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The MEF2A and MEF2D Isoforms Are Differentially Regulated in Muscle and Adipose Tissue during States of Insulin Deficiency*

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ABSTRACT

Previously we have demonstrated that striated muscle GLUT4 gene expression decreased following streptozotocin-induced diabetes due to a loss of MEF2A transcription factor expression without any significant effect on the MEF2D isoform (Mora, S. and J. E. Pessin (2000) *J Biol Chem*, 275:16323–16328). In contrast to both cardiac and skeletal muscle, adipose tissue displays a selective decrease in MEF2D expression in diabetes without any significant alteration in MEF2A protein content. Adipose tissue also expresses very low levels of the MEF2 transcription factors and nuclear extracts from white adipose tissue exhibit poor *in vitro* binding to the MEF2 element. However, addition of *in vitro* synthesized MEF2A to adipose nuclear extracts results in the formation of the expected MEF2/DNA complex. More importantly, binding to the MEF2 element was also compromised in the diabetic condition. Furthermore, *in vivo* overexpression of MEF2A selectively in adipose tissue did not affect GLUT4 or MEF2D expression and was not sufficient to prevent GLUT4 down-regulation that occurred in insulin-deficient states. (*Endocrinology* **142:** 1999–2004, 2001)

THE GLUT4 facilitative glucose transporter is the major insulin-responsive glucose transporter and is predominantly expressed in adipose and striated muscle tissues (1–3). In the basal state, this transporter slowly recycles between a poorly described intracellular compartment and the plasma membrane such that in the steady-state this carrier is localized mainly inside the cell (3–7). Upon insulin stimulation, this transporter redistributes to the plasma membrane, thereby increasing the number of functional glucose transporters at the cell surface and enhancing glucose uptake (1, 3, 5, 6, 8–10).

In addition to this acute regulation of GLUT4 subcellular localization, the expression of the GLUT4 gene is also under dynamic regulatory control. In insulin-deficient states such as fasting or streptozotocin [STZ (1)]-induced diabetes, both GLUT4 messenger RNA and protein content are severely down-regulated in adipose, cardiac, and skeletal muscle (11–16). Although the loss of GLUT4 protein expression can occur at multiple levels, there is a near complete transcriptional inhibition in STZ-induced diabetes (17).

Promoter studies performed in both tissue culture cell lines and in transgenic mice have revealed the presence of two critical cis-DNA elements in the transcriptional control of this gene. Oshel *et al.* (18) have identified a regulatory element (Domain I) located between −712 and −772 of the human GLUT4 promoter that binds a 70-kDa protein. Other studies have also identified a MEF2 binding element located between −473 and −464 (19). Mutational analysis of the MEF2 element has demonstrated its functional necessity for tissue-specific and hormonal/metabolic regulation (20, 21). More recently, we have demonstrated that cardiac and skeletal muscle expression of GLUT4 is dependent upon the binding of a MEF2A-MEF2D heterodimer complex to this element (22). STZ-induced insulin deficiency resulted in a specific decrease in MEF2A protein content in striated muscle, which in turn resulted in a decreased binding to the MEF2 element (22). These data demonstrated that the MEF2A isoform was a critical factor in muscle-specific regulation of GLUT4 gene expression.

In our continuous efforts to analyze the functional role of MEF2 in GLUT4 transcriptional regulation, we have now observed that in contrast to striated muscle, STZ-induced diabetes resulted in a selective loss of MEF2D expression in adipose tissue without any significant effect on the MEF2A isoform. Furthermore, overexpression of MEF2A in adipose tissue was not sufficient to prevent GLUT4 down-regulation.

Materials and Methods

Generation of transgenic mice

Human MEF2A full complementary DNA was cloned into the *Mlu*I and *Not*I sites of pSTEC vector (obtained from Dr. Curt Sigmund, The University of Iowa) and the AP2 promoter (kindly provided by Dr. Barbara Kahn, Harvard Medical School) was cloned upstream into the *Hin*dIII and *Sph*I sites. Constructs were then linearized, purified, and injected into the male pro-nucleus followed by implantation into a pseudopregnant female mice. Positive founders were identified by PCR and Southern blot analysis. All procedures were reviewed and approved by the University of Iowa Committee for the Care and Use of Animals.

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Animal maintenance

Animals were housed in 12-h light, 12-h dark cycle and fed *ad libitum*. Experimental diabetes was performed by a single ip injection of STZ, 200 mg/kg for mice and 85–100 mg/kg for rats. Following 3–4 days after the STZ injection, blood tail samples were analyzed for glucose concentration using a One Touch glucometer (Lifescan, Milpitas, CA). Mice with glycemia higher than 400 mg/dl and rats with glycemia higher than 300 mg/dl were considered diabetic. Three to five days following the STZ injection, animals were killed by CO_2 asphyxiation. One set of diabetic rats were subsequently treated with insulin for 7 days as previously described (22).

Preparation of tissue extracts and Western blotting

Adipose tissue from individual mice was carefully minced in a buffer containing 30 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM NaF, 2 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinin, 2 μg/ml pepstatin A, and 2 μg/ml leupeptin. Samples were subsequently homogenized with 20 strokes in a Dounce homogenizer in ice and incubated for 15 min at 17 C. Samples were then centrifuged at 13,000 $\times$ *g* for 10 min at 4 C to remove insoluble materials. Protein content in the supernatants was assayed by the BCA method (Pierce Chemical Co., Rockford, IL). Samples were then run in an SDS-PAGE and transferred onto a nitrocellulose filter and subsequently immunoblotted with various antibodies as indicated in the individual figure legends.

Nuclear extracts from adipocytes

Epididymal fat pads from four to five Sprague Dawley rats were pooled together, minced, and digested with type I collagenase (Harlan, Inc., Indianapolis, IN) (Worthington Biochemical Corp., Lakewood, NJ) in a Krebs-Ringer-HEPES (KRHB) buffer (30 mM HEPES, pH 7.4, 120 mM NaCl, 4 mM KH_2PO_4, 1 mM $MgSO_4$, 75 μM $CaCl_2$, 10 mM $NaHCO_3$, 1% BSA), for 45 min to 1 h at 37 C with gentle agitation. After digestion, adipocytes were filtered through a cotton mesh and washed 4 times with KRHB buffer and twice quickly in hypotonic buffer (10 mM HEPES, pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 2 μg/ml each aprotinin, pepstatin A, leupeptin, and 6 μg/ml each TPCK and TLCK). After the last wash, cells were incubated at room temperature for 10 min in 10 ml of this buffer and subsequently homogenized in the same buffer using a glass Dounce homogenizer on ice. Nuclei were pelleted by centrifugation at 3,000 $\times$ *g* for 20 min at 4 C. To obtain a crude total membrane preparation, the supernatant was centrifugated at 200,000 $\times$ *g* for 90 min.

Nuclear protein extracts were obtained by incubating the isolated nuclei in one volume of nuclear extraction buffer (10 mM HEPES, pH 7.6, 400 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol) supplemented with 1/10 of a volume of 4 M KCl (~725 mM KCl final), and the extraction was allowed to proceed for 30 min at 4 C. After extraction, the insoluble nuclei were precipitated by centrifugation at 13,000 $\times$ *g* for 15 min. The nuclear extract was then dialyzed against a buffer containing 25 mM HEPES, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, 2 μg/ml each of pepstatin, aprotinin, leupeptin, and 6 μg/ml each of TLCK and TPCK for 2–3 h at 4 C. The extracts were quantified for protein content using the BCA method (Pierce Chemical Co.) and frozen in small aliquots at −70 C.

Electrophoretic mobility shift assay (EMSA)

EMSA analysis was performed as previously described using oligonucleotides carrying the MEF2 element (22). Control EMSA using a PPAR specific oligonucleotide was performed using a Gelshift kit from Geneka Biotechnology (Montréal, Canada).

Results

Previously, we determined that the MEF2A-MEF2D heterodimer is the primary complex that binds to the GLUT4 MEF2 cis-DNA element CTAAAAATAG (22). In these studies, insulin-deficient diabetes was found to specifically result in a loss of MEF2A protein in striated muscle. To compare the expression pattern of MEF2A and MEF2D in adipose tissue, Western blots were performed on nuclear protein extracts isolated from cardiac muscle and adipose tissue (Fig. 1). As previously reported, STZ-induced diabetes resulted in a decrease in cardiac GLUT4 protein levels, which recovered following insulin therapy. In parallel with the loss of GLUT4 protein, the MEF2A isoform also declined in STZ-diabetes and recovered following insulin treatment. However, MEF2D protein expression levels were unaffected. The changes in MEF2A in the heart were in marked contrast to that in adipose tissue. Even though STZ-diabetes resulted in a marked decrease in GLUT4 protein, there was essentially no change in the expression levels of MEF2A. Instead, STZ-diabetes resulted in a dramatic decline in the protein levels of MEF2D, which fully recovered following insulin treatment. It is important to note that the relative levels of MEF2A and MEF2D between tissues cannot be inferred from these immunoblots as longer exposure times were required to visualize these bands in the adipose tissue extracts.

To determine whether the loss of MEF2D expression had any effect on the binding to the MEF2 site, we next performed EMSA on heart and adipose tissue nuclear extracts (Fig. 2A). As expected, a specific MEF2 binding complex was detected

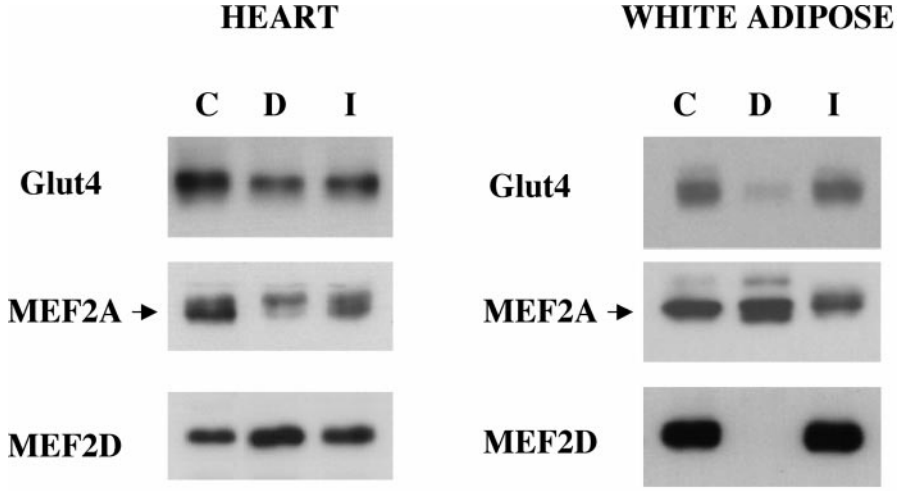

FIG. 1. MEF2A and MEF2D expression are differentially regulated in cardiac muscle and white adipose tissue. Nuclear extracts were obtained from either the heart or epididymal white adipose tissue of control (C), STZ-induced diabetic (D), or insulin-treated diabetic (I) rats as described in *Materials and Methods*. The extracts (30–50 μg) were then subjected to Western blotting using MEF2A or MEF2D specific antibodies. Total cell membrane preparations were immunoblotted for GLUT4. These are representative blots from two independent experiments. The exposure times for the adipose tissue extracts were approximately four times longer than that for the heart extracts.

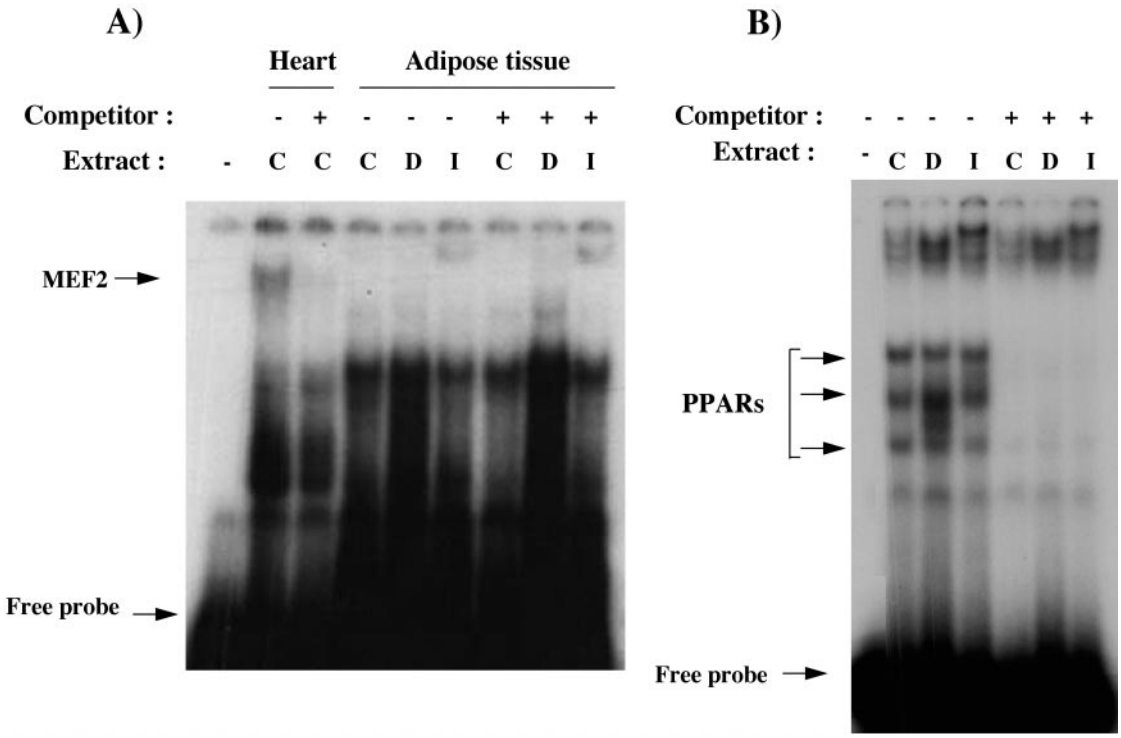

FIG. 2. White adipose tissue nuclear extracts have very low MEF2 binding activity. A, Nuclear extracts (10 μg) obtained from white adipose tissue isolated from control (C), diabetic (D), or insulin-treated diabetic animals (I) were incubated with ^{32}P-labeled MEF2 consensus oligonucleotide and subjected to gel electrophoresis as described in *Materials and Methods*. Ten micrograms of nuclear extracts isolated from heart were used as a positive control. For competition studies the extracts were incubated with 40-fold of the unlabeled oligonucleotide. B, Nuclear extracts (5 μg) obtained from white adipose tissue isolated from control (C), diabetic (D) or insulin-treated diabetic animals (I) were incubated with ^{32}P-labeled PPAR consensus oligonucleotide and subjected to gel electrophoresis as described in *Materials and Methods*. For competition studies the extracts were incubated with 100-fold of the unlabeled oligonucleotide.

in heart nuclear extracts and was specifically competed by excess of unlabeled MEF2 oligonucleotide. Surprisingly however, we were unable to detect any specific MEF2 binding complex in control, diabetic or insulin-treated diabetic nuclear extracts from adipose tissue. This was not due to the preparation of nonfunctional adipose tissue nuclear extracts as they were fully capable of displaying specific binding to a control PPAR cis-DNA element with the three bands detected corresponding to the three different PPAR (α,β,γ) isoforms (Fig. 2B). These data suggest that the level of the MEF2A-MEF2D heterodimeric protein expression in adipose tissue nuclear extracts is either too low to be detected in this assay or alternatively, the endogenous MEF2 transcription factors are repressed in the adipose tissue extracts, thereby preventing their binding to this element.

If the relative functional levels of MEF2 were too low to be detected by these assay, then the addition of *in vitro* translated MEF2A should restore binding to the MEF2 element. As can be seen in Fig. 3A, a specific MEF2 binding complex was detected in heart nuclear extracts but was not present in the adipose tissue nuclear extracts. Addition of *in vitro* translated MEF2A to the adipose tissue nuclear extracts resulted in the appearance of specific MEF2 binding complexes. One of these bands matched with the mobility of the MEF2A-MEF2D heterodimer band observed in heart (Fig. 3A) and was competed upon the addition of cold oligonucleotide (Fig. 4). In addition, reduced binding activity was observed in the adipose tissue extracts from diabetic animals compared with the control or following insulin treatment (Fig. 3A). Moreover when *in vitro* synthesized MEF2D protein was added to the control or diabetic adipose nuclear extracts, we could not detect the band matching the mobility of the MEF2A-MEF2D heterodimer (Fig. 3B). Instead, a band of higher mobility was observed that was competed with the addition of cold oligonucleotide. Furthermore, the apparent mobility of the *in vitro* synthesized MEF2D homodimer was less than the MEF2A homodimer but greater than that observed upon the addition of MEF2D to the adipocyte extracts (Fig. 4). These data indicate that in the absence of MEF2A, MEF2D can either homodimerize as a nonfunctional complex and/or also interacts with other factors.

In any case, if MEF2A was potentially a limiting factor then overexpression of this isoform might be expected to induce the expression of GLUT4. Thus, to further examine the physiologic role of the MEF2A isoforms in the *in vivo* expression of the GLUT4 gene, we used the AP2 promoter to drive the adipose tissue specific expression of the MEF2A protein (Fig. 5A). The increased expression of MEF2A protein was observed in two independently isolated transgenic lines. In both cases, however, the increased expression of MEF2A had no significant effect on the expression of GLUT4 as assessed by Western blot analysis (Fig. 5A). As typically observed, STZ-induced diabetes reduced GLUT4 protein levels in adipose tissue. The insulin-responsive aminopeptidase (IRAP) is known to colocalize with GLUT4 and undergoes an identical pattern of insulin-stimulated translocation to the plasma

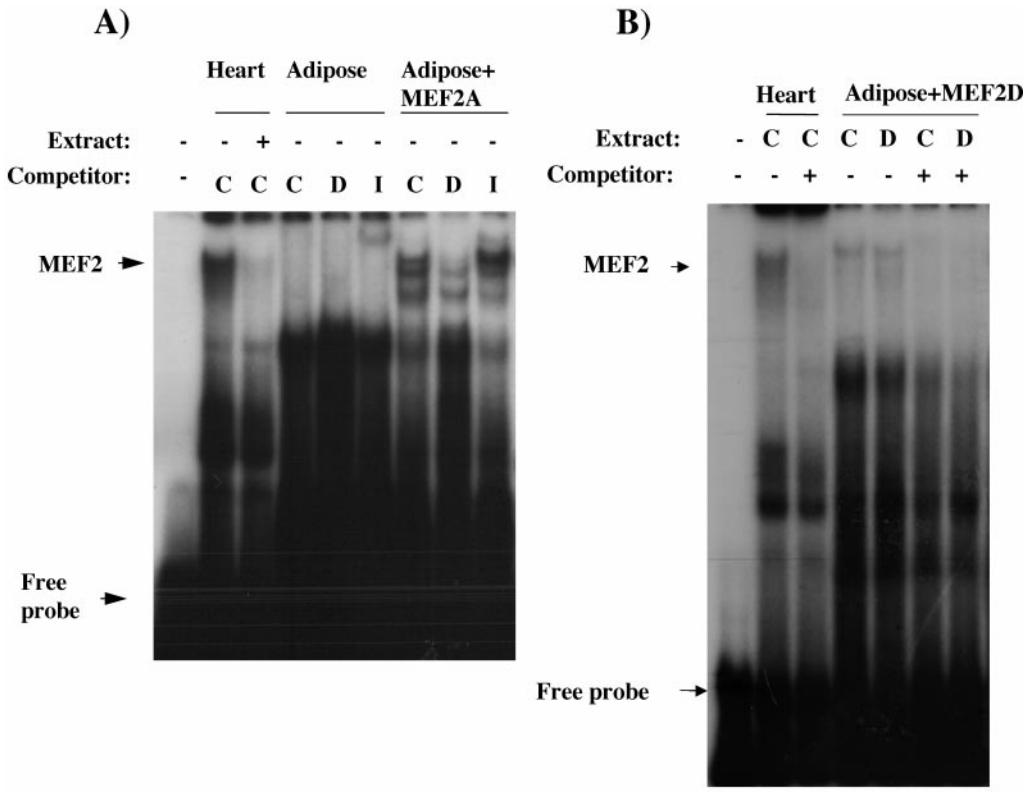

FIG. 3. Addition of MEF2A to white adipose nuclear extracts restores MEF2 binding activity. A, Nuclear extracts (20 μg) obtained from white adipose tissue isolated from control (C), diabetic (D), and insulin-treated diabetic (I) rats were preincubated with (Adipose + MEF2A) or without (Adipose) *in vitro* synthesised MEF2A (0.2 μl of reaction) and subsequently with ^{32}P-labeled MEF2 consensus oligonucleotide and subjected to gel electrophoresis as described in *Materials and Methods*. Ten micrograms of nuclear extracts isolated from heart were used as a positive control. B, Nuclear extracts from adipose tissue isolated from control (C) and diabetic (D) mice were preincubated with 0.2 μl of *in vitro* synthetised MEF2D (Adipose + MEF2D). Extracts were subsequently incubated with ^{32}P-labeled MEF2 consensus oligonucleotide and subjected to gel electrophoresis as described in *Materials and Methods*. Ten micgrams of nuclear extracts isolated from heart were used as a positive control. For competition studies the extracts were incubated with 40-fold of the unlabeled oligonucleotide.

membrane (23–26). Interestingly, in contrast to the loss of GLUT4 protein expression in STZ-diabetes, there was no significant change in IRAP protein levels. Importantly, the increase in MEF2A expression did not prevent the decline in GLUT4 protein levels. Furthermore, increased expression of MEF2A did not affect the expression of the MEF2D protein or its decline in STZ-induced diabetes (Fig. 5B). Similarly, insulin deficiency induced by fasting also resulted in a marked decrease in adipose tissue GLUT4 expression with little effect on IRAP expression levels (Fig. 6). Although fasting had no effect on MEF2A protein levels, there was a significant reduction in the expression of MEF2D. Similar to STZ-induced diabetes, increased MEF2A expression did not prevent the fasting-dependent decline in either GLUT4 or MEF2D protein levels.

Discussion

It is well established that one of the major acute actions of insulin is the recruitment of the GLUT4 glucose transporter protein from intracellular storage sites to the plasma membrane in striated muscle and adipose tissue (1, 2, 8, 27, 28). However, in addition to this acute action of insulin, the expression levels of GLUT4 messenger RNA and protein are also regulated by a variety of more chronic hormonal/nutritional/metabolic states. For example, GLUT4 expression is markedly up-regulated during muscle and adipose differentiation and following muscle exercise/contraction (29–31). In contrast, states of insulin deficiency such as Type I diabetes or fasting result in decreased GLUT4 expression due to transcriptional repression (12, 15–17, 32).

Recent promoter analyses have identified two potential cis-DNA sequence elements in the GLUT4 promoter that are necessary for both tissue-specific and hormonal/metabolic regulation (18–20). The specific binding of the MEF2 transcription factor to the GLUT4 consensus MEF2 binding site (−473/−464) is down-regulated in STZ-induced insulin deficiency but is fully restored following insulin treatment (21, 22). Although there are four MEF2 isoforms, this site appears to interact with a heterodimeric complex composed of the MEF2A-MEF2D isoforms (22). In addition, insulin deficiency appears to result in a selective decrease in MEF2A expression in both cardiac and skeletal muscle (22). In the present study, we have recapitulated this finding that insulin deficiency results in a selective loss of the MEF2A isoform in striated muscle. However, white adipose tissue displays a markedly different pattern of MEF2 isoform regulation. In this tissue, STZ-induced diabetes results in a selective decrease in MEF2D expression without any significant alteration in MEF2A expression.

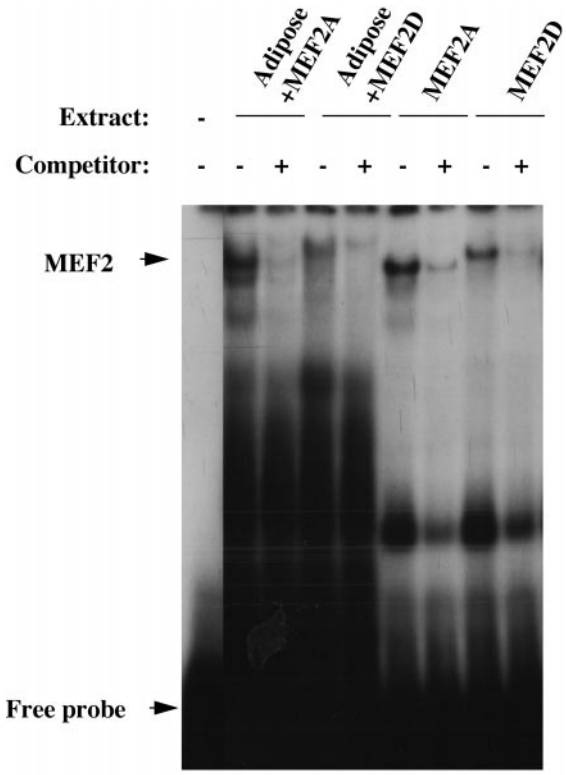

FIG. 4. MEF2A but not MEF2D is rate limiting in adipose tissue from control animals to form a MEF2A-MEF2D complex. Nuclear extracts from adipose tissue isolated from control animals were preincubated with either 0.2 μl of *in vitro* synthesized MEF2A (Adipose + MEF2A) or MEF2D (Adipose + MEF2D) and compared with the mobility of *in vitro* synthesized MEF2A alone (MEF2A) or MEF2D alone (MEF2D). Extracts were subsequently incubated with ^{32}P-labeled MEF2 consensus oligonucleotide and subjected to gel electrophoresis as described in *Materials and Methods*. For competition studies the extracts were incubated with 40-fold of the unlabeled oligonucleotide.

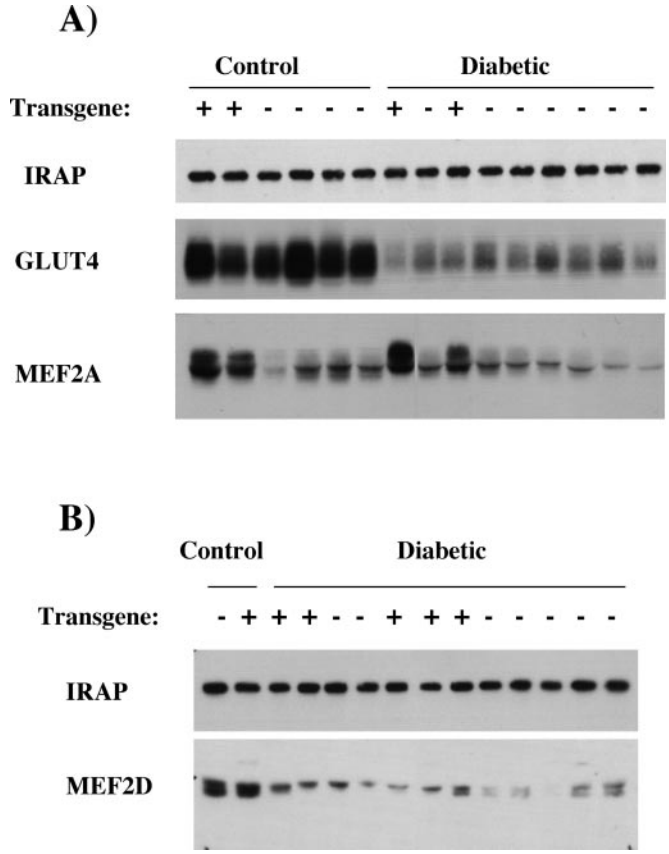

FIG. 5. MEF2A overexpression does not prevent GLUT4 down-regulation in STZ-induced diabetes. Transgenic mice with adipose tissue-specific MEF2A expression were prepared as described in *Materials and Methods*. Wild-type and transgenic mice were either left untreated (Control) or made diabetic by STZ treatment (Diabetic). Whole tissue extracts were then prepared from white adipose tissue and 10–50 μg of protein subjected to Western blotting with specific antibodies directed against GLUT4, IRAP, MEF2A, and MEF2D.

In either case, the net outcome in both diabetic muscle and adipose tissue would be the same, rendering a low content of the MEF2A-MEF2D heterodimer. Surprisingly however, adipose tissue nuclear extracts exhibited very poor binding to the MEF2 element, suggesting that either the levels of the MEF2A-MEF2D heterodimer are extremely low and/or the DNA binding activity was repressed. Nevertheless, addition of MEF2A to these extracts restored DNA binding and demonstrated a decreased binding activity in adipose tissue extracts from diabetic animals. In contrast, addition of *in vitro* synthesized MEF2D to either control or diabetic nuclear extracts did not result in the formation of the MEF2A-MEF2D heterodimer but instead generated a complex with a slower mobility. This band more likely corresponded to an heterodimer composed of MEF2D with an unidentified protein, as the mobility seen in several gels appears to be different from the MEF2D homodimer. These data are consistent with a low level of MEF2A protein in adipose tissue compared with striated muscle and is probably rate limiting for the formation of the MEF2A-MEF2D complex in this tissue. In this context, down-regulation of MEF2D content by diabetes/fasting would further compromise the formation of the MEF2A-MEF2D complex. At present, the molecular mechanism(s) responsible for this apparent tissue-specific isoform regulation remains unknown as the elements controlling MEF2 gene expression have not been determined. Several studies have reported that phosphorylation by p38 mitogen-activated protein kinase, the Big mitogen-activated protein (MAP) kinase (BMK1), calmodulin-dependent protein kinase (CaMK) and PI3-kinase can transcriptionally activate both MEF2A and MEF2C isoforms (33–37). In addition, MEF2 can interact with histone deacetylases 4 and 5, resulting in the repression of the transcriptional activity of MEF2. CaMK kinase appears to induce the dissociation of MEF2 from these histone deacetylases, thereby relieving the transcriptional inhibition (38, 39). Further studies will be necessary to determine whether any of these mechanisms are responsible for the regulation of binding and transcriptional activation of the MEF2A-MEF2D heterodimer.

In summary, our data demonstrate that the MEF2A and MEF2D isoforms undergo differential regulation in white adipose tissue and striated muscle *in vivo*. The loss of MEF2D expression in adipose tissue cannot be compensated for by increased expression of MEF2A. Future studies will be necessary to determine whether forced MEF2D expression can directly compensate and maintain GLUT4 gene expression in adipose tissue of insulin-deficient diabetic mice.

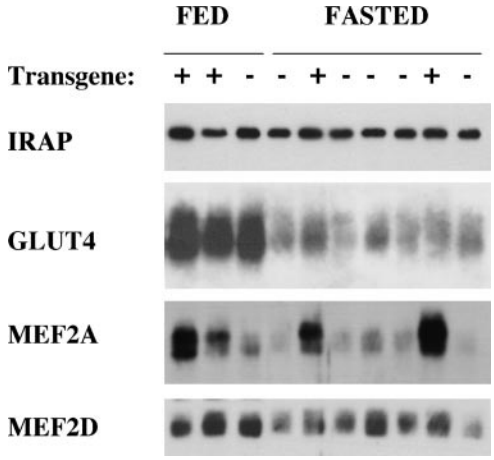

FIG. 6. MEF2A overexpression does not prevent GLUT4 downregulation during fasting. Transgenic mice with adipose tissue-specific MEF2A expression were prepared as described in *Materials and Methods*. Wild-type and transgenic mice were either left untreated (FED) or fasted for 48 h (FASTED). Whole tissue extracts were then prepared from white adipose tissue and 10–50 μg of protein was subjected to Western blotting with specific antibodies directed against GLUT4, IRAP, MEF2A, and MEF2D.

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