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The Transcription Factor Nuclear Factor I Mediates Repression of the GLUT4 Promoter by Insulin*

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Insulin represses GLUT4 expression in 3T3-L1 adipocytes through an insulin response element located at bases −706 to −676 in the 5′-flanking sequence. Nuclear proteins related to the nuclear factor I (NF1) family of transcription factors bind to this insulin response element. Mutations that disrupt binding of NF1 proteins to the insulin response element impair the insulin response in reporter gene assays. Insulin treatment of 3T3-L1 adipocytes induces a rapid change in the level of phosphorylation of NF1 proteins, providing a potential mechanism for insulin's ability to regulate gene expression through NF1. Another as yet unidentified protein, not related to NF1, also binds to the GLUT4 insulin response element and is able to mediate partial repression of the GLUT4 promoter in reporter gene assays.

Glucose enters cells through the activity of glucose transporters, a family of membrane-spanning proteins (for reviews, see Refs. 1 and 2). GLUT4, the insulin-responsive glucose transporter, is present in adipose and muscle cells, the two tissues that respond to insulin stimulation with a large and rapid increase in glucose uptake. Insulin increases glucose uptake in these cells by stimulating the translocation of GLUT4 from a sequestered site in the intracellular microsomal compartment to the plasma membrane, where it can then transport glucose into the cell. Glucose uptake is the rate-limiting step for glucose metabolism, so the level of GLUT4 protein at the plasma membrane is the ultimate determinant of glucose utilization in adipose and muscle cells. Muscle and, to a lesser degree, adipose tissue are the primary sites of glucose disposal after a meal; therefore, alterations in GLUT4 expression affect the ability of the whole animal to metabolize a glucose load. This has been well demonstrated in transgenic mice, where overexpression of GLUT4 increases insulin-stimulated glucose uptake and overall glucose disposal (3, 4). Even modest increases in GLUT4 expression have been shown to ameliorate insulin resistance in the db/db mouse (5) and to completely alleviate the insulin resistance that develops in mice fed a high fat diet (6). Decreased expression of GLUT4 has also been shown to affect insulin sensitivity, since targeted disruption of the GLUT4 gene in mice causes a decrease in insulin-stimulated glucose uptake (7) and mice that are heterozygous for the GLUT4 knockout develop a diabetic phenotype (8).

GLUT4 expression in adipose tissue is invariably decreased in states of insulin resistance (9, 10). This decrease in GLUT4 protein and mRNA levels is closely correlated with the decrease in insulin-stimulated glucose uptake into these tissues. No decrease in the expression of GLUT4 in muscle tissue has been observed in insulin-resistant states. However, a number of mechanisms have been proposed to explain how adipocyte metabolism could affect the insulin sensitivity of other tissues (11). Free fatty acids (12) and tumor necrosis factor-α (13) are both factors that are secreted by adipocytes and can cause insulin resistance in other tissues. Thus, changes in lipid metabolism or changes that alter tumor necrosis factor-α production in the adipocyte may underlie the ability of alterations in adipocyte metabolism to affect insulin sensitivity of other tissues. Whatever the mechanism, evidence for the ability of GLUT4 expression levels in the adipocyte to affect whole body glucose disposal comes from studies with transgenic mice; if GLUT4 is specifically overexpressed only in adipose tissue, there is an increase in the insulin sensitivity of the whole animal with an increase in whole body glucose disposal and decreased basal insulin levels (14).

The 3T3-L1 adipocyte is a reliable *in vitro* model of tissue adipocytes. Just as in adipose tissue, insulin acutely increases GLUT4 activity at the plasma membrane in 3T3-L1 adipocytes. However, prolonged treatment with insulin represses GLUT4 gene expression in 3T3-L1 adipocytes (15), with a rapid decrease occurring in the rate of GLUT4 transcription. Because circulating insulin levels are increased in obesity and type 2 diabetes mellitus, the repression of adipocyte GLUT4 expression by insulin may play a role in the insulin resistance of obesity and type 2 diabetes mellitus.

Using promoter-reporter gene constructs, we previously identified a cis-acting element in the GLUT4 gene that mediates the repression of GLUT4 by insulin in 3T3-L1 adipocytes (16). A potential insulin response element was identified between bases −706 and −676 in the 5′-flanking region by DNase I footprint analysis. A reporter gene construct in which bases −706 to −676 were deleted was not repressed by insulin treatment, confirming that this sequence is necessary for the repression of the GLUT4 promoter by insulin in 3T3-L1 adipocytes. This sequence does not show homology to previously described insulin response elements and thus represents a distinct mechanism of gene regulation by insulin. In this report, we identify nuclear proteins that bind to this insulin response element (IRE)[1] as members of the nuclear factor I (NF1) family of transcription factors.

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[1] The abbreviations used are: IRE, insulin response element; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; bp, base pair(s); CTF, CCAAT-binding transcription factor; NF1, nuclear factor I.

EXPERIMENTAL PROCEDURES

Cell Culture—3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% calf serum and induced to differentiate into adipocytes by treating confluent cells with 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, and 167 nM insulin in 10% fetal bovine serum for 2 days, followed by treatment with 167 nM insulin in 10% fetal bovine serum for 2 days. The cells were then maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and fed every other day. 8 days after induction of differentiation, a medium change was made, and cells were studied on day 9.

Plasmid Constructs—The −785-GLUT4/CAT plasmid (wild type) contained 785 bp of the 5′-flanking region of the murine GLUT4 gene, the GLUT4 transcription initiation site, 171 bp of GLUT4 5′-untranslated sequence, and the coding sequence for the bacterial chloramphenicol acetyltransferase (CAT) gene (16). Mutations in the GLUT4 IRE were produced by digesting the −785-GLUT4/CAT plasmid with *Hin*dIII and *Sma*I to remove bases −785 to −469 of the GLUT4 promoter. This sequence was then replaced with an insert generated by the polymerase chain reaction using *Taq* and *Pwo* DNA polymerase (Expand; Roche Molecular Biochemicals) to amplify the insert in a two-step technique as described by Chen and Przybyla (17). In the first reaction, the following oligonucleotides containing the desired mutations were used as 5′ primers: M1, 5′-CACCTGTCCCTTAAGTCCCCTCCAAGAACCAGTGTAG-3′; M2, 5′-CACCTGTCCCTTGGGTCCCCTTTAAGAACCAGTGTAG-3′; M3, 5′-CACCTGTCCCTTGGGTCTTCTCCAAGAACCAGTGTAG-3′.

These primers, except for the underlined base changes, were complementary to bases −673 to −710 in the 5′-flanking region of the GLUT4 gene. 5′-AGCTTGCGAAATTTCTGAAAGAATTG-3′, complementary to bases −248 to −269 in the 5′-flanking region of the GLUT4 gene (excluding the underlined bases), was used as the 3′ primer. The −785-GLUT4/CAT plasmid was used as the template. The product from the first amplification reaction was purified from an agarose gel using Qiaquick (Qiagen) and was used as the 3′ primer in the second reaction. The M13 reverse primer (5′-CAGGAAACAGCTATGAC-3′) was used as the 5′ primer, and −785-GLUT4/CAT was again used as the template. This product was digested with *Hin*dIII and *Sma*I. The 316-bp fragment was purified from an agarose gel using Qiaquick (Qiagen) and was inserted into the *Hin*dIII/*Sma*I-digested −785-GLUT4/CAT plasmid. The plasmids were sequenced to confirm the ligated ends, the generation of the mutation, and the polymerase chain reaction (PCR)-amplified sequence.

Stable Transfections—Transfection was performed using the calcium phosphate co-precipitation method (18). Subconfluent 3T3-L1 preadipocytes were transfected with 25 μg of the reporter construct and 2.5 μg of the pSV2Neo plasmid. The cells were incubated with the calcium phosphate-DNA precipitate for 4 h and then shocked with 10% glycerol in phosphate-buffered saline for 2 min. Cells were selected and maintained in 300 μg/ml G418 beginning 24 h after transfection. After 7 days, resistant foci of clones were pooled and maintained for further study. Two or more independent pools of 20–50 foci were studied for each construct.

Quantitation of mRNA—Cells were lysed in 5 M guanidinium isothiocyanate, 0.5% sarcosyl, and 5% β-mercaptoethanol, and total RNA was isolated by centrifugation over a 5.7 M CsCl cushion. For quantitation of the endogenous GLUT4 message, 10 μg of total RNA was separated by electrophoresis through a 1.2% agarose gel containing 6.5% formaldehyde. The RNA was transferred to a nylon membrane (Hybond-N; Amersham Pharmacia Biotech), fixed by UV irradiation, and hybridized with a 1.7-kilobase pair murine GLUT4 cDNA (2×10^6 cpm/ml) that was labeled by random hexamer priming (Decaprime; Ambion, Inc.). Hybridization was performed in a solution containing 50% formamide, 4× SSC, 5× Denhardt's solution, 50 mM phosphate buffer, pH 7.0, 100 μg/ml yeast tRNA, 0.5 mg/ml sodium pyrophosphate, and 1% SDS at 42 °C for 16 h. The filter was washed at high stringency (0.1× SSC, 0.1% SDS at 60 °C) for 1 h. Band intensity of the 2.7-kilobase pair GLUT4 mRNA was quantitated on a Fuji BAS2000 bioimaging autoanalyzer. To quantitate the reporter mRNA levels, a quantitative reverse transcriptase PCR assay was used as described previously (16), except that the cellular RNA was not digested with DNase I prior to the reverse transcriptase PCR. Instead, a control was performed to document that any DNA contamination of the cellular RNA was quantitatively insignificant compared with the reporter mRNA. To determine this, a known quantity of competitor cRNA was reverse transcribed. The reverse transcriptase was then heat-inactivated before adding an aliquot of the RNA sample, followed by amplification by PCR using the same conditions as for the competitive reverse transcriptase PCR.

Gel Mobility Shift Assay—Nuclear extract was prepared from day 9 3T3-L1 adipocytes following the method described by Lavery and Schibler (19). Except when noted, the following were added to each of the solutions during purification as inhibitors of protein phosphatase activity: 30 mM β-glycerophosphate (Sigma), 1 mg/ml *p*-nitrophenyl phosphate (Sigma), and 5 mM sodium vanadate (Fisher). The protein concentration of the nuclear extract was quantitated using the BCA protein assay reagent (Pierce).

A 41-bp double-stranded GLUT4 IRE oligonucleotide probe (probe A), corresponding to bases −710 to −674 of the 5′-flanking region of the GLUT4 gene plus GATC on the 3′-end for labeling, was synthesized and labeled with [α-^{32}P]dATP by a fill-in reaction (Megaprime; Ambion). Mutated GLUT4 IRE probes (probes M1, M2, and M3) were also synthesized. These probes were identical to the wild-type GLUT4 IRE probe (probe A) except for two base changes introduced as indicated by the boldface capital letters in Table I. 50–100 $\times 10^3$ cpm of labeled probe was incubated with 3 μg of nuclear extract in a 30-μl solution containing 0.33× NUN buffer (1× NUN: 0.3 M NaCl, 1 M urea, 1% Nonidet P-40, 25 mM Hepes (pH 7.9), and 1 mM dithiothreitol), 8.3% glycerol, 20 mM HEPES (pH 7.6), 2 mM dithiothreitol, 10 μg of bovine serum albumin, and 2 μg of poly(dI-dC). The binding reaction was incubated at room temperature for 30 min and then separated by electrophoresis on a polyacrylamide gel in 0.5× TBE (45 mM Tris borate, 1 mM EDTA). For competition reactions, 100 ng of unlabeled oligonucleotide (approximately 100-fold excess over the labeled probe) was added to the binding reaction. The −710 to −674 GLUT4 double-stranded oligonucleotide (probe A) was used as a specific competitor, and a 24-bp double-stranded oligonucleotide including the unrelated DNA binding sequence corresponding to bases −437 to −421 of the stearoyl-CoA desaturase 2 5′-flanking region was used as a nonspecific competitor (competitor S). A 27-bp double-stranded oligonucleotide containing the NF1 binding site from adenovirus 2 (N in Table I) was also used as a competitor. For the "supershift" assays, 4 μl of an anti-CCAAT-binding transcription factor (CTF)/NF1 antibody raised against recombinant CTF-2 (generously provided by N. Tanese, New York University) or 4 μl of nonimmune rabbit serum was added to the binding reaction. Autoradiography was performed at −80 °C with Kodak X-Omat AR film (Eastman Kodak Co.) and an intensifying screen for the indicated times.

Western Immunoblot—15 μg of nuclear extract was boiled in an SDS sample loading buffer (2% SDS, 10% glycerol, 100 mM dithiothreitol, 50 mM Tris-HCl, pH 6.8) for 5 min. The samples were loaded on a denaturing SDS-polyacrylamide gel, using a 12.5% stacking gel and a 7% resolving gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore Corp.). The filter was blocked in 5% nonfat milk in 0.05% Tween 20, 25 mM Tris-HCl, 150 mM NaCl (TTBS) and then incubated with antibody in 1% nonfat milk in TTBS. The filter was washed with TTBS and then probed with peroxidase-conjugated anti-rabbit IgG (Sigma), washed with TTBS, and then developed using an enhanced chemiluminescent reagent (Amersham Pharmacia Biotech). Primary antibodies used include the anti-CTF/NF1 antibody (generously provided by N. Tanese), and an anti-NF1 antibody raised against the common N terminus of the NF1 isoforms (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Phosphatase Treatment—Nuclear extract from 3T3-L1 adipocytes was prepared using the method of Lavery and Schibler (19) as described above, except that the inhibitors of protein phosphatase activity were not included. 15 μg of nuclear extract was treated with alkaline phosphatase (from calf intestine, Roche Molecular Biochemicals) in 1× phosphatase buffer, 0.3× NUN buffer, and 2 units of alkaline phosphatase at 37 °C for 3 h. In some samples, 1% SDS was added to the reaction mixture, as noted. Inactive alkaline phosphatase (Δ) was prepared by incubating in 25 mM EDTA at 70 °C for 4 h. Control nuclear extract was incubated in the reaction buffer at 37 °C with no enzyme added.

RESULTS

DNA Binding Properties of 3T3-L1 Adipocyte Nuclear Proteins That Bind to the GLUT4 Insulin Response Element—Our earlier experiments identified the nucleotide sequence from bases −676 to −706 in the 5′-flanking region of the GLUT4 gene as necessary for repression of the GLUT4 promoter by insulin in 3T3-L1 adipocytes (16). Examination of this sequence revealed the presence of the consensus binding site sequence for the NF1 family of transcription factors, TGGN$_7$CCA at bases −687 to −699. In order to determine if the

TABLE I

Nucleotide sequence of the wild type and mutated GLUT4 insulin response element probes

Also shown are the adenovirus 2 NF1 binding sequence probe (N) and the consensus binding sequence for NF1 proteins.

Probe	Sequence
GLUT4 IRE	cacctgtcccttgggtcccctccaagaaccagtgtaggatc
M1	cacctgtcccttTTgtcccctccaagaaccagtgtaggatc
M2	cacctgtcccttgggtccccAAaagaaccagtgtaggatc
M3	cacctgtcccttgggtccAAtccaagaaccagtgtaggatc
N	ttttggattgaagccaatatgataaaa
NF1 consensus	TGG CCA

nuclear factors that bind to this region are related to the NF1 family, a series of mutations were made to specifically disrupt the NF1 consensus sequence (Table I). A gel mobility shift assay was performed using these mutated oligonucleotides in direct binding experiments and as competitors of the wild-type GLUT4 IRE oligonucleotide (probe A). When incubated with 3T3-L1 adipocyte nuclear extract, the wild-type GLUT4 IRE produced two major protein-DNA complexes, bands 1 and 2 (Fig. 1*A*, *lanes 3*, *10*, and *18*). Closer inspection of the gels, particularly when the gels were run for a longer time (Fig. 1*B*, *lane 1*), revealed that band 1 is actually composed of two protein-DNA complexes, forming bands 1a and 1b. (In some experiments, an additional protein-DNA complex migrating between bands 1a and 1b is also seen). As seen in Fig. 1, 3T3-L1 nuclear proteins have altered affinities for mutants 1 and 2, which have base changes that disrupt the 5′- or 3′-half of the consensus NF1 binding sequence. None of the proteins that form the major protein-DNA complexes identified by the wild-type sequence bind to mutant 1 (Fig. 1*A*, *lane 11*, and Fig. 1*B*, *lane 7*), and mutant 1 does not compete with the wild-type sequence for the binding of any of these proteins (Fig. 1*A*, *lane 7*, and Fig. 1*B*, *lane 4*). The protein that forms the fastest migrating complex (band 2) does not bind to mutant 2 (Fig. 1*A*, *lane 12*, and Fig. 1*B*, *lane 9*), and mutant 2 does not compete with the wild-type sequence for the binding to this protein (Fig. 1*A*, *lane 8*, and Fig. 1*B*, *lane 5*). A protein that produces a protein-DNA complex that has a similar migration to the slower migrating complex identified by the wild-type sequence does bind to mutant 2. 3T3-L1 adipocyte nuclear protein(s) binds to mutant 2 and forms a protein-DNA complex with a mobility at position 1a but does not form a complex with a mobility at position 1b (Fig. 1*A*, *lane 12*, and Fig. 1*B*, *lane 9*). Similarly, mutant 2 competes with the wild-type sequence for the proteins that form band 1a but does not compete for binding to the proteins that form band 1b (Fig. 1*A*, *lane 8*, and Fig. 1*B*, *lane 5*). Mutant 3 has a two-base substitution in the middle of the NF1 consensus binding sequence that should not affect binding of NF1 transcription factors. 3T3-L1 adipocyte nuclear proteins bind to mutant 3 and form the same protein-DNA complexes as the wild-type sequence (Fig. 1*A*, *lane 13*, and Fig. 1*B*, *lane 11*), and mutant 3 can compete with the wild-type sequence for binding to all of the proteins that bind to it (Fig. 1*A*, *lane 9*, and Fig. 1*B*, *lane 6*). Finally, an oligonucleotide that contains the adenovirus 2 NF1 binding site (*N*) was used as a competing oligonucleotide. The NF1 oligonucleotide competes with the wild-type GLUT4 IRE for binding to the 3T3-L1 adipocyte nuclear proteins that form bands 1b and 2 but does not compete with the wild-type sequence for binding to the protein(s) that forms band 1a (Fig. 1*A*, *lanes 6* and *14*, and Fig. 1*B*, *lane 3*) Taken together, these results indicate that the 3T3-L1 adipocyte nuclear proteins that bind to the GLUT4 IRE include proteins that have similar binding characteristics as NF1 family members, forming bands 1b and 2. In addition, there are proteins that bind to this region that have binding properties different from NF1 family members, forming band 1a.

Nuclear Proteins in the NF1 Family Bind to the GLUT4 Insulin Response Element—Antibody raised against recombinant CTF/NF1 (generously provided by N. Tanese) was used in the gel mobility shift assay to determine whether the proteins in the 3T3-L1 adipocyte nuclear extract that bind to the GLUT4 IRE contain NF1. As shown in Fig. 1*A* (*lanes 1* and *2*) and Fig. 1*C*, antibody to CTF/NF1 (*N*), but not preimmune serum (*C*), disrupts formation of complexes 1b and 2, and produces a "super-shifted" band when the 3T3-L1 nuclear proteins are bound to the GLUT4 IRE. The anti-NF1 antibody does not disrupt band 1a. These results confirm those of the mutational analysis, *i.e.* that bands 1b and 2 contain proteins in the NF1 family and that band 1a does not contain an NF1 protein.

To confirm which protein classes bind to the mutated GLUT4 insulin response elements, the NF1 oligonucleotide was used to compete for binding of 3T3-L1 adipocyte nuclear proteins to the M1, M2, and M3 oligonucleotides in the gel mobility shift assay. The mutant 1 bound neither the NF1 nor the non-NF1 proteins (Fig. 1*A*, *lanes 11* and *15*, and Fig. 1*B*, *lanes 7* and *8*). The protein-DNA complex formed with mutant 2 (Fig. 1*A*, *lane 12*, and Fig. 1*B*, *lane 9*) was not competed away by the NF1 oligonucleotide (Fig. 1*A*, *lane 16*, and Fig. 1*B*, *lane 10*), confirming that this mutant does not bind NF1 proteins. Some of the protein-DNA complexes formed with the mutant 3 probe (Fig. 1*A*, *lane 13*, and Fig. 1*B*, *lane 11*) are competed away by the NF1 oligonucleotide, but the complex forming band 1a remains (Fig. 1*A*, *lane 17*, and Fig. 1*B*, *lane 12*). Table II summarizes the classes of proteins that bind to the wild type and mutated GLUT4 insulin response elements based on the results of these gel mobility shift assays.

The NF1 Binding Site in the GLUT4 Promoter Is Necessary for Full Repression of Reporter Gene Expression by Insulin—GLUT4 promoter/CAT reporter constructs were produced that contained mutations 1, 2, and 3 in the context of 785 base pairs of 5′-flanking sequence. The wild type and mutant constructs were stably transfected into 3T3-L1 preadipocytes. After the cells were differentiated into adipocytes, the responses of the reporter genes were assessed when the cells were treated with 1 μM insulin for 8 h. Insulin repressed endogenous GLUT4 expression by 44–54% in all of the stable cell lines expressing the reporter constructs, similar to the suppression observed in untransfected 3T3-L1 adipocytes. As shown in Fig. 2, expression of the wild type −785-GLUT4/CAT reporter construct was repressed by insulin to a similar degree as the endogenous GLUT4 gene, as was seen previously (16). Expression of the reporter construct containing the M3 mutation, which does not disrupt any of the protein-DNA interactions detected in the gel mobility shift assay, was also repressed by insulin to a similar degree as both the endogenous GLUT4 gene and the wild type reporter construct. In contrast, expression of the reporter construct that contains the M1 mutation, which disrupts all of the major protein-DNA interactions detected in the gel mobility shift assay, showed no repression by insulin treatment. Finally, expression of the reporter construct containing the M2 mutation, which disrupts NF1 protein binding but does not disrupt binding of the non-NF1 nuclear protein(s), showed partial repression by insulin treatment. However, this repression is sig-

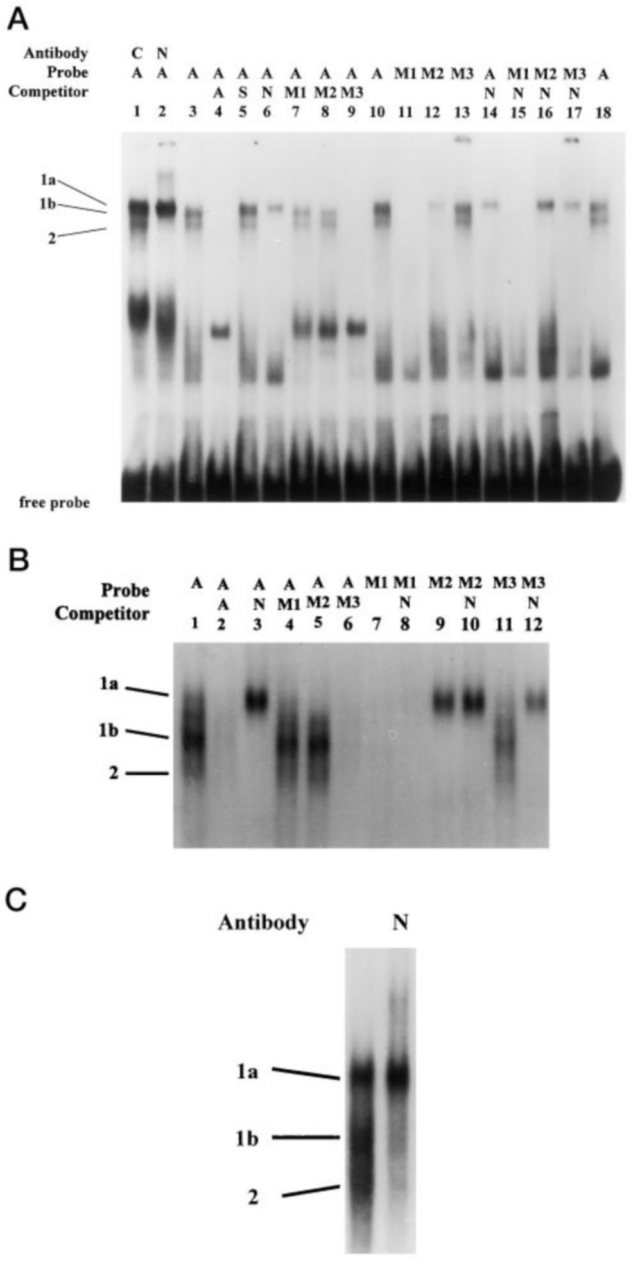

FIG. 1. **Analysis of protein binding to the GLUT4 insulin response element.** *A*, 3 μg of nuclear extract from 3T3-L1 adipocytes was incubated with [α-^{32}P]dATP-labeled double-stranded oligonucleotide and separated by electrophoresis on a 6% polyacrylamide gel for 2 h. The gel was exposed to x-ray film at −80 °C overnight. Oligonucleotides used as probes were the GLUT4 IRE containing bases −710 to −684 of the 5′-flanking region of the GLUT4 gene (probe A) and the GLUT4 IRE containing mutations M1, M2, and M3 (see Table I). A 100-fold excess of unlabeled oligonucleotide was added as a competitor, as indicated. In addition to the GLUT4 IRE and mutant oligonucleotides, an NF1 binding oligonucleotide (*N*, see Table I) was used as a competitor, and an unrelated DNA binding oligonucleotide (stearoyl-CoA desaturase 2, *S*) was used as a nonspecific competitor. 4 μl of anti-NF1 (*N*) or nonimmune control (*C*) antiserum was added as indicated. *1a*, *1b*, and *2* indicate specific protein-DNA complexes. *B* and *C*, electrophoresis was run on 5% polyacrylamide gels for 4 h.

TABLE II
3T3-L1 adipocyte nuclear proteins bound to wild type and mutated GLUT4 insulin response element sequences

IRE	Proteins bound	
	NF1	Non-NF1
Wild type	+	+
M1	−	−
M2	−	+
M3	+	+

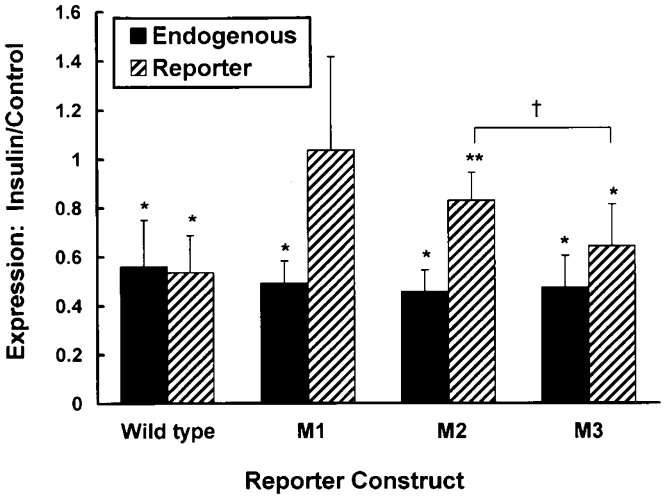

FIG. 2. **Effect of insulin on endogenous GLUT4 and GLUT4/CAT reporter gene mRNA levels.** Stable 3T3-L1 cell lines expressing GLUT4/CAT reporter gene constructs containing 785 bp of the 5′-flanking region of the GLUT4 gene with the wild type sequence or containing mutations M1, M2, and M3 of the IRE (bases −706 to −676) were prepared. The cells were induced to differentiate into cells expressing the adipocyte phenotype, and on day 9 of the differentiation protocol, insulin was added to treated cells to a concentration of 1 μM. Cells were harvested after 8 h, and RNA was isolated. Endogenous GLUT4 mRNA was analyzed by Northern analysis. GLUT4/CAT reporter mRNA was quantitated by a competitive reverse transcriptase PCR technique (see "Experimental Procedures"). Results are expressed as a ratio of the expression level in treated cells to the expression level in control cells (mean ± S.D. of six independent experiments.) *, $p <$ 0.005 different from 1.0; **, $p < 0.02$ different from 1.0; †, $p < 0.05$. The absolute expression level in control cells for each of the constructs (10^{-6} pg of reporter/pg of total RNA) was as follows: wild type, 0.60 ± 0.32; M1, 1.7 ± 0.75; M2, 1.9 ± 0.51; M3, 1.7 ± 0.48.

nificantly less than that of the wild type ($p < 0.005$) or M3 mutant ($p < 0.05$) constructs.

Insulin Treatment Causes a Change in the Mobility of the NF1-containing Protein-DNA Complexes—3T3-L1 adipocytes were treated with 1 μM insulin for 0, 10, 20, or 60 min. Nuclear extracts were prepared from these cells and studied by gel mobility shift assay using the GLUT4 IRE as the probe. The gels were run for a longer period of time to allow for better separation of the bands. While this prolonged electrophoresis resulted in loss of sharpness of the bands, it revealed that insulin treatment of the cells caused a decreased mobility of certain of the protein-DNA complexes (Fig. 3). While this change in mobility is subtle, it is reproducible, having been observed in numerous experiments using four preparations of nuclear extract. This mobility change was seen in nuclear extract prepared from cells treated with insulin for 10 min. However, by 20 min the mobility change began to diminish, and by 60 min the mobility of the protein-DNA complexes reverted to that of cells not treated with insulin.

The bands whose mobility was shifted by insulin treatment correspond to bands 1b and 2, *i.e.* the bands that contain the NF1 proteins (see above). Note that the exposure for Fig. 3 was chosen to best discern band 1b; the mobility shift of band 2 is better seen with other exposures (not shown). The identity of the shifted bands as containing NF1 proteins was verified by examining the mobility of the non-NF1 proteins that bind to the GLUT4 IRE. Fig. 4 shows the results of a gel mobility shift assay with nuclear extracts incubated with the radiolabeled GLUT4 IRE probe (probe A) in the presence of an excess of an unlabeled NF1 consensus binding site oligonucleotide to com-

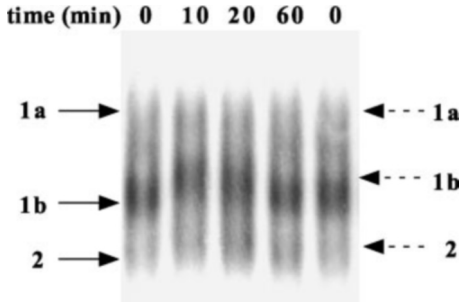

FIG. 3. **Binding of nuclear proteins from control or insulin-treated 3T3-L1 adipocytes to the GLUT4 insulin response element.** 3 μg of nuclear extract from 3T3-L1 adipocytes treated with 1 μM insulin for the indicated times was incubated with [α-^{32}P]dATP-labeled double-stranded oligonucleotide containing bases -710 to -684 of the 5′-flanking region of the GLUT4 gene (probe A). The protein-DNA mixtures were separated by electrophoresis on a 5% polyacrylamide gel. The gel was exposed to x-ray film at -80 °C overnight. These results were confirmed with four different preparations of nuclear extracts. The *arrows* show the positions of protein-DNA complexes from untreated 3T3-L1 adipocytes, and the *dashed arrows* show the position of the same complexes from cells treated with insulin for 10 min.

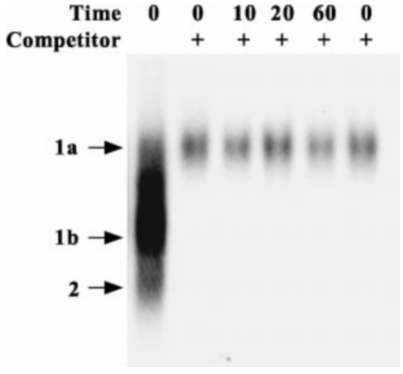

FIG. 4. **Binding of nuclear proteins from control or insulin-treated 3T3-L1 adipocytes to the GLUT4 insulin response element.** 3 μg of nuclear extract from 3T3-L1 adipocytes treated with 1 μM insulin for the indicated times was incubated with [α-^{32}P]dATP-labeled double-stranded oligonucleotide containing bases -710 to -684 of the 5′-flanking region of the GLUT4 gene (probe A). A 100-fold excess of unlabeled NF1 consensus binding site oligonucleotide was added as a competitor as indicated. The protein-DNA mixtures were separated by electrophoresis on a 5% polyacrylamide gel. The gel was exposed to x-ray film at -80 °C overnight. *1a*, *1b*, and *2* indicate specific protein-DNA complexes (see "Results").

pete away binding of NF1 proteins. There is no difference in the mobility of the remaining non-NF1-containing complex (band 1a) that is formed from nuclear extract from 3T3-L1 adipocytes that were treated with insulin for 0, 10, 20, or 60 min. In addition, incubating the 3T3-L1 nuclear extract with CTF/NF1 antibody in the gel mobility shift binding reaction confirmed that the protein-DNA complexes that had altered mobility when prepared from insulin-treated cells contained NF1 proteins.[2]

Since 1 μM is a supraphysiologic insulin concentration, the effect of more physiologic concentrations of insulin on the gel mobility shift pattern was examined. Nuclear extract was prepared from 3T3-L1 adipocytes treated with increasing concentrations of insulin for 10 min. As shown in Fig. 5, insulin at a concentration as low as 10 nM altered the mobility of the protein complexes that bind to the GLUT4 IRE, and treatment with 25 nM insulin induced a maximal shift in mobility.

The change in mobility of the NF1-containing protein-DNA complexes induced by insulin observed in the gel mobility

[2] D. W. Cooke and M. D. Lane, unpublished data.

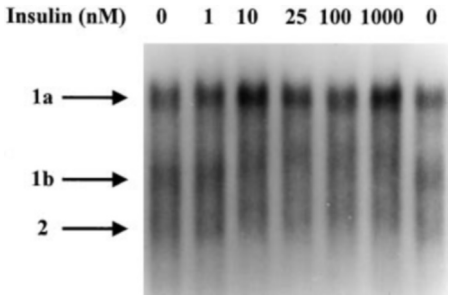

FIG. 5. **Binding of nuclear proteins from control or insulin-treated 3T3-L1 adipocytes to the GLUT4 insulin response element.** 3 μg of nuclear extract from 3T3-L1 adipocytes treated with the indicated concentration of insulin for 10 min was incubated with [α-^{32}P]dATP-labeled double-stranded oligonucleotide containing bases -710 to -684 of the 5′-flanking region of the GLUT4 gene (probe A). The protein-DNA mixtures were separated by electrophoresis on a 5% polyacrylamide gel. The gel was exposed to x-ray film at -80 °C overnight. These results were confirmed with four different preparations of nuclear extracts. The *arrows* show the position of protein-DNA complexes from untreated 3T3-L1 adipocytes.

shift assay could be due to various causes, including 1) insulin treatment causing new proteins to bind to the DNA probe, either in addition to or in place of the proteins bound in untreated cells, or 2) insulin treatment inducing a covalent modification of the proteins that bind to the DNA probe. To determine whether insulin induces a covalent modification of the NF1 proteins in 3T3-L1 cells, Western immunoblot analysis was performed on nuclear proteins isolated from control and insulin-treated cells. As shown in Fig. 6*A*, anti-CTF/NF1 antibody detects three protein isoforms in 3T3-L1 adipocytes. When the adipocytes are treated with insulin, there is a rapid disappearance of the fastest migrating protein band. This band disappears in nuclear extract from adipocytes treated with insulin for 10 min and reappears in the nuclear extract from cells treated with insulin for 60 min. When 3T3-L1 adipocytes are treated with increasing concentrations of insulin for 10 min, the fastest migrating protein band begins to disappear with 10 nM insulin treatment and completely disappears with 25 nM insulin treatment. Concomitant with the disappearance of the fastest migrating protein, there is an increased amount of the protein band corresponding to the slowest migrating protein (better seen in Fig. 7). Similar results are obtained when whole cell lysates are prepared from the 3T3-L1 adipocytes instead of nuclear extract,[2] so the change in mobility is not due to a change in subcellular localization of the proteins. When Western immunoblot analysis is performed on these same samples using another anti-NF1 antibody (Santa Cruz Biotechnology), proteins of different molecular mass than those detected by the CTF/NF1 antibody of Tanese are detected, presumably representing other NF1 isoforms. The NF1 isoforms detected by this antibody also show a mobility shift in response to insulin treatment (Fig. 6*B*), with insulin treatment inducing the appearance of two new protein isoforms. Coincident with this appearance is a decrease in the quantity of the isoforms detected in the basal state. Thus, insulin treatment of 3T3-L1 adipocytes induces a modification of multiple NF1 isoforms that alters their mobility on SDS-polyacrylamide gel electrophoresis.

Insulin Treatment Induces a Change in Phosphorylation of NF1—The insulin-induced mobility shift of the NF1 proteins on SDS-polyacrylamide gel electrophoresis may be due to a change in their extent of phosphorylation. To investigate this, 3T3-L1 adipocyte nuclear extracts from control and insulin-treated cells were treated with alkaline phosphatase followed by SDS-polyacrylamide gel electrophoresis and Western immu-

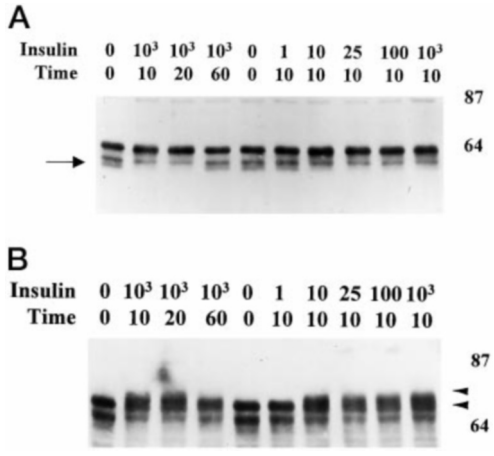

FIG. 6. **Analysis of NF1 proteins from 3T3-L1 adipocytes treated with insulin.** 15 μg of nuclear extracts from 3T3-L1 adipocytes treated with insulin for the indicated times and concentrations were separated on a 7% SDS-polyacrylamide gel. After transfer to polyvinylidene difluoride membrane, the proteins were probed with an anti-NF1 antibody and then developed using an enhanced chemiluminescent assay. The *numbers* to the *right* show the mobility of molecular mass standards. *A*, antibody to CTF/NF1 generously provided by N. Tanese was used. The *arrow* shows the fastest migrating protein that is recognized by the anti-NF1 antibody that disappears with insulin treatment. *B*, antibody to the N-teminus of NF1 (Santa Cruz Biotechnology) was used. The *arrowheads* indicate the protein isoforms induced upon exposure of the cells to insulin.

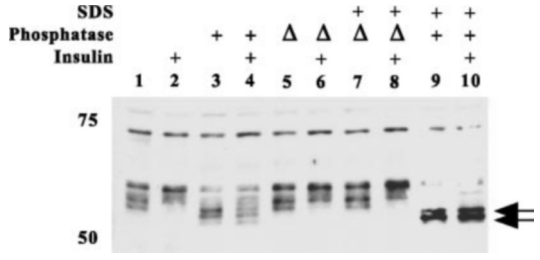

FIG. 7. **Phosphatase treatment of NF1 proteins from 3T3-L1 adipocytes.** 3T3-L1 adipocytes were treated with 1 μM insulin for 10 min, and nuclear extract was prepared from control or insulin-treated (+) cells. 15 μg of nuclear extract was treated with alkaline phosphatase (+) or heat-inactivated alkaline phosphatase (Δ) in the presence of 1% SDS as indicated. The extracts were then separated on a 7% SDS-polyacrylamide gel. After transfer to polyvinylidene difluoride membrane, the proteins were probed with an anti-CTF/NF1 antibody (generously provided by N. Tanese) and developed using an enhanced chemiluminescent assay. The *arrows* show the fully dephosphorylated proteins recognized by the anti-CTF/NF1 antibody. The *numbers* to the *left* show the mobility of molecular mass standards.

noblotting using the CTF/NF1 antibody. As a control, nuclear extract was treated with alkaline phosphatase that had been heat-inactivated (Δ, Fig. 7). There was no change in the mobility of the NF1 proteins that were treated with the heat-inactivated phosphatase. As shown in Fig. 7, phosphatase treatment resulted in an increase in the mobility of the proteins identified by the CTF/NF1 antibody. When 1% SDS was included in the incubations, virtually all of the NF1 proteins were converted to two faster migrating species, presumably representing fully dephosphorylated forms. Moreover, while there was a change in the mobility of the NF1 proteins from insulin-treated cells compared with control cells, this difference was abolished when the proteins were fully dephosphorylated. Partial dephosphorylation of nuclear extract from insulin-treated cells (Fig. 7, *lane 4*) resulted in the apparent regeneration of the isoform that disappeared with insulin treatment. These results strongly suggest that exposure of 3T3-L1 adipocytes to insulin causes a rapid phosphorylation of NF1 proteins.

DISCUSSION

In a previous paper (16), we demonstrated that the region from bases −676 to −706 in the 5′-flanking sequence of the GLUT4 gene contains a cis-element, which mediates the repression of the gene by insulin in 3T3-L1 adipocytes. Several lines of evidence now implicate isoforms of the NF1 family of transcription factors as trans-acting factors that mediate this effect. A subset of 3T3-L1 adipocyte nuclear proteins that bind to the GLUT4 IRE have a sequence specificity that corresponds to that of NF1 proteins, and their binding is specifically competed by an NF1 consensus binding site oligonucleotide (Fig. 1). These same proteins are recognized by a polyclonal anti-NF1 antibody, as shown by the supershift experiments using the gel mobility shift assay (Fig. 1). The importance of NF1 proteins in mediating the down-regulation of the GLUT4 gene by insulin was confirmed by the reporter gene studies using constructs that carried specific mutations that disrupt the NF1 binding site (Fig. 2). Full down-regulation by insulin occurred only with the construct that maintained NF1 binding.

Another protein not related to the NF1 family also binds to the GLUT4 IRE. GLUT4 promoter-reporter gene studies suggest that this protein is also important for the regulation of GLUT4 expression in 3T3-L1 adipocytes by insulin. Expression of a promoter-reporter construct that contains a mutation that disrupts NF1 binding but maintains binding of this other protein (the M2 mutation) is still significantly repressed by insulin treatment (Fig. 2). However, the level of repression of the M2 construct is significantly less than that of either the wild-type reporter construct or the construct containing the mutation that does not disrupt binding of either the NF1 protein(s) or the non-NF1 protein (mutant M3). Therefore, although NF1 binding is necessary to mediate the full effect of insulin, the non-NF1 protein is able to mediate a partial repression of the GLUT4 promoter.

The NF1 family of transcription factors consists of more than a dozen isoforms. Early studies by Jones *et al*. (20) found that CTF was identical to NF-I (now referred to as NF1), which had been identified as a factor required for the initiation of adenovirus DNA replication. The nucleotide sequence to which NF1 binds is the same for its action as a transcription factor or as a DNA replication factor. Four distinct genes have been identified (NF1/CTF, NF1-L, NF1/Red1, and NF1/X), with multiple products from each of these genes produced by alternative splicing. The predicted molecular masses of the isoforms range from 27 to 62 kDa. Larger isoforms have been identified (20), most likely due to post-translational modification of these proteins (21, 22). NF1 family members bind to DNA both as homo- and heterodimers (23), adding another level of diversity to this protein family. There is nearly complete amino acid sequence identity in the N-terminal regions of the NF1 isoforms, with more diversity in their C-terminal portions. These transcription factors bind through their N-terminal domain to the sequence $TGGN_7CCA$ and regulate transcription through their proline-rich C-terminal domain. The DNA binding domains of the NF1s show no obvious sequence similarity to any of the known classes of DNA-binding domains such as zinc finger, leucine zipper, or helix-loop-helix motifs. Most NF1 isoforms appear to be expressed constitutively, but at least some tissue-specific isoforms appear to exist, as reported for brain (24) and bone (25). NF1 has been implicated in adipocyte gene expression, since an NF1 binding site was shown to be necessary to direct tissue-specific expression mediated by the 422/aP2 promoter to adipocytes (26). In addition, an NF1 binding site is necessary for the transcriptional activation of the stearoyl-CoA desaturase 1 promoter during differentiation of 3T3-L1 cells into adipocytes (27). It is not yet known which isoforms are

expressed by 3T3-L1 adipocytes or in adipose tissue. The antibodies used in the present study are not isoform-specific; moreover, specific isoforms cannot be identified based on molecular mass. However, since Western analysis using two different anti-NF1 antibodies identified multiple proteins, it appears that several isoforms are expressed by 3T3-L1 adipocytes.

Because of the rather ubiquitous expression of NF1 isoforms, they were first believed to be basal transcription factors that contribute to the expression of unregulated "housekeeping" genes. Recently, however, data has accumulated that demonstrate various roles for NF1 in mediating regulated gene expression. NF1 functions as an accessory factor for gene regulation by a number of other transcription factors, such that mutations that abolish NF1 binding either eliminate or greatly diminish regulation by the other transcription factors. These factors have included the cAMP response element-binding protein (28) and members of the steroid hormone receptor superfamily, including the glucocorticoid (29), estrogen (30), androgen (31), and vitamin D (25) receptors. The studies presented here, however, are the first demonstration that NF1 mediates the regulation of a gene by insulin. Thus, NF1 joins a list that so far includes HNF-3 (32), Ets-related proteins (33, 34), sterol-regulatory element-binding proteins (35), upstream stimulatory factors (36, 37), and possibly Sp1 (38) and CCAAT/enhancer-binding protein family members (39) as mediators of gene regulation by insulin. It will be of interest to determine the role NF1 plays in the regulation of other genes by insulin, since a number insulin-responsive genes have been found to have NF1 binding sites. Importantly, an NF1 binding site has been identified near the insulin response element of the fatty acid synthase gene (40). Further studies will be required to determine if NF1 binding to this element is necessary for the stimulation of fatty acid synthase expression by insulin.

Early studies in Tjian's laboratory found that NF1 can undergo post-translational modification, including *O*-glycosylation (22) and phosphorylation (21). The existence of such modification raises the possibility that the function of NF1 may be regulated by post-translational modification. Indeed, Yang *et al.* demonstrated that overexpression of Myc resulted in the phosphorylation of NF1 and suggested that this may be the mechanism through which Myc represses the expression of a number of genes (41). We have shown that insulin induces a change in the mobility of NF1 proteins on SDS-polyacrylamide gel electrophoresis and that this change in mobility is abolished if the proteins are dephosphorylated by phosphatase treatment (Fig. 7). This provides compelling evidence that insulin induces a change in the level of phosphorylation of NF1 and is the first demonstration of this phenomenon.

Although the results presented in this paper support the importance of NF1 in mediating the repression of the GLUT4 promoter by insulin, there is an apparent inconsistency regarding the role that phosphorylation of NF1 plays in this regulation; *i.e.* the change in phosphorylation in response to insulin treatment is transient, while repression of GLUT4 expression by insulin is more prolonged (15). It is possible that the loss of the insulin-induced phosphorylation is due to the difficulty of maintaining the phosphorylation states of proteins *in vitro*. Insulin has been shown to activate protein phosphatases (42, 43). If NF1 is exposed to one of these phosphatases during the preparation of nuclear extract, the insulin-induced phosphorylation could be lost in extracts from the later time points when the phosphatase has been activated. It is also possible that a transient phosphorylation of NF1 could initiate a cascade that results in a sustained repression of GLUT4 expression. For example, phosphorylation of NF1 could facilitate the binding of another repressive transcription factor that remains bound even if NF1 becomes dephosphorylated. Future studies will be needed to identify the phosphorylation sites of NF1 affected by insulin and to demonstrate that the change in NF1 phosphorylation induced by insulin is necessary for the repression of GLUT4 expression by insulin in 3T3-L1 adipocytes. However, the concentration of insulin required to induce a change in phosphorylation of NF1 (Fig. 6) correlates well with the concentration of insulin required to suppress GLUT4 transcription (15), supporting a relationship between the two findings.

In summary, we have found that two classes of nuclear proteins bind to the insulin response element of the GLUT4 gene. One of these is a protein(s) related to the NF1 family of transcription factors. Furthermore, binding of this NF1 protein(s) is necessary for full repression of the GLUT4 promoter by insulin, since mutations that specifically abolish binding of NF1 impair the insulin response. Finally, we find that treatment of 3T3-L1 cells with insulin results in a rapid phosphorylation of NF1 proteins in 3T3-L1 adipocytes. Thus, we have identified a new role for the NF1 family of transcription factors in mediating gene regulation by insulin. This regulation appears to involve insulin inducing a phosphorylation of NF1 proteins, which will be a new mechanism of acute gene regulation by insulin.

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