THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 21, Issue of May 26, pp. 16323–16328, 2000

Printed in U.S.A.

The MEF2A Isoform Is Required for Striated Muscle-specific Expression of the Insulin-responsive GLUT4 Glucose Transporter*

Received for publication, December 20, 1999, and in revised form, March 10, 2000
Published, JBC Papers in Press, March 15, 2000, DOI 10.1074/jbc. M910259199

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Previously, we have demonstrated that an MEF2 consensus sequence located between −473/−464 in the human GLUT4 gene was essential for both tissue-specific and hormonal/metabolic regulation of GLUT4 expression (Thai, M. V., Guruswamy, S., Cao, K. T., Pessin, J. E., and Olson, A. L. (1998) *J. Biol. Chem.* 273, 14285–14292). To identify the specific MEF2 isoform(s) responsible for GLUT4 expression, we studied the pattern of expression of the MEF2 isoforms in insulin-sensitive tissues. Both heart and skeletal muscle were found to express the MEF2A, MEF2C, and MEF2D isoforms but not MEF2B. However, only the MEF2A protein was selectively downregulated in insulin-deficient diabetes. Co-immunoprecipitation with isoform-specific antibodies revealed that, in the basal state, essentially all of the MEF2A protein was presented as a MEF2A-MEF2D heterodimer without any detectable MEF2A-MEF2A homodimers or MEF2A-MEF2C and MEF2C-MEF2D heterodimers. Electrophoretic mobility shift assays revealed that nuclear extracts from diabetic animals had reduced binding to the MEF2 binding site compared with extracts from control or insulin-treated animals. Furthermore, immunodepletion of the MEF2A-MEF2D complex from control extracts abolished binding to the MEF2 element. However, addition of MEF2A to diabetic nuclear extracts fully restored binding activity to the MEF2 element. These data strongly suggest that the MEF2A-MEF2D heterodimer is selectively decreased in insulin-deficient diabetes and is responsible for hormonally regulated expression of the GLUT4 gene.

The major insulin-responsive facilitative glucose transporter GLUT4[1] is predominantly expressed in striated muscle and adipocytes, tissues that display insulin-stimulated glucose uptake (1–3). In the basal state, this transporter slowly recycles between a poorly described intracellular storage compartment(s) and the plasma membrane such that the steady-state distribution favors intracellular localization (3, 4). However, following insulin stimulation, there is a dramatic increase in the rate of GLUT4 exocytosis with a smaller decrease in the rate of plasma membrane endocytosis (4). This redistribution of pre-existing GLUT4 protein provides the major mechanism accounting for the acute insulin-stimulated glucose uptake that occurs in the post-prandial state (1, 3, 5, 6). In addition to this acute regulation of GLUT4-containing vesicle trafficking, the expression of GLUT4 is transcriptionally regulated in a variety of persistent altered metabolic states. For example, states of insulin deficiency induced by either streptozotocin (STZ) treatment or nutritional restriction results in decreased GLUT4 mRNA and protein in adipose tissue and cardiac and skeletal muscle (7–9). The decrease in GLUT4 protein levels directly correlates with a decrease in GLUT4 mRNA and the rate of GLUT4 gene transcription (10). Promoter analysis in tissue cultured 3T3L1 adipocytes has recently identified an insulin-responsive element (−706/−676) located in the 5′-flanking region of the murine GLUT4 gene (11, 12). In addition, analysis of tissue-specific GLUT4 gene expression in the cultured C2C12 muscle cell line demonstrated the necessary requirement for a myocyte enhancer factor 2 (MEF2) cis-DNA regulatory element (13). The importance of the MEF2 site was further supported by expression analysis in transgenic mice that demonstrated the essential function of the human GLUT4 MEF2 site (−473/−464) in both tissue-specific and hormonal/metabolic regulation (14). Importantly, *in vitro* MEF2 sequence-specific binding activity was found to decrease following STZ-induced diabetes (15).

Currently, there are four known members of the mammalian MEF2 family termed MEF2A, MEF2B, MEF2C, and MEF2D (16, 17). These DNA-binding proteins are important transcription factors in both the maintenance and induction of the muscle differentiated phenotype (18–21). These factors bind DNA as homo- and heterodimers but can also form protein-protein interactions with the thyroid hormone receptor (22), and members of the basic helix-loop helix family of transcription factors, such as MyoD or myogenin (18, 20, 22–25).

Although the MEF2 element appears to be essential for GLUT4 expression, the specific MEF2 isoform(s) regulating the tissue-specific and hormonal/metabolic regulation of the GLUT4 gene has not been determined. In this report, we demonstrate that cardiac and skeletal muscle expression of GLUT4 is dependent upon a MEF2A-MEF2D heterodimer. STZ-induced insulin deficiency results in a specific decrease in expression of the MEF2A mRNA and protein. Furthermore, the addition of *in vitro* synthesized MEF2A protein to diabetic nuclear extracts restored the binding activity to a comparable level found in control or insulin-treated animals.

EXPERIMENTAL PROCEDURES

Rats—Male Sprague-Dawley rats (180–200 g) were obtained from Harlan. These animals were either left untreated or made diabetic by an intraperitoneal injection of STZ (90–100 mg/kg body weight) following an overnight fast. Three days following the injection, blood tail samples were checked for glucose concentration using a One-Touch®

* This work was supported in part by Research Grants DK33823 and DK25295 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a postdoctoral fellowship (Formacion de Personal Investigador) from the Ministerio de Educacion y Cultura, Spain.

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[1] The abbreviations used are: GLUT4, muscle/adipose-specific glucose transporter; MEF, myocyte enhancer factor; STZ, streptozotocin; PMSF, phenylmethylsulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone.

glucometer (Lifescan, Milpitas, CA). Animals with a glycemia ≥300 mg/dl were considered diabetic and sacrificed 3–5 days following the STZ injection by CO_2 asphyxiation for removal of tissues. A set of diabetic animals were also treated with human insulin for 7 days with a daily dose of 3 units of regular insulin (Humulin-R) and 2 units of long-acting insulin (Humulin-N) as described previously (26, 27). All tissues were snap-frozen in liquid nitrogen and kept at −80 °C until used. All procedures were reviewed and approved by the University of Iowa Committee for the Care and Use of Animals.

RNA Isolation and Northern Blot Analysis—RNA was extracted with guanidinium thiocyanate method using the RNAzol reagent from TelTest (Friendswood, TX) and following the manufacturer's instructions. For Northern blots, 30 μg of total RNA was fractionated in a 1% formaldehyde agarose gel. The samples were then transferred to a Hybond-N nylon membrane (Amersham Pharmacia Biotech) overnight and prehybridized at 65 °C for 3–4 h in a solution containing 20% formamide, 4× SSPE, 5× Denhardt's, 5% SDS, 10% dextran sulfate, 40 mM HCl, 0.4 mg/ml salmon sperm DNA, and 0.2 mg/ml yeast tRNA. The same solution was used for overnight hybridization at 65 °C with 10^6 cpm/ml ^{32}P-random primed labeled probe corresponding to full-length human α-actin, rat GLUT4, human MEF2A, and mouse MEF2B, MEF2C, and MEF2D cDNAs. The cDNA probes were labeled using [α-^{32}P]dCTP and the Rediprime labeling kit (Amersham Pharmacia Biotech) following manufacturer's directions. After hybridization membranes were washed thoroughly and exposed to film overnight at −70 °C.

Preparation of Nuclear Extracts and Total Membranes—Nuclear extracts from heart and skeletal muscle were obtained as described by Thai *et al*. (15) with minor modification. Briefly, frozen tissues were pulverized in liquid nitrogen and then resuspended in homogenization buffer A (250 mM sucrose; 10 mM Hepes, pH 7.6; 25 mM KCl; 1 mM EDTA; 10% glycerol; 0.15 mM spermine; 0.5 mM spermidine; 0.1 mM PMSF; 2 μg/ml each aprotinin, leupeptin, and pepstatin A; and 6 μg/ml each TLCK and TPCK. The tissues were homogenized with 10 strokes of a Teflon pestle and filtrated through gauze and centrifuged at 3,900 × *g* for 10 min at 4 °C. The supernatant was then centrifuged at 200,000 × *g* for 1 h at 4 °C to obtain a pellet of crude total membranes. The pellet from the low speed centrifugation was resuspended in 10 ml of homogenization buffer A and homogenized in a loose-type Dounce homogenizer. The homogenate was then layered over 0.5 volume of buffer B (1 M sucrose; 10 mM Hepes, pH 7.6; 25 mM KCl; 1 mM EDTA; 10% glycerol; 0.15 mM spermine; 0.5 mM spermidine; 0.1 mM PMSF; 2 μg/ml each aprotinin, leupeptin, and pepstatin A; and 6 μg/ml each TLCK and TPCK, followed by centrifugation at 3,900 × *g* for 10 min at 4 °C. The pellet was then resuspended in buffer A/glycerol (9:1, w/w) and layered over one-third buffer B:glycerol (9:1). The gradient was centrifuged at 48,000 × *g* for 30 min at 4 °C. The semi-purified nuclear pellet was resuspended in 1 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). Nuclear proteins were extracted at 4 °C for 30 min and insoluble nuclei precipitated by centrifugation at 13,000 rpm in a microcentrifuge for 15 min. Supernatant was dialyzed against a buffer containing 25 mM Hepes, pH 7.5; 100 mM KCl; 0.1 mM EDTA; 10% glycerol; 1 mM dithiothreitol; 0.1 mM PMSF; 2 μg/ml pepstatin, aprotinin, and 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 BCA method (Pierce) and frozen in small aliquots at −70 °C.

Western Blot Analysis—Nuclear extracts (30–50 μg) were resolved in a 10% SDS-polyacrylamide gel electrophoresis. The fractionated proteins were transferred to nitrocellulose membranes in a buffer containing 25 mM Tris, 190 mM glycine, pH 8.5, at 0.6 A for 6 h at 4 °C. Filters were then blotted for 60 min with 5% nonfat dry milk in Tris-buffered saline/Tween buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.2% Tween 20). The nitrocellulose membranes were then probed with a 1:2000 dilution of MEF2A/C or a MEF2D polyclonal antibodies (kindly provided by Dr. Ron Prywes, Columbia University, New York, NY) or a polyclonal GLUT4-specific antibody generated in our laboratory. In addition, we have prepared a polyclonal MEF2A-specific antibody (IA-17) generated against amino acid residues 88–131 of MEF2A and a polyclonal MEF2C-specific antibody (IA-14) generated against amino acid residues 311–351 of MEF2C. The filters were then washed three times with Tris-buffered saline/Tween buffer at room temperature for 10 min and probed for 1 h at room temperature with a goat anti-rabbit horseradish peroxidase-conjugated antibody (Pierce). Membranes were then washed as before and proteins visualized by enhanced chemiluminescence (Pierce).

In Vitro Translation of MEF2—In vitro transcription and translation was performed using a TNT reticulocyte lysate system from Promega (Madison, WI) following manufacturer's instructions. For each reaction 1 μg of template DNA corresponding to full-length cDNA of MEF2A, MEF2B, MEF2C, or MEF2D was used.

Immunoprecipitation—Nuclear extracts (50–100 μg) were incubated with 5 μg of MEF2D monoclonal antibody (Transduction Laboratories, San Diego, CA) coupled to goat anti-mouse IgG-agarose beads (Sigma) in 25 mM Hepes, pH 7.6; 100 mM KCl; 0.1 mM EDTA; 10% glycerol; 1% Nonidet P-40; 1 mM sodium vanadate; 1 mM PMSF; 2 μg/ml of pepstatin, aprotinin, and leupeptin; and 6 μg/ml each of TLCK and TPCK. Samples were microcentrifuged for 1 min and the pellets washed five times in the above buffer. The samples were then resuspended in Laemli sample buffer, heated for 5 min at 100 °C, and subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis. For immunoprecipitation with the MEF2A antibody, 2 ml of the polyclonal IA-17 antibody were cross-linked to protein A beads with dimethyl pimelimidate reagent using the Immunopure protein A IgG kit (Pierce). Immunoprecipitation was then performed at 4 °C as described for the MEF2D antibody above.

Electrophoretic Mobility Shift Assays—For electrophoretic mobility shift assays, we used a Gelshift kit obtained from Geneka Biotechnology (Montreal, Canada). For each reaction, 4–10 μg of nuclear extracts were incubated in DNA binding buffer at 4 °C for 20 min and subsequently with the labeled oligonucleotide probe corresponding to the MEF2 consensus binding site (Santa Cruz Biotechnology, Santa Cruz, CA) for an additional 20 min at 4 °C. The samples were then run in a 5% acrylamide:bisacrylamide gel (38:2) in TGE buffer (250 mM Tris, 1.9 M glycine, and 10.5 mM EDTA). Labeling of the oligonucleotide probes was performed as described by Ausubel *et al*. (28). For immunodepletion experiments, 50–80 μg of nuclear extract were immunoprecipitated as described above using the immunoprecipitation buffer without detergent.

RESULTS

Insulin-deficient Diabetes Results in a Selective Decrease in MEF2A Protein Expression in Cardiac and Skeletal Muscle—Previously, we have demonstrated that the MEF2 consensus cis-DNA element is essential for both tissue-specific and hormonal/metabolic regulation of the GLUT4 gene (15). In addition, nuclear extracts from STZ-induced diabetes bind poorly to this element compared with control tissue extracts. In order to identify the specific MEF2 isoform(s) responsible for the loss of MEF2 binding and GLUT4 transcriptional activity, we initially performed Northern blot analysis of total RNA isolated from control, STZ-induced diabetic, and STZ-diabetic animals treated with insulin for 1 week (Fig. 1). As typically observed, STZ-induced diabetes resulted in decreased GLUT4 mRNA levels in both the heart and hindlimb skeletal muscle. The reduction in GLUT4 mRNA was fully reversible following insulin treatment and was specific as there was no significant change in α-actin expression. Similarly, the two MEF2D transcripts were also unaffected in STZ-induced diabetes, whether or not the animals were treated with insulin. In contrast, insulin deficiency resulted in decreased levels of the MEF2A and MEF2C mRNA that were also recovered following insulin therapy. As expected, we were unable to detect the presence of any MEF2B transcripts in adult striated muscles consistent with this isoform only expressed at significant amounts during embryonic development (29, 30) (data not shown).

Since previous studies have observed that the relative levels of the MEF2 transcripts do not always correlate with protein expression (31, 32), it was necessary to perform specific Western blots of nuclear extracts. However, due to the high degree of sequence identity between the MEF2 proteins, antibodies selective for the MEF2A and MEF2C isoforms have not been available. Therefore, to distinguish between the MEF2A and MEF2C proteins, we generated specific polyclonal antibodies as described under "Experimental Procedures." To demonstrate the specificity of several MEF2 antibodies, we *in vitro* translated the three MEF2 isoforms (MEF2A, MEF2C, and MEF2D), followed by immunoblotting (Fig. 2). In the absence of any template, none of the antibodies detected any immunore-

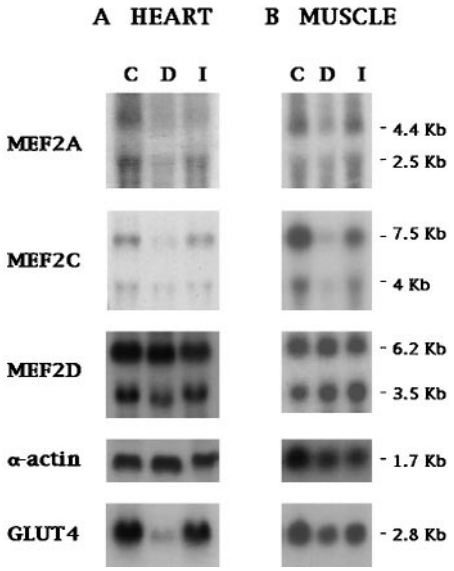

FIG. 1. **MEF2A, MEF2C, and GLUT4 mRNA levels are decreased in striated muscle of insulin-deficient diabetic rats.** Total heart (*A*) and hindlimb skeletal muscle (*B*) RNA (30 μg) was isolated from control (*C*), STZ-diabetic (*D*), and insulin-treated STZ-diabetic rats (*I*). The RNA was resolved in an agarose denaturing gel, transferred to a nylon membrane, and probed with full-length ^{32}P-labeled cDNAs corresponding to MEF2A, MEF2C, MEF2D, GLUT4, or α-actin genes as described under "Experimental Procedures." This is a representative experiment independently performed two times.

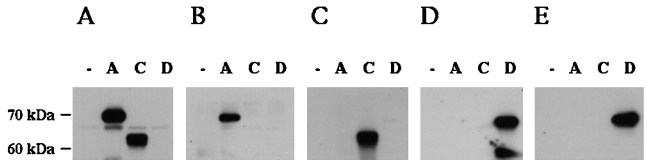

FIG. 2. **Western blot analysis of *in vitro* transcribed and translated MEF2 isoforms.** The empty vector (−), MEF2A (*A*), MEF2C (*C*), and MEF2D (*D*) cDNAs were *in vitro* translated and resolved by 10% SDS-polyacrylamide gel electrophoresis. The samples were then transferred to a nitrocellulose membrane and immunoblotted with the MEF2A polyclonal antibody provided by Dr. Ron Prywes (*A*), the MEF2A IA-17 polyclonal antibody (*B*), the MEF2C IA-14 polyclonal antibody (*C*), the MEF2D polyclonal antibody obtained from Dr. Ron Prywes (*D*), and the MEF2D monoclonal antibody obtained from Signal Transduction Laboratories (*E*) as described under "Experimental Procedures." This is a representative experiment independently performed two times.

active protein. As reported previously (33), an antibody prepared against MEF2A kindly provided by Dr. Ron Prywes cross-reacted with both the MEF2A and MEF2C isoforms but did not detect MEF2D (Fig. 2*A*). In contrast, the IA-17 antibody was specific for MEF2A, whereas the IA-14 was specific for MEF2C (Fig. 2, *B* and *C*). Furthermore, the two MEF2D antibodies (one polyclonal kindly provided by Dr. Ron Prywes, and one monoclonal from Signal Transduction Laboratories) were both specific for MEF2D and did not cross-react with either MEF2A or MEF2C (Fig. 2, *D* and *E*). In addition, although the IA-17 MEF2A antibody was capable of immunoprecipitating the native protein, the IA-14 MEF2C was only able to react in Western blots and was incapable of immunoprecipitating the native MEF2C protein (see Fig. 5 and data not shown).

In any case, having established the specificity of these antibodies, we next assessed the expression of the MEF2 isoforms in cardiac and skeletal muscle nuclear extracts from control, diabetic, and insulin-treated diabetic animals (Fig. 3). Immunoblotting with the MEF2A/C antibody demonstrated that one and/or both of these isoforms was decreased in streptozotocin-induced diabetes. The decrease in MEF2A/C immunoreactivity

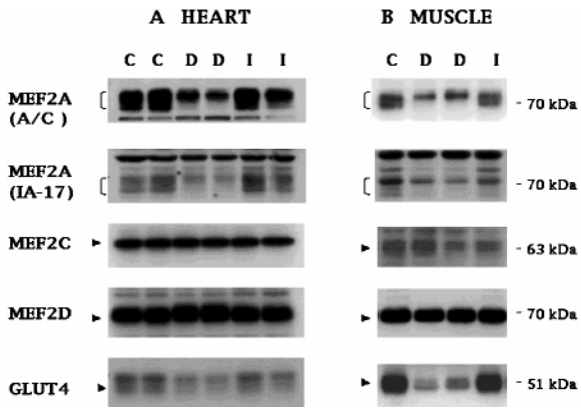

FIG. 3. **MEF2A protein is specifically decreased in striated muscle of insulin-deficient diabetic rats.** Nuclear extracts were isolated from the heart (*A*) and hindlimb skeletal muscles (*B*) of control (*C*), STZ-diabetic (*D*), and insulin-treated STZ-diabetic (*I*) rats. The protein samples (30 μg) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were then immunoblotted with the MEF2A polyclonal antibody provided by Dr. Ron Prywes, the MEF2A IA-17 polyclonal antibody, the MEF2C IA-14 polyclonal antibody, and the MEF2D monoclonal antibody obtained from Signal Transduction Laboratories as described under "Experimental Procedures." This is a representative experiment independently performed three times.

was specifically due to a loss of MEF2A protein without any effect on the MEF2C protein as detected by the IA-17 and IA-14 antibodies. Furthermore, the diabetic state had no effect on MEF2D protein levels. The changes in MEF2A protein levels directly correlated with the changes in GLUT4 protein. Together, these data suggest that the expression of the MEF2A nuclear protein isoform is selectively down-regulated in insulin-deficient diabetes.

MEF2A Is Specifically Complexed with MEF2D—The MEF2 family of transcription factors bind to DNA as either homodimeric or heterodimeric complexes (16). To examine the association state of MEF2A, we initially immunoprecipated nuclear extracts with the specific MEF2D antibody and determined the amount of co-immunoprecipitated MEF2 isoforms (Fig. 4). In the absence of the MEF2D antibody, all the MEF2A, MEF2C, and MEF2D proteins remained in the supernatant without any protein detected in the immunoprecipitated pellets. In contrast, incubation with the MEF2D antibody resulted in the quantitative depletion of the MEF2D protein from the supernatant and appearance in the immunoprecipitated pellet. Western blots of the MEF2D immunoprecipitates demonstrated that all the MEF2A isoform was co-immunoprecipitated with MEF2D, whereas there was essentially no co-immunoprecipitated MEF2C protein, which was completely retained in the supernatant.

To further confirm the specific interaction of MEF2A with MEF2D, nuclear extracts were immunoprecipitated with the MEF2A-specific antibody (Fig. 5). Under these conditions, the MEF2A protein was completely immunodepleted from the cell extracts. This resulted in the co-immunoprecipitation of approximately 33% of the MEF2D protein, as observed both by its depletion from the supernatant and appearance in the immunoprecipitated pellet. Furthermore, essentially none of the MEF2C protein was immunodepleted from the supernatant following MEF2A immunoprecipitation. Although we were unable to immunoblot the MEF2A immunoprecipitates with the MEF2C antibody due to heavy chain cross-reactivity, these data are consistent with the absence of a MEF2A-MEF2C complex. It should also be noted that, since the MEF2C antibody (IA-14) was unable to immunoprecipitate the native MEF2C protein, we could not examine the co-immunoprecipi-

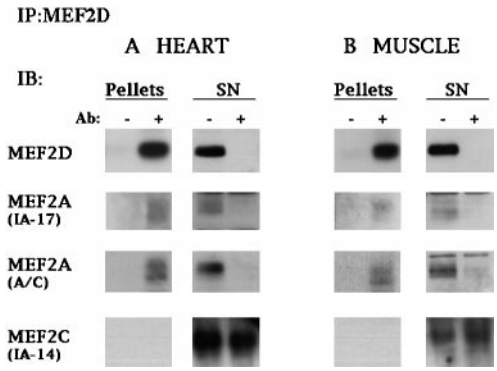

FIG. 4. **Immunoprecipitation of MEF2D results in the co-precipitation of MEF2A in striated muscle nuclear extracts.** Heart (*A*) and hindlimb skeletal muscle (*B*) were obtained from control rats. The nuclear extracts (50 μg) were immunoprecipitated with the MEF2D monoclonal antibody, and the resultant pellets and supernatants (*SN*) were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were then immunoblotted with the MEF2D polyclonal antibody the MEF2A IA-17 polyclonal antibody, the MEF2A polyclonal antibody (MEF2A/C) and the MEF2C IA-14 polyclonal antibody as described under "Experimental Procedures." This is a representative experiment independently performed three times for each tissue.

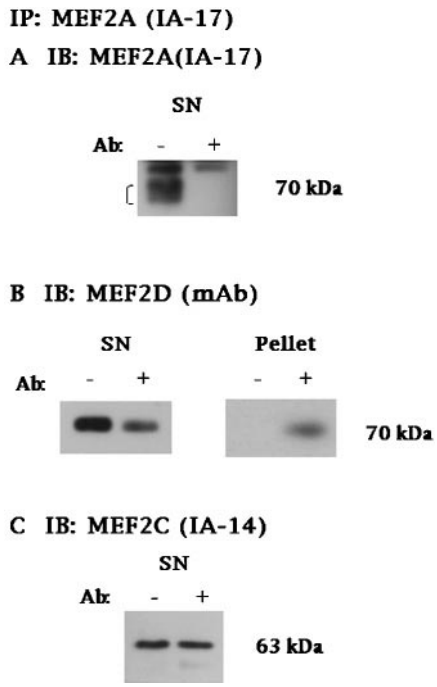

FIG. 5. **Immunoprecipitation of MEF2A results in the partial co-precipitation of MEF2D in heart nuclear extracts.** Heart nuclear extracts (50 μg) were immunoprecipitated with the MEF2A IA-17 polyclonal antibody linked to protein A beads as described under "Experimental Procedures." Equal volumes of samples were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membranes. *A*, the resulting supernatant (*SN*) was immunoblotted with the MEF2A IA-17 antibody. *B*, the resulting supernatant (*SN*) and pellet were immunoblotted with the MEF2D monoclonal antibody. *C*, the resulting supernatant (*SN*) was immunoblotted with the MEF2C IA-14 polyclonal antibody as described under "Experimental Procedures." This is a representative experiment independently performed two times.

tation of MEF2C with MEF2A or MEF2D. Nevertheless, these data indicate that the majority of the MEF2A protein existing as a heterodimeric complex with MEF2D, whereas MEF2D is found in both a heterodimeric complex with MEF2A and as a MEF2D-MEF2D homodimeric complex. Importantly, MEF2C is apparently not complexed with either MEF2A or MEF2D

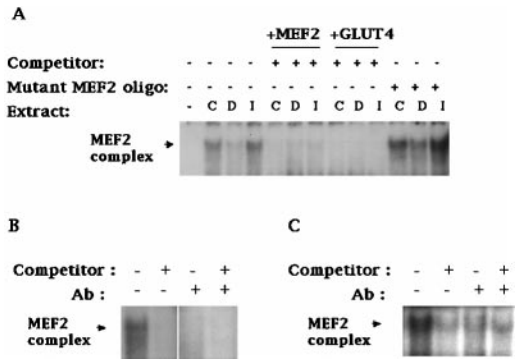

FIG. 6. **Insulin-deficient diabetes results in a decrease DNA binding activity to the MEF2 consensus site.** *A*, heart nuclear extracts (10 μg) from control (*C*), STZ-diabetic (*D*), or insulin-treated STZ-diabetic (*I*) rats were incubated with a ^{32}P-labeled 25-base pair MEF2 consensus double-stranded oligonucleotide as described under "Experimental Procedures." Specific binding was determined by electrophoretic mobility shift assay in the absence or presence of a 100-fold molar excess of unlabeled muscle creatine kinase (+*MEF2*) oligonucleotide, a 18-base pair oligonucleotide corresponding to the MEF2 site in the GLUT4 gene (+*GLUT4*), or a mutated MEF2 oligonucleotide. *B*, the heart nuclear extracts were first immunodepleted with the MEF2D monoclonal antibody and equal volumes of supernatants were subjected to electrophoretic mobility shift assays in the absence and presence of 100-fold excess cold competitor oligonucleotide. *C*, the heart nuclear extracts were first immunodepleted with the MEF2A IA-17 polyclonal antibody and equal volumes of the resultant supernatants were subjected to electrophoretic mobility shift assays in the absence and presence of 100-fold excess cold competitor oligonucleotide. This is a representative experiment independently performed two times for each antibody.

and therefore must necessarily be present as a homodimeric MEF2C-MEF2C complex, or perhaps complexed to another transcription factor.

The MEF2A-MEF2D Heterodimeric Complex Interacts with the GLUT4 MEF2-responsive Element—To assess the functional binding properties of the MEF2 dimeric complexes from heart nuclear extracts, we next examined MEF2 DNA binding by electrophoretic mobility shift assays (Fig. 6). Using a consensus MEF2 double-stranded oligonucleotide, we observed that heart nuclear extracts resulted in a specific decrease in the mobility of the labeled probe. In agreement with previous findings (15), the MEF2-DNA complexed band was substantially reduced in extracts isolated from diabetic animals compared with controls rats but which fully recovered following insulin treatment (Fig. 6*A*). This binding activity was specifically blocked in the presence of either the myosin creatine kinase or GLUT4 unlabeled MEF2 element but not by a mutant MEF2 oligonucleotide with two substitutions (C → G and A → C), in the consensus core sequence (mutant oligonucleotide sequence: 5′-GATCGCTGTAAACATAACCCTGTCG-3′), that impair MEF2 binding (15). Furthermore, the MEF2 sequence-dependent binding activity was completely lost when the nuclear extracts were immunodepleted with either the MEF2D- or MEF2A-specific antibody (Fig. 6, *B* and *C*). These data indicate that the heterodimer MEF2A-MEF2D interacts with the MEF2-responsive element.

Addition of MEF2A to Nuclear Extracts from Diabetic Animals Restores Binding Activity to the MEF2 Site—Since nuclear extracts from diabetic animals have reduced levels of MEF2A but have normal amounts of MEF2D, re-addition of MEF2A protein should restore binding activity to this site. To this end, we prepared different dilutions of *in vitro* translated MEF2A protein and compared these to the endogenous MEF2A protein present in control extracts (Fig. 7*A*). The amount of *in vitro* translated MEF2A protein present in 0.15–0.3 μl of transcription reaction was found to be similar to the endogenous

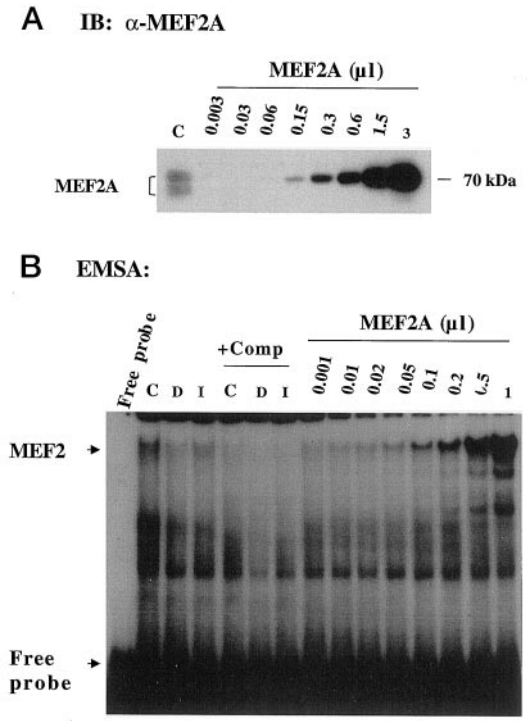

FIG. 7. **Addition of *in vitro* translated MEF2A protein restores DNA binding activity in nuclear extracts from diabetic rats.** *A*, control heart nuclear extracts from (30 μg) and increasing amounts of *in vitro* translated MEF2A protein (0.003, 0.03, 0.06, 0.15, 0.3, 0.6, 1.5, and 3 μl of the reaction) were subjected to Western blotting as described under "Experimental Procedures." *B*, nuclear extracts (10 μg) obtained from control (*C*), STZ-diabetic (*D*), and insulin-treated STZ-diabetic rats (*I*) were subjected to electrophoretic mobility shift assay using the ^{32}P-labeled 25-base pair MEF2 consensus double-stranded oligonucleotide in the absence or presence of 100-fold excess unlabeled competitor oligonucleotide. The diabetic extracts (10 μg) were also pre-mixed with increasing amounts of *in vitro* translated MEF2A (0.001, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1 μl of the reaction), in the same proportion of the samples used for Western blot in *panel A*. This is a representative experiment independently performed two times.

level of MEF2A in 30 μg of control nuclear extracts. The same dilutions were proportionally added to nuclear extracts from diabetic animals and tested for binding activity to the MEF2 site (Fig. 7*B*). The addition of similar amounts of MEF2A to the diabetic nuclear extracts restored binding up to a level comparable to that observed in the control extract. In fact, further addition of excess MEF2A resulted in increased binding, probably due to both MEF2A-MEF2D complex formation and the presence of MEF2A-MEF2A homodimers. Taken together, these data demonstrate that the MEF2A-MEF2D complex is responsible for binding to the GLUT4 MEF2 site and that the down-regulation of MEF2A protein levels in insulin-deficient diabetes accounts for the lack of GLUT4 gene expression in striated muscle.

DISCUSSION

It has been well established that insulin stimulates the recruitment or translocation of pre-formed GLUT4 glucose transporter proteins from intracellular storage sites to the cell surface membrane (1–3, 5).This process is necessary for normal tissue (striated muscle and adipose) insulin sensitivity and the maintenance of whole body glucose homeostasis. However, in addition to the acute stimulation of GLUT4 protein intracellular trafficking, the tissue-specific expression levels of GLUT4 are also regulated by a variety of hormonal/nutritional/metabolic states. For example, GLUT4 expression is markedly up-regulated during muscle and adipose differentiation and following muscle exercise/contraction (34–36). In contrast, states of relative insulin deficiency (type I diabetes and fasting) result in decreased GLUT4 expression (7, 26). Furthermore, although there was no effect on muscle GLUT4 levels in patients with non-insulin-dependent diabetes, there was a selective loss of GLUT4 protein in adipose tissue (37).

Based upon these data, we and others have begun to identify the cis-regulatory elements that are necessary for the tissue-specific and regulated expression of the GLUT4 gene. Recently, we have identified a consensus MEF2 binding sequence located at (−473/−464) in the human GLUT4 gene (13, 15). Deletions or point mutations within this element completely prevented tissue-specific and hormonally/metabolically regulated expression in both differentiating culture muscle C2C12 cells and more importantly in transgenic mice (15). Furthermore, DNA binding activity to this sequence was markedly reduced in nuclear extracts from insulin-deficient diabetic rats but was fully restored following insulin therapy. Thus, these data provide compelling evidence that the MEF2 binding site is, at least, one essential functional element in the expression of the GLUT4 gene.

There are four known members of the mammalian MEF2 family of transcription factor, and since MEF2B expression is restricted to early embryo development (29, 30), the other MEF2 isoforms were likely candidates responsible for the loss of DNA binding activity in nuclear extracts from diabetic animals. To this end, Western blotting using isoform-specific antibodies demonstrated that only the level of MEF2A protein was down-regulated in both skeletal muscle and heart of insulin-deficient diabetic rats. Interestingly, the mRNA of MEF2C was also decreased; however, this did not result in any significant change in protein expression. Previous studies have also observed a discordance in the levels of MEF2 mRNA *versus* protein, suggesting the presence of post-transcriptional control mechanism(s) (31, 32). In any case, the decrease in MEF2A expression correlated with the decrease in GLUT4 gene expression and reduction in nuclear extract binding activity to the MEF2 consensus sequence. These findings were confirmed by the addition of *in vitro* translated MEF2A protein to diabetic nuclear extracts, which at the appropriate endogenous amount restored the DNA binding activity to the same level as found in control nuclear extracts.

At present, the mechanism(s) regulating MEF2 gene expression and protein levels remains largely unknown. All the MEF2 genes contain large 5′-noncoding regions with multiple spliced exons and large introns. In *Drosophila*, two D-MEF enhancers have been described, one that binds the cardiac homeodomain protein Tinman (38) and another controlled by the basic helix-loop helix transcription factor Twist (39). However, no cis-regulatory sequences have yet been described for any vertebrate MEF2 gene. Alternatively, MEF2A and also MEF2C can be transcriptionally activated by phosphorylation through the p38 mitogen-activated protein kinase (40–43) and protein kinase C isoforms (41). In addition, MEF2C has been reported to undergo nuclear export into the cytoplasm following transforming growth factor-β stimulation (44).

Although we cannot exclude these possibilities for the loss of MEF2A expression in muscles of insulin-deficient diabetic rats, an additional mechanism may be envisaged based upon the dimerization of the MEF2 isoforms. In this study, we have also observed that the MEF2A antibody will co-immunoprecipitate approximately one-third of the MEF2D protein, whereas the MEF2D antibody will co-immunoprecipitate essentially all of the MEF2A protein. These data suggest that all the MEF2A protein exists as an MEF2A-MEF2D heterodimer. Since MEF2C is not co-immunoprecipitated with MEF2D, these data

further indicate that the excess MEF2D is present in an MEF2D-MEF2D homodimer. The presence of an MEF2A-MEF2D heterodimer is consistent with studies in the HeLa cell system but is somewhat in disagreement with cultured C2C12 cells (45). In any case, decreased MEF2A expression would therefore increase the relative proportion of MEF2D-MEF2D homodimers. Although it has been suggested that MEF2D homodimers can function as a transcriptional inhibitor (45), we do not feel that this is likely for the GLUT4 promoter. The fact that decreased levels of MEF2A, either in diabetic nuclear extracts or by MEF2A antibody immunodepletion, markedly reduces DNA binding strongly suggests the MEF2D homodimer has a relatively low affinity for the GLUT4 MEF2 element.

In summary, our data demonstrate that MEF2A protein levels are selectively reduced in striated muscle of insulin-deficient diabetic rats. This loss of MEF2A expression accounts for the reduction in DNA binding activity and directly correlates with the decrease in GLUT4 gene expression. Future studies are now needed to determine whether the down-regulation of MEF2A expression occurs at the transcriptional or post-transcriptional level. In addition, further analysis will be necessary to determine the specific functional roles of the MEF2A-MEF2D heterodimer and MEF2D-MEF2D homodimer in the control of GLUT4 transcriptional activity.

Acknowledgment—We thank Dr. Ron Prywes for kindly providing the MEF2A/C and MEF2D antibodies.

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