Z line. With the use of a confocal microscope it was possible to localize FBPase with higher precision on both sides of the Z line, in the isotropic regions of myocytes. Similar patterns have been reported for some glycolytic enzymes (Beitner, 1985). However, investigation of FBPase:α-actinin and

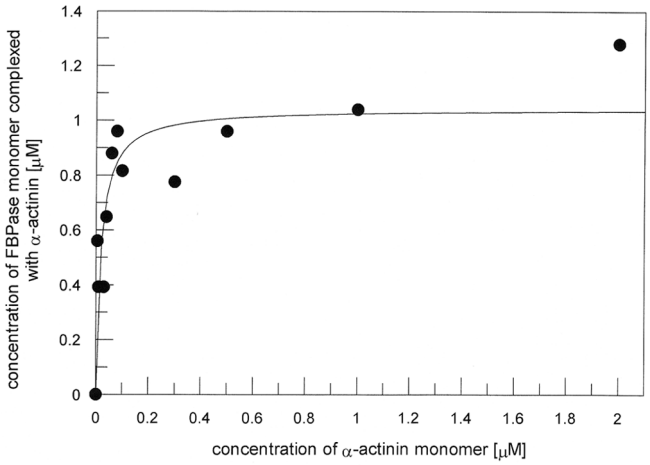

Fig. 5. Saturation of muscle FBPase (1.36 μm monomer concentration) with muscle α-actinin. The experiment was performed as described in Materials and methods.

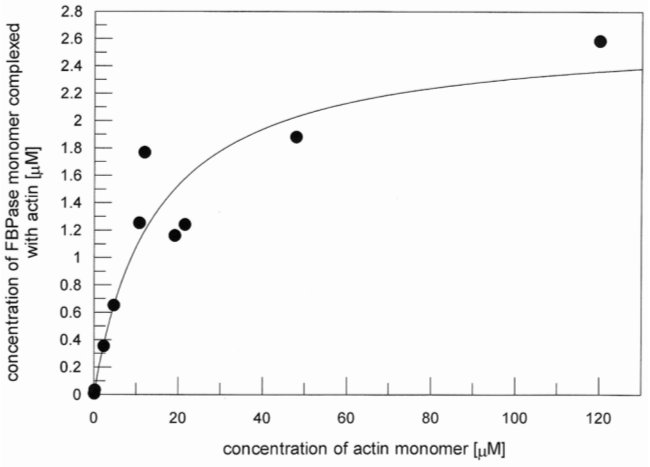

Fig. 6. Saturation of muscle FBPase (2.72 μm monomer concentration) with muscle F-actin. The experiment was performed as described in Materials and methods.

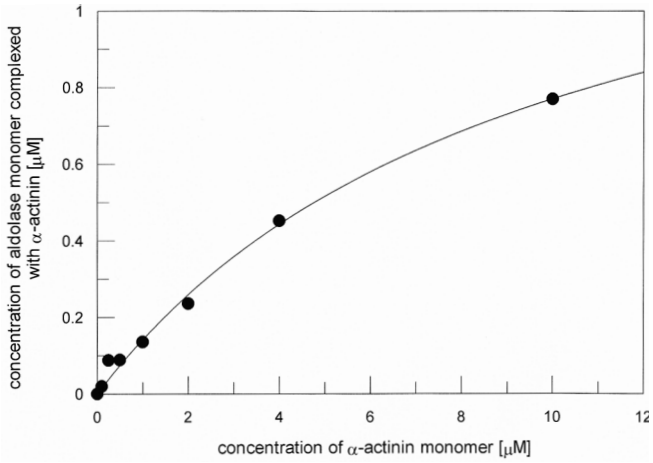

Fig. 7. Saturation of muscle aldolase (1.25 μm monomer concentration) with α-actinin. The experiment was performed as described in Materials and methods.

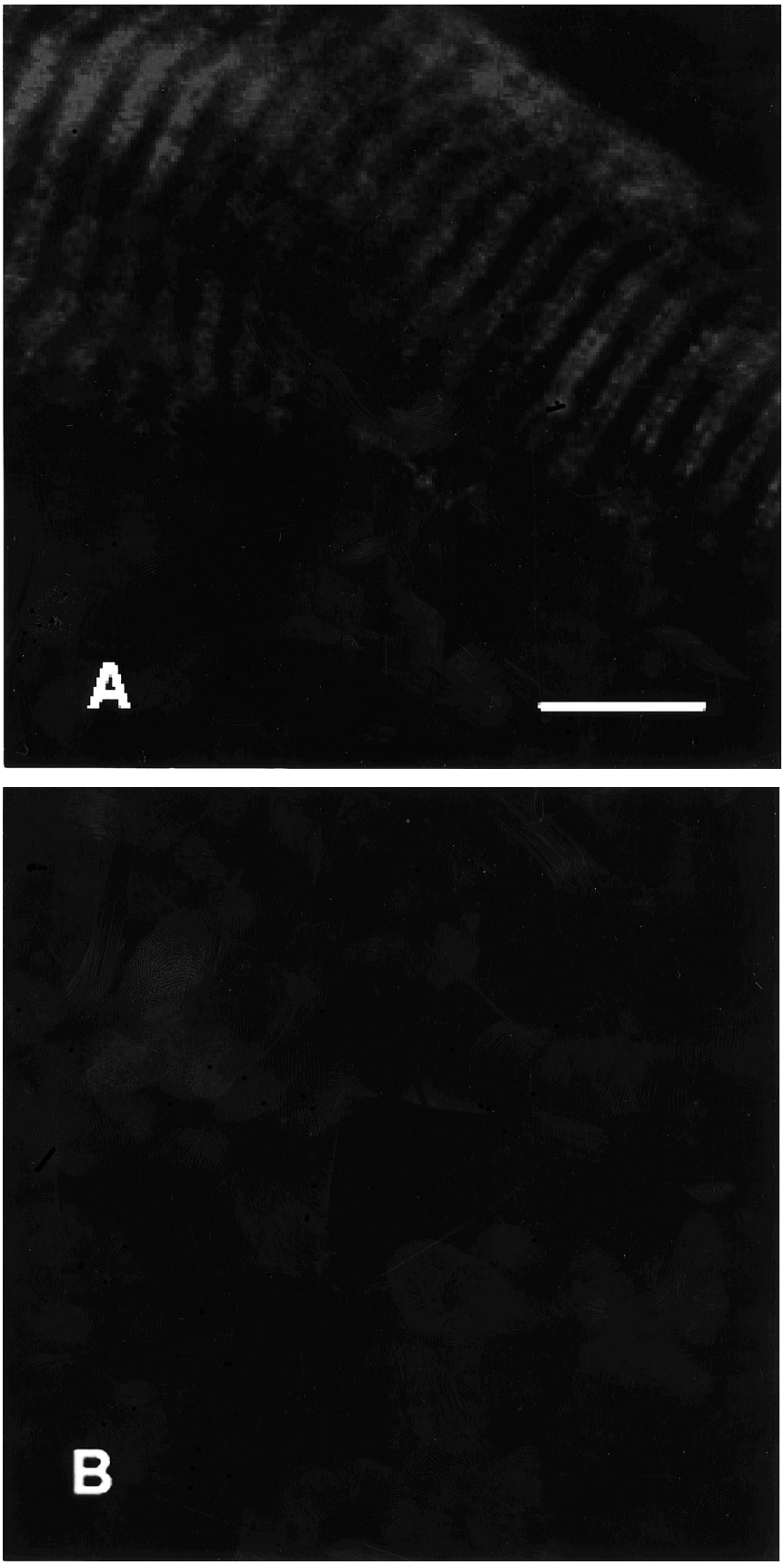

Fig. 8. Confocal visualization of human muscle FBPase. **A.** Tissue stained with anti-FBPase antibodies and FITC conjugated secondary antibodies. **B.** Control reaction (with omission of the specific antibodies). Bar: 10 μm.

FBPase:F-actin interactions revealed almost one thousand higher affinity of FBPase to the former, which supports the hypothesis that *in vivo* FBPase binds to Z line.

It has been postulated that FBPase participates in the regulation of glycolysis via futile cycle (Newsholme and Start, 1976). Since glycogen synthesis from noncarbohydrate precursors in striated muscle has been reported, FBPase must participate in this pathway. Muscle FBPase is highly sensitive to synergistic inhibition by AMP and fructose-2,6-bisphosphate. $I_{0.5}$ for AMP inhibition is close to 0.1 μm and at physiological concentration of AMP (i.e. 10-20 μm) muscle FBPase in vivo should be almost completely inhibited (Skalecki et al., 1995). Recently we have presented evidence that interaction of muscle aldolase with muscle FBPase results in desensitization of the latter towards AMP (Rakus and Dzugaj, 2000). It is feasible that in muscle cells, FBPase participates in formation of at least ternary complex with α-actinin and aldolase. Although the binding constant of FBPase to aldolase ($K_A \approx 1x10^6$ M^{-1}) is ten times lower than K_A for FBPase:α-actinin complex, it is still ten times higher than KA for aldolase:·-actinin ($K_A=1x10^5$ M^{-1}). Furthermore, affinity of aldolase to FBPase is not lower than affinity of aldolase to such cytoskeletal structures and proteins as: actin - $K_A=4x10^5$ M^{-1} ~ $2x10^6$ M^{-1} (Walsh et al., 1980; Lakatos and Minton, 1991) or microtubules - $K_A=5x10^4$ M^{-1} (Vertessy et al., 1997). Therefore, although muscle aldolase was localized on the structural proteins of skeletal muscle, tubulin (Carr and Knull, 1993) and recently on sarcoplasmic reticulum (Xu and Becker, 1998), the additional complex of aldolase and FBPase localized on the Z line is not excluded. Especially if we take into account that concentration of aldolase in muscle cells (ca 130 μm) is about 200 times higher than concentration of FBPase (ca 0.7 μm). Our results are in agreement with immunocytochemical study of Lane (Lane et al., 1989) who found that glycogen synthase is associated with Z line in rabbit myocytes. These results support hypothesis that glyconeogenic enzymes in striated muscle form a metabolic complex on both sides of Z line.

The hypothesis, however, asks for additional investigation.

Acknowledgements. We wish to thank Prof. U. Grossbach and Prof. J.R. Wisniewski from III. Zoologisches Institut-Entwicklungsbiologie, University of Goettingen, for enabling us to work with the confocal microscope.

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Accepted September 26, 2002