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 down-regulated 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 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™
glucomer (Lifescan, Milpitas, CA). Animals with a glycerin ≥300 mg/dl were considered diabetic and sacrificed 3–5 days following the STZ injection by CO₂ asphyxiation for removal of tissues. A set of diabetic animals were also treated with human insulin for 7 days with a daily dose of 5 units of regular insulin (Humulin-R) and 2 units of long-acting insulin (Humulin-N) coupled to the previous regimen. The 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 Tel-Test (Friendswood, TX) and further purified by standard procedures. 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) 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; 100 mM KCl; 0.1 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 a TES buffer (10 mM Hepes, pH 7.6; 400 mM KCl; 3 mM MgCl₂; 0.1 mM EDTA; 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 10⁶ cpm/ml 32P-random primed labeled probe corresponding to full-length 5.8S rRNA. Following hybridization, the membranes were washed with 1% SDS, 0.1% Nonidet P-40, and 0.1% 3-(N-morpholino)propanesulfonic acid (MOPS) buffer, followed by incubation 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.

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% polyacrylamide gel electrophoresis and Western blot analysis. For immunoprecipitation with the MEF2A antibody, 2 μl 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.

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 1:20,000 dilution of MEF2A or 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 88–131 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.

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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. 2A). In contrast, the IA-17 antibody was specific for MEF2A, whereas the IA-14 was specific for MEF2C (Fig. 2B). In addition, 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 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 immunoprecipitated 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 MEF2D protein was immunodepleted from the supernatant following MEF2A immunoprecipitation. Although we were unable to immuno blot 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 membrane. 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.

Addition 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
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. 7B). 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 contrast, the 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**

The presence of GLUT4 protein in different cell types and its role in insulin-stimulated glucose uptake are well established. GLUT4 is a glucose transporter protein that is expressed in skeletal muscle, adipose tissue, and liver, and its expression is regulated by insulin. The GLUT4 gene contains several transcriptional regulatory elements, including MEF2 binding sites. MEF2 is a family of transcription factors that are essential for the regulation of muscle gene expression.

MEF2 proteins are composed of four amino-terminal MADS domains and a carboxyl-terminal transactivation domain. The MADS domains contain a 100-bp DNA binding motif that is conserved among eukaryotic species and is responsible for the binding to the MEF2 consensus sequence.

**Experimental Procedures.**

**A**  
[IB: α-MEF2A  MEF2A (μl)]

**B**  
[EMSA: MEF2 (μl)]

**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 (0.006, 0.06, 0.06, 0.15, 0.2, 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 3P-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.

Addition of in vitro translated MEF2A protein restores DNA binding activity in nuclear extracts from diabetic rats. These results are consistent with the hypothesis that the down-regulation of MEF2A protein levels in diabetic muscle is responsible for the decreased expression of GLUT4 gene. The data also suggest that the binding of MEF2 to the GLUT4 MEF2 site is necessary for the transcriptional activation of the GLUT4 gene.

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...
Regulation of GLUT4 Expression by MEF2 Isoforms

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 GLUT4 binding 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.

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