Insulin-responsive Nuclear Proteins Facilitate Sp1 Interactions with the Insulin-like Growth Factor-I Gene*

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The diabetes-induced decrease in insulin-like growth factor-I transcription appears to be mediated by footprint region V in exon 1. Since region V contains both an Sp1 site and an AT-rich element that recognizes an insulin-responsive binding protein (IRBP), we tested the hypothesis that Sp1 interactions are facilitated by an IRBP. Binding of nuclear extracts to region V probes was reduced by mutational or chemical interference with the AT-rich element. Blocking the AT site also reduced interactions of Sp1 with region V in vitro and blunted transactivation of region V reporter constructs by Sp1 in vivo. Sp1 binding was enhanced by small quantities of hepatic nuclear extracts, but enhancement was reduced by the AT mutation and abolished by a 5-base pair insertion between the AT-rich and GC-rich sites, and transactivation by Sp1 in vivo was diminished by inserting bases between the AT-rich and GC-rich elements. However, treating cells with insulin increased the ability of nuclear extracts to enhance Sp1 binding. These findings indicate that the presence of the AT-rich element is essential for the actions of Sp1 in vitro and in vivo, and the combination of both spacing requirements and insulin responsiveness suggests that IRBP may interact directly with Sp1.

Insulin-like growth factor-I (IGF-I)† is critical for normal growth and development. A small protein similar to insulin in structure (1), IGF-I plays a broad role in cellular proliferation and differentiation and overall tissue growth and has anabolic effects on metabolism (reviewed in Ref. 2). IGF-I is expressed throughout the body and acts via autocrine, paracrine, and endocrine pathways; the majority of circulating (endocrine) IGF-I is produced by the liver (3). Abrogation of hepatic IGF-I production in liver-specific knockout mice resulted in a 75% drop in serum IGF-I levels, and normal growth was maintained only by a 6-fold increase in circulating growth hormone levels, presumably via stimulation of local IGF-I production in extrahepatic tissues (4).

Expression of the IGF-I gene is stimulated by growth hormone, neurotrophic status, and insulin (5–7). Diabetic animals display lower levels of IGF-I protein and mRNA than normal animals, but IGF-I levels are normalized by insulin treatment (8, 9). IGF-I expression appears to be regulated largely at the level of gene transcription, as nuclear run-on studies show that IGF-I gene transcription is diabetes-responsive in rats and insulin-responsive in cultured hepatocytes (9, 10).

Most IGF-I transcripts originate in exon 1 (11, 12), and the IGF-I gene contains a number of DNase I-footprint protein binding sites within 300 bp upstream and downstream of the major transcription initiation sites (13). One of the footprinted areas, downstream region V, appears to be critical for IGF-I regulation by insulin and diabetes status, as deletion of this region abolishes the differential expression of IGF-I observed in invitro transcription assays with hepatic nuclear extracts from normal and diabetic animals (13). Region V is a 24-bp sequence that contains a GC-rich Sp1 binding site (14) adjacent to an AT-rich sequence with homology to the binding site for the homeodomain family of transcription factors (reviewed in Ref. 15).

Sp1 and the related factors Sp3 and Sp4 bind to the 6-bp sequence 5′-CCGCC (16–18). A ubiquitous factor that is relatively unregulated, Sp1 is involved in the expression of numerous genes, including tumor necrosis factor α, DNA polymerase, Smad7, and fatty acid synthase (19–22). These genes encode diverse proteins with a wide variety of functions, and Sp1 actions alone are not likely to control expression. Rather, Sp1 appears to act in concert with other transcription factors that are more specific, such as Elk-1 (19), E2F (20), AP1 (21), and sterol response element binding protein (22), to elicit the proper level of expression for these genes.

The homeodomain family of proteins binds to the core consensus sequence 5′-ATTA, such as that found in IGF-I region V; homeodomain proteins are typically involved in developmental regulation of gene expression (reviewed in Ref. 15). The region V sequence recognizes several factors in nuclear extracts, including both Sp1 and an insulin-responsive binding protein (IRBP), that interacts with the AT-rich element (14, 23). In this study, we provide evidence supporting the hypothesis that IRBP interacts with Sp1 to stimulate binding to region V and expression of the IGF-I gene.

EXPERIMENTAL PROCEDURES

Reagents—Mediatech Cellgro cell culture medium was obtained from Fisher, and fetal bovine serum, antibiotics, and recombinant human insulin were from Life Technologies, Inc. [γ-32P]ATP (3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech. Oligonucleotides corresponding to IGF-I region V were obtained from Life Technologies, Inc., berenil (diminazene aceturate), protease inhibitors, and other chemicals were purchased from Sigma, and recombinant human Sp1 (rhSp1) was purchased from Promega (Madison, WI). DNA modifying enzymes were from New England Biolabs (Beverly, MA).

Plasmids for Transfection Experiments—Reporter plasmids con-
tained tandem copies of IGF-1 footprint region V in pGL3-promoter (Promega, Madison, WI). The wtV, VmAT, and VmSp1 constructs have previously been described (14), and the VcSp1, V+2, V+5, and V+10 reporters were generated in the same manner. Briefly, double-stranded oligonucleotides containing the sequences listed in Table I were phosphorylated with T4 polynucleotide kinase, self-ligated with T4 DNA ligase, and separated on a 3% agarose gel. Bands representing 4-mers were eluted, treated with Tag DNA polymerase to add terminal deoxynucleotides, subcloned into pCR2.1 (a TA cloning vector from Invitrogen, Carlsbad, CA), and then cloned into pGL3 promoter at the KpnI and XhoI sites. The pPacSp1 expression plasmid, containing the strong actin promoter, was provided by Dr. Robert Tjian (24); we generated the pPac vector control by removing the 2.1-kb Sp1 fragment from pPacSp1.

Cell Culture and Transfection—CHO cells and HepG2 cells were obtained from American Type Culture Collection (Manassas, VA). CHO cells are used in many laboratories, whereas HepG2 cells are liver-derived and, thus, more likely to be metabolically relevant to studies of “endocrine” production of IGF-1. CHO cells were maintained in F-12 medium containing 10% fetal bovine serum, whereas HepG2 cells were cultured in Eagle’s minimal essential medium with 10% serum. Cells were generally cotransfected at 50–60% confluence using 2 µg of reporter plasmid and 0.5 µg of expression plasmid per 35-mm well. CHO cells were transfected by the calcium phosphate method (5 Prime Inc., Boulder, CO). Medium was replaced 2–3 h after transfection and again after 24 h. Cells were harvested for luciferase assays 48 h after transfection. When included, berenil was used at 500 nm. HepG2 cells were transfected overnight with the Targefect F2 reagent (Targetting Systems, San Diego, CA) and harvested for luciferase measurement ~36 h after transfection. Results were normalized to total protein concentration.

Hepatic Nuclear Extract Preparation—The Emory University Animal Care and Use Committee approved all animal use. Male Sprague-Dawley rats were obtained from Charles River Laboratories (Raleigh, NC). Nuclear extracts from livers of normal rats were prepared using a modification of the method previously described (25). Briefly, livers were homogenized in an anaerobic tissue processor in 2M sucrose with addition of 10 µg/ml leupeptin, and 0.77 µg/ml pepstatin A. Nuclei were pelleted, resuspended in storage buffer (20 mM Tris-Cl, pH 7.9, 85 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM phenylmethylsulfonyl fluoride, and 50% glycerol) and repelleted, followed by lysis in NUN buffer (1.1 M urea, 0.33 M NaCl, 1.1% Nonidet P-40, 27.5 mM HEPES, pH 7.6, 11% glycerol, 1% dry milk and filtered through cheesecloth, and nuclei were pelleted of the AT-rich element not only for interactions with IRBP (in initiation site 3 (11)) and contains both an AT-rich sequence and an adjacent GC-rich element that is recognized by Sp1 (Table I and Ref. 14). Gel shift studies with hepatic nuclear extracts and a wild type region V probe (wtV, Table I) revealed three complexes, B1, B2, and B3 (Fig. 1A, lane 1); we previously demonstrated that B2 contains Sp1 and B3 contains Sp3 and that both interact with the GC-rich element (14). B1 contains IRBP, an insulin-responsive binding protein(s) that is distinct from Sp1 and binds to the AT-rich element (23). To investigate the functional importance of the AT-rich sequence, we conducted gel shift analyses using a region V probe with a 4-bp mutation in the AT-rich region (VmAT, Table I). This mutation completely abolished B1 binding and greatly reduced interactions with Sp1 and Sp3 (Fig. 1A, lane 2). Since Sp1 and Sp3 interact directly with the GC-rich element, these observations provided the first evidence that Sp1 and Sp3 binding depends in part on either (a) the presence of neighboring DNA sequence elements, and/or (b) the presence of IRBP, presumably bound to the neighboring AT-rich element. As a separate method of evaluating interactions involving the AT-rich element, we performed gel shift experiments in which the probe was treated with berenil before the addition of nuclear extracts. Berenil binds in a non-intercalative fashion to AT-rich stretches of DNA that are at least 4–6 bp long and blocks interactions of proteins with such elements (27). As shown in Fig. 1B, berenil almost completely prevented the binding of all three major complexes, indicating the importance of the AT-rich element not only for interactions with IRBP (in B1), but apparently also with Sp1 and Sp3.

TABLE I

| Region V oligonucleotide sequences | wtV | VmAT | VmSp1 |
|-----------------------------------|-----|------|-------|
| AT-rich                            | TTTGCCCTATTATCCGCCCAACATTCA | TTTGCCCTACCGCTCTGCCCAACATTCA | TTTGCCCTATTATCCGCCCAACATTCA |
| GC-rich                            | TTTGCCCTATTATCCGCCCAACATTCA | TTTGCCCTATTATCCGCCCAACATTCA | TTTGCCCTATTATCCGCCCAACATTCA |

Berenil was incubated with the probe for 30 min before the addition of nuclear extracts or 30 min before the addition of berenil before the addition of rhSp1 also resulted in diminished binding (Fig. 2B). Mutation of the AT-rich element blunted the effect of berenil on Sp1 binding to region V, as seen with control probes containing the consensus Sp1 site but a mutated AT-rich element (VmATcSp1 in Table I, data not shown). Thus, the AT-rich site is critical not only for interactions with IRBP (in B1) but also for efficient binding of Sp1.

We then evaluated the importance of the AT-rich element for
Sp1 transactivation via region V in vivo. CHO cells were co-transfected with reporter constructs containing three copies of the wtV, VmAT, and VmSp1 sequences (Table I) along with an expression vector containing Sp1 and the appropriate empty vector controls. The wtV reporter exhibited 2-fold stimulation by Sp1, and the VmSp1 reporter displayed no stimulation, as expected (Fig. 3A). However, the VmAT reporter resulted in reduced activation by Sp1 (21% less than the wild type reporter, \(p < 0.003\)). In other experiments, CHO cells were treated with berenil after transfection. Blocking AT-rich sequences in this manner also prevented activation of the wtV reporter by Sp1 (Fig. 3B). Taken together, these findings indicate that the AT-rich site is critical for Sp1 interactions with region V both in vitro and in vivo.

Facilitation of Sp1 Binding Occurs Even If the GC-rich Element Is a Consensus Sp1 Site—The GC-rich element in IGF-I region V contains a 1-bp mutation from the consensus Sp1 site (16) with a T substituted for a C (Table I). We examined binding with region V probes mutated to contain the consensus Sp1 site both with an intact AT-rich element and with a mutated AT-rich element (VcSp1 and VmATcSp1, Table I). As expected, rhSp1 interacted more strongly with the VcSp1 probe than with the wtV probe in gel shift studies (Fig. 5A, lanes 2 and 3). Similar to the results obtained with the wtV and VmAT probes, binding of rhSp1 to the VmATcSp1 probe mutated in the AT-rich site was weaker than binding to the VcSp1 probe with the intact AT-rich site (Fig. 5B). Although Sp1 bound more strongly to the VcSp1 probe, facilitation by a factor(s) in nuclear extracts was still observed with VcSp1 (Fig. 5C, lane 3), demonstrating that the presence of a consensus Sp1 site did not eliminate the potential of other factors to enhance Sp1 binding.

Positioning of the AT-rich and GC-rich Elements Is Critical for Facilitation—Facilitation of Sp1 binding to the GC-rich element may be mediated by physical interactions of Sp1 with a factor such as IRBP that binds to the AT-rich element; such interactions could promote or stabilize Sp1 binding and action. To investigate this possibility, we performed gel shift studies...
with region V probes containing 2-, 5-, and 10-bp insertions between the AT-rich and GC-rich elements (V/H11001^2, V/H11001^5, and V/H11001^10, Table I). As shown in Fig. 6, facilitation of Sp1 binding by a factor(s) in nuclear extracts occurred as long as the AT-rich and GC-rich sites were positioned close to their native positions with regard to the face of the DNA helix; the insertion of either 2 or 10 bp into region V did not inhibit facilitation, whereas a 5-bp insertion that introduced a 180° alteration in the relative positioning of the two sites completely abolished facilitation. The spatial relationship of the AT-rich and GC-rich elements is thus important for the interaction between the sites that occurs in vitro.

The importance of the spacing between the two sites was also tested in transient transfection studies. HepG2 hepatoma cells were cotransfected with pGL3 luciferase reporter constructs containing three tandem copies of the wtV, VmAT, or VmSp1 sequences together with either a pPac expression vector encoding Sp1 or the empty vector control. Samples were harvested 48 h after transfection and assayed for luciferase activity. Panel A, transfected cells were incubated with 500 nM berenil for 48 h after transfection, and extracts were assayed for luciferase activity. Data represent the means ± S.E. for three separate experiments.

The AT-rich element is important for region V activation by Sp1 in transfection studies. Panel B, CHO cells were co-transfected with pGL3 luciferase reporter constructs containing three tandem copies of the wtV, VmAT, or VmSp1 sequences together with either a pPac expression vector encoding Sp1 or the empty vector control. Samples were harvested 48 h after transfection and assayed for luciferase activity. Panel B, transfected cells were incubated with 500 nM berenil for 48 h after transfection, and extracts were assayed for luciferase activity. Data represent the means ± S.E. for three separate experiments.

Fig. 3. The AT-rich element is important for region V activation by Sp1 in transfection studies. Panel A, CHO cells were co-transfected with pGL3 luciferase reporter constructs containing three tandem copies of the wtV, VmAT, or VmSp1 sequences together with either a pPac expression vector encoding Sp1 or the empty vector control. Samples were harvested 48 h after transfection and assayed for luciferase activity. Panel B, transfected cells were incubated with 500 nM berenil for 48 h after transfection, and extracts were assayed for luciferase activity. Data represent the means ± S.E. for three separate experiments.

Fig. 4. Hepatic nuclear extracts facilitate the interaction of Sp1 with region V. Trace amounts of either nuclear extracts (NE), rhSp1, or both were incubated with the wtV and VmAT probes and subjected to electrophoresis. Similar results were obtained in at least three additional experiments.

10^{-6} M insulin for 24 h. The facilitation of Sp1 binding was ~4-fold greater with extracts from insulin-treated cells (Fig. 8, lanes 4 and 5, p < 0.04), suggesting that IRBP may be the factor that binds to the AT-rich sequence and facilitates Sp1 binding to the adjacent GC-rich element.

DISCUSSION

Diabetes-related regulation of IGF-I gene transcription appears to involve region V, a DNase I footprint site located downstream from the major transcription initiation sites in exon 1 (13). Region V contains both a GC-rich element that is a functional Sp1 site (14) and an AT-rich element immediately adjacent to the GC-rich element. Our examination of insulin-mediated IGF-I expression led to the detection of IRBP, an insulin-responsive complex that binds to the AT-rich element (23). The present studies show that binding of hepatic nuclear extracts to region V probes is diminished either by mutations in the AT-rich element or by the addition of berenil, which blocks DNA-protein interactions involving AT-rich sequences. The AT-site mutations and berenil also reduce both binding of recombinant Sp1 to region V probes in vitro and the ability of Sp1 to transactivate IGF-I reporter constructs in vivo. Sp1 binding to region V is therefore at least partially dependent on the presence of the AT-rich element.

The requirement of neighboring sequences to facilitate transcription factor binding is well recognized (28). However, our studies of facilitation of Sp1 binding by nuclear extracts indicate that the presence of the AT-rich element may also be...
important in permitting binding of a nuclear factor(s) such as IRBP, which in turn enhances Sp1 binding. The possibility of physical interaction between Sp1 and a factor such as IRBP was shown by the spatial dependence of facilitation; insertion of 5 bp between the AT-rich and GC-rich elements abolished facilitation, but facilitation persisted with 2- and 10-bp insertions that would maintain the AT-rich and GC-rich elements on the same side of the DNA helix. Since the insertion of 2, 5, or 10 additional base pairs between the AT-rich and GC-rich sequences abrogated the ability of Sp1 to activate reporter constructs in vivo, it is likely that facilitation of Sp1 activation or

FIG. 5. Sp1 binds more strongly if region V contains the consensus Sp1 binding site. Gel shift analysis of interactions between various region V sequences and rhSp1. Panel A, rhSp1 was incubated with probes corresponding to its consensus binding site (cSp1), region V containing a consensus Sp1 site (VcSp1), wtV, and VmAT. Panel B, gel mobility shift studies with rhSp1 and probes containing the consensus Sp1 sequence in the context of either wtV or VmAT. Panel C, facilitation studies using trace amounts of nuclear extracts and/or rhSp1 incubated with the VcSp1 probe. Similar results were obtained in at least two additional experiments.

FIG. 6. The spatial relationship between the AT-rich and GC-rich elements is critical for facilitation of Sp1 binding to region V. Gel shift reactions contained trace amounts of hepatic nuclear extracts (NE) and/or rhSp1 incubated with the wtV probe or probes containing 2-, 5-, and 10-bp insertions between the AT-rich and GC-rich sites. Similar results were obtained in two additional experiments.

| Probe       | wtV   | V+2   | V+5   | V+10  |
|-------------|-------|-------|-------|-------|
| Sp1         |       |       |       |       |
| NE          | +     | +     | +     | +     |
| rhSp1       |       |       |       |       |
| VcSp1       | +     | +     | +     | +     |
| VmATcSp1    |       |       |       |       |

FIG. 7. The spacing between the AT-rich and GC-rich elements affects activation by Sp1 in transfection assays. HepG2 cells were cotransfected with reporter plasmids containing four tandem repeats of region V sequences as indicated together with the Sp1 expression construct or the empty vector control. Cells were harvested 36 h post-transfection and assayed for luciferase activity. Results were normalized to total protein and then normalized to the pGL3 vector control. Data are the means ± S.E. for at least five separate transfections.

FIG. 8. Nuclear extracts (NE) from insulin-treated cells exhibit stronger facilitation of rhSp1 binding. CHO-IR cells were incubated in F-12 medium with or without 10⁻⁶ M insulin for 24 h, and nuclear extracts were prepared. Gel shifts were conducted using the wtV probe and trace amounts of either the nuclear extracts, rhSp1, or both. Similar results were obtained in three additional experiments using different nuclear extract preparations.
Facilitation of Sp1 Binding to the IGF-I Gene

recruitment of nuclear factors that contribute to the transcriptional machinery may have more stringent requirements than facilitation of Sp1 DNA binding alone. Furthermore, a physical interaction between Sp1 and a factor such as IRBP is supported by the observation that facilitation of Sp1 binding to region V is greater with nuclear extracts from insulin-treated cells than with extracts from untreated cells.

Sp1 has been implicated in the expression of a large number of genes, most often in conjunction with other transcription factors that provide specificity. For example, stimulation of gene transcription by the sterol response element binding protein, which mediates the expression of a number of genes involved in cholesterol biosynthesis and uptake and fatty acid synthesis (reviewed in Ref. 29), often requires the presence of additional factors, including Sp1 (30–32). In addition, Sp1 has been shown to cooperate with a plethora of other transcription factors such as API, E2F, c-Jun, NF-Y, Elk-1, and the Smad proteins to control the expression of a diverse group of genes (19, 20, 21, 34). Thus, Sp1 appears to have a wide role as a cofactor in the transcriptional regulation of many genes; our data support the hypothesis that IGF-I is another example of such a gene.

The consensus binding site for Sp1 consists of the sequence 5’-CCGCCCC-3’ (16), whereas the Sp1 binding site within region V contains the sequence 5’-CTGGCCC-3’. We hypothesize that this 1-bp difference from the consensus site may render Sp1 binding and activity more dependent on the presence of a factor(s) such as IRBP, which binds to the neighboring AT-rich element; when insulin levels are high, IRBP binding at the AT-rich element could promote interactions with Sp1 and stimulate IGF-I expression. Consistent with our hypothesis, we observed greater binding of Sp1 to a region V probe containing a consensus Sp1 site as compared with the wild type sequence, and although facilitation of Sp1 binding by nuclear extracts was still observed with the VcSp1 probe, facilitation was less than with the VtSp1 probe. It is possible that nuclear extracts could facilitate Sp1 binding by altering the status of Sp1 phosphorylation. Depending on the gene, phosphorylation can result in either increased or decreased binding to DNA probes (35–38). Daniel et al. (33) demonstrate that Sp1 is involved in glucose-mediated regulation of the acetyl-CoA carboxylase gene and that dephosphorylation of Sp1 upon glucose treatment results in greater DNA binding and transcriptional activation. To test the possibility that facilitation in the present studies reflects changes in Sp1 phosphorylation, we conducted gel shift studies using nuclear extracts or rhSp1 treated with either alkaline phosphatase or the phosphatase inhibitor okadaic acid. Since we were unable to demonstrate a consistent effect of either phosphorylation or dephosphorylation on the binding of Sp1 to region V (data not shown), it is unlikely that the potential role of insulin in modulating Sp1 effects on IGF-I transcription involves changes in Sp1 phosphorylation status. However, since some (weaker) facilitation was observed with probes lacking the AT-rich element (Fig. 4), it remains possible that some facilitation may occur via protein-protein interactions and/or post-translational modifications that do not involve binding to the AT-rich element.

Although our data are suggestive, we do not know whether the facilitation of Sp1 binding to region V is due to IRBP or instead reflects the contributions of another factor. However, binding experiments showing that nuclear extracts from CHO-IR cells treated with 10−6 M insulin for 24 h provide greater facilitation than nuclear extracts from untreated cells (Fig. 8) are operationally consistent with a role for IRBP. The observation of insulin-responsive facilitation combined with insulin-regulated IGF-I transcription and IRBP binding to region V support the need for identification of IRBP for future mechanistic studies.
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