Insulin-like growth factor-binding protein (IGFBP)-5 is a secreted protein that binds to IGFs and modulates IGF actions. IGFBP-5 is also found in the nuclei of cultured cells and has transactivation activity. Here we report the nuclear localization of endogenous IGFBP-5 in mouse embryonic skeletal cells. Chromatin immunoprecipitation experiments indicated that IGFBP-5 interacts with the nuclear histone-DNA complex. Using a series of deletion mutants, the transactivation domain of IGFBP-5 was mapped to its N-terminal region. Intriguingly, the transactivation activity of IGFBP-5 is masked by negative regulatory elements located in the L- and C-domains. Among the other IGFBPs, the N-domains of IGFBP-2 and -3 also had strong transactivation activity, whereas those of IGFBP-1 and -6 had no activity. The IGFBP-4 N-domain had modest activity. Sequence analysis revealed several amino acids in the IGFBP-5 N-domain that are not present in IGFBP-1. The activities of mutants in which these residues were changed to the corresponding IGFBP-1 sequence were determined. Mutations that changed acidic residues to neutral residues (e.g. E8A, D11S, E12A, E30S/P31A, E43L, and E52A) or a polar to a basic residue (e.g. Q56R) significantly reduced transactivation activity. The E8A/D11S/E12A triple mutant and E52A/Q56R double mutants showed further reduced activity. The combinatorial mutants had essentially no transactivation activity. Taken together, our results indicate that there are several conserved residues in the IGFBP-5 N-terminal region that are critical for transactivation and that IGFBP-2 and -3 also have strong transactivation activity in their N-domains.

The insulin-like growth factor (IGF) system, consisting of two ligands (IGF-I and -II), two receptors (the IGF-I receptor and IGF-II receptor), and six high affinity IGF-binding proteins (IGFBPs), converges on a conserved signaling pathway that plays fundamental roles in vertebrate development and physiology and is implicated in several human diseases (1). The bioavailability and bioactivity of IGFs are regulated by their interactions with various members of the IGFBP family. IGFBPs all have a highly cysteine-rich N-terminal (N)-domain, a cysteine-rich C-terminal (C)-domain, and a middle linker (L)-domain with no cysteine residues except in IGFBP-4. The N- and C-domains are highly conserved within the IGFBP family, whereas the L-domain varies both within the family and across species.

Besides binding to IGF and modulating its actions, IGFBP-5 has been reported to regulate cell proliferation (2, 3), migration (4–6), and apoptosis/survival (7–11) independent of IGF. Although the ligand-dependent actions of IGFBP-5 are attributed to its interactions with the IGF ligand and other proteins (1), the mechanistic basis of the ligand-independent actions of IGFBP-5 is not yet understood.

Andress et al. (12) used IGFBP-5 affinity chromatography to purify a 420-kDa membrane protein from human osteoblast cells and proposed that it was an IGFBP-5 receptor. The same study reported that IGFBP-5 stimulated serine/threonine phosphorylation of this putative receptor in vitro, which in turn phosphorylated casein, a known serine/threonine kinase substrate. However, the molecular nature of this putative IGFBP-5 receptor remains elusive, and whether this protein is present in other cell types is unknown.

An additional mechanism underlying the actions of IGFBP-5 is suggested by its localization in the nucleus. When added to cultured human bone tumor and breast cancer cells, exogenous IGFBP-5 has been shown to be capable of cellular and nuclear entry (13). Likewise, a peptide corresponding to the nuclear localization sequence of IGFBP-5 (residues 201–218) fused to enhanced green fluorescent protein and transfected into Chinese hamster ovary cells targeted enhanced green fluorescent protein to the nucleus (14). These experimental observations are consistent with the presence of a consensus nuclear localization sequence in IGFBP-5 and IGFBP-3 (15). It has also been reported that IGFBP-5 may interact with Four and a Half LIM protein 2 (FHL2) and retinoid X receptor, nuclear proteins known to be involved in transcriptional regulation (16, 17). Recently, we have shown that 1) endogenous IGFBP-5 is localized in the nuclei of cultured porcine vascular smooth muscle cells; 2) several basic residues in the IGFBP-5 C-domain are necessary and sufficient for nuclear localization of the intact protein; and 3) the IGFBP-5 N-domain activates transcription independent of IGF (18). These findings suggest that IGFBP-5 is present in the nucleus and may affect gene expression independent of the IGF ligand.

The objectives of this study are to 1) determine whether IGFBP-5 interacts with the histone-DNA complex; 2) define the IGFBP-5 transactivation domain and determine the specific amino acids critical for its transactivation activity; and 3) to investigate whether other IGFBPs have similar transactivation activity. Our results indicate a physical association of IGFBP-5 with the histone-DNA complex in the nucleus. We have identified several key residues in the IGFBP-5 N-terminal region that are critical for its transactivation activity. In addition to IGFBP-5, the N-domains of IGFBP-3 and IGFBP-2 also possess strong transactivation activity.
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EXPERIMENTAL PROCEDURES

Materials—All chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA) unless noted otherwise. Fetal bovine serum, Dulbecco’s modified Eagle’s medium, penicillin, streptomycin, and trypsin were purchased from Invitrogen. The IGFBP-5 polyclonal antibody raised in guinea pig was a generous gift from Dr. David R. Clemmons, University of North Carolina at Chapel Hill. The M2 anti-FLAG antibody was purchased from Sigma, and Cy3-conjugated second antibodies were from Jackson ImmunoResearch (West Grove, PA).

Plasmid Constructs—To generate Gal4DNA binding domain (DBD) and IGFBP fusion protein constructs, DNA fragments encoding various portions of human IGFBP-1, -2, -4, -5, and -6 and bovine IGFBP-3 were generated by PCR and subcloned into the BamHI/NotI sites of the pBind vector (Promega, Madison, WI) to fuse the IGFBP in-frame to the C terminus of Gal4DBD. C-terminal deletion or point mutants were generated by PCR using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). For this, pBind-IGFBP-5 or pBind-IGFBP-5N was used as template. 0.2 mM of each PCR primer, 0.2 mM of dNTP, 2.5 units of Pfu Turbo DNA polymerase, and Pfu DNA polymerase reaction buffer were used to mutagenize the template DNA by PCR (95 °C for 1 min; 95 °C for 50 s, 60 °C for 50 s, and 68 °C for 7 min, for 18 cycles; and then 68 °C for 7 min) in a total volume of 10 μl.

To generate the IGFBP-5:FLAG expression construct, DNA encoding mature human IGFBP-5 was amplified by PCR (forward primer: ATGGCGGCGCAACCTAGGCACTGGCTCTTCTGGTCAC; reverse primer: TAGGATCCCTATCGTCGTCATCTTGATTCCTCAACGTTGCTGCTGTC), and subcloned into pCMV-tag1 (Stratagene) using ATGGATCCACCATGCTCAACGAAAAGC (forward primer) and TAGGATCCCTATCGTCGTCATCTTGATTCCTCAACGTTGCTGCTGTC (reverse primer), and subcloned into pCMV-tag1 using the BamHI site. The purified plasmids were sequenced at the University of Michigan DNA Sequencing Core Facility.

Cell Culture and One-hybrid Transcription Activation Assay—Human embryonic kidney (HEK) 293 cells and U2 osteosarcoma (OS) cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin in a humidified-air atmosphere containing 5% CO2. For transfection, 0.5 × 105 HEK293 cells were seeded into each well of 6-well plates (Falcon, Corning, NY). 500 ng of pBind or pBind-IGFBP DNA and 500 ng of Gal4 reporter (pG5-luc) DNA were transfected into cells as described previously (18). For co-transfection experiments, a mixture of pBind or Gal-IGFBP-5N (500 ng) and pCMV-tag1, IGFBP5-FLAG, or IGFBP5L:FLAG. One day after the transfection, the cells were washed three times with phosphate-buffered saline, fixed in 4% paraformaldehyde in phosphate-buffered saline for 30 min, and blocked with 0.5% bovine serum albumin plus 0.1% Triton X-100 for 1 h at room temperature. The cells were then incubated with a mouse anti-FLAG antibody (Sigma) in blocking buffer at 4 °C overnight. After washing, the cells were incubated with Cy3-conjugated anti-mouse IgG (Jackson ImmunoResearch) in blocking buffer for 2 h at room temperature. The cells were washed, counterstained with 0.5 μg/ml 4’,6-diamidino-2-phenylindole, and examined under a fluorescence microscope.

Chromatin Immunoprecipitation Assays—To determine the possible association of IGFBP-5 with the histone-DNA complex, wild-type or U2OS cells stably transfected with the FLAG:IGFBP-5 construct were fixed in fresh 5 mM dimethyl-3,3’-dithiobispropionimidate-2HCl for 30 min at room temperature. After rinsing with 100 mM Tris-HCl and 150 mM NaCl, the cells were further fixed in 1% formaldehyde/phosphate-buffered saline for 10 min at 37 °C and lysed using nuclear lysis buffer (50 mM Tris-Cl, pH 8.1, 10 mM EDTA, 1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). After breaking the chromatin into average length of 2 kb by using a sonicator (Fisher Scientific model 60), the cell lysates were centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was diluted 10-fold using ChIP dilution buffer (0.01% SDS, 0.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl, pH 8.1, and 167 mM NaCl) and precleared using 20 μl of protein G-agarose (Upstate, Lake Placid, NY). Subsequently, 2 μg of anti-FLAG antibody (Sigma), anti-histone H3 antibody (Abcam, Cambridge, MA), mouse IgG, or rabbit IgG (Jackson ImmunoResearch) was added. After incubation overnight at 4 °C, 20 μl of protein G beads was added, and the mixture was incubated for another 2 h. The beads were spun down and rinsed twice. The immunoprecipitated complexes were eluted by boiling in Laemmli buffer and analyzed by Western immunoblot using the anti-FLAG or anti-Histone H3 antibody.

Statistical Analysis—Values are presented as mean ± S.E. Differences between groups were analyzed by one-way analysis of variance followed by Fisher’s protected least significance difference test using Statview (Abacus Concept, Inc.). Statistical significance was defined as p < 0.05.

RESULTS

IGFBP-5 Is Physically Associated with the Histone-DNA Complex in the Nucleus—To determine whether endogenous IGFBP-5 is present in the nucleus in an in vivo setting, immunocytochemical analysis was performed in mouse embryo costal cartilage sections using a specific IGFBP-5 polyclonal antibody. The sections were counterstained by SYTO13, a nuclear dye. As shown in Fig. 1A, IGFBP-5 immunoreactivity is clearly detected in the nuclei, indicating that endogenous IGFBP-5 is localized in the nuclei of cartilage cells in mouse embryos. To investigate whether IGFBP-5 interacts with DNA in the nucleus, ChIP assays were carried out in U2OS cells stably transfected with FLAG:
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IGFBP-5 When introduced into U2OS cells, FLAG:IGFBP5 signal was detected exclusively in the nucleus, co-localized with the histone H3 signal (Fig. 1B). As shown in the upper panel in Fig. 1C, the anti-histone H3 antibody was able to immunoprecipitate FLAG:IGFBP-5 (lane 4) in the FLAG:IGFBP-5 transfected but not the wild-type control cells (lane 8). This interaction was specific, because neither rabbit IgG nor mouse IgG was able to immunoprecipitate FLAG:IGFBP-5 (lanes 5 and 6) and the anti-FLAG antibody was able to pull down IGFBP-5 only in the transfected cells (lane 3). Likewise, the anti-FLAG antibody specifically pulled down histone 3 (Fig. 1C, lower panel) in the transfected cells (lane 4) but not in the control cells (lane 8). These results suggest that IGFBP-5 is localized in the nucleus and that the nuclear IGFBP-5 is physically associated with the histone-DNA complex.

IGFBP-5 Transactivation Domain Is Located in the N-terminal Region and Is Masked by Negative Regulatory Elements in the L- and C-domains—To map the location of the transactivation domain in human IGFBP-5, a series of truncated IGFBP-5 fragments were generated and fused to Ga4DBD. The transactivation activities of these fusion proteins were measured after transfection into HEK293 cells together with a Ga4 reporter construct. As shown in Fig. 2, the human IGFBP-5 N-domain (IGFBP-5-(1–80)) fusion protein caused a Ga4-dependent transactivation 21.72 ± 1.07-fold greater than the pBind control group ($n = 5$, $p < 0.01$). pBind-IGFBP-5-(1–60) caused a similar increase (26.27 ± 1.49, $n = 5$, $p < 0.01$). This activity is evolutionarily conserved because zebrafish IGFBP-5 N-domain, but not human or zebrafish IGFBP-1 N-domain, had significant transactivation activity (18). Intriguingly, the longer fragments of IGFBP-5-(1–100) and IGFBP-5-(1–120) only had modest transactivation activity (5- to 6-fold), whereas IGFBP-5-(1–169) had no transactivation activity. Western immunoblot analysis confirmed the expression of these fusion proteins (Fig. 2B).

When the activity of full-length human IGFBP-5-(1–252) was tested, it had no transactivation activity. In fact, it significantly reduced the reporter activity to 0.17 ± 0.02-fold, equating to a 5.9-fold repression ($p < 0.01$, Fig. 3A). These data suggest that the IGFBP-5 transactivation domain is located within the N-terminal 60 amino acids and that transactivation activity is suppressed or masked by negative regulatory elements located in the L- and C-domains. To test this idea further, IGFBP-5:FLAG and IGFBP-5:FLAG-L+C constructs were generated. When introduced into HEK293 cells, IGFBP-5:FLAG and IGFBP-5:FLAG-L+C were detected exclusively in the nucleus (Fig. 4A). As shown in Fig. 4 (B and C), nuclear expression of neither IGFBP-5:FLAG nor IGFBP-5:FLAG-L+C significantly inhibited the activity of pBind-IGFBP-5N.
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Several Conserved Residues in the IGFBP-5 N-terminal Region Are Important for Its Transactivation Activity—Although the N-domains of IGFBP-5 and -1 share high sequence identity, only the N-domain of IGFBP-5 has transactivation activity, and this activity is evolutionarily conserved (Ref. 18; see also Fig. 7). Taking advantage of this finding, we compared the amino acid sequences of these two highly homologous proteins to map the sequence determinants of the transactivation activity of the IGFBP-5 N-domain. Transactivation domains are often rich in acidic (20–22) or proline residues (23, 24). Sequence analysis of the N-terminal region of IGFBP-1 and -5 revealed several conserved proline and acidic residues unique to IGFBP-5 (Fig. 5A). For instance, Pro31 and Pro33 are conserved between human and zebrafish IGFBP-5, but are not present in human IGFBP-1. Pro22 in particular is located in a cluster of three proline residues, Pro19-Pro20-Ser21-Pro22. Several acidic residues, including Glu8, Asp11, Glu30, and Glu43, are found in human and zebrafish IGFBP-5, whereas the corresponding residues in human IGFBP-1 are neutral (Fig. 5A). Likewise, Glu42 and Glu43 in human IGFBP-5 are neutral amino acids in IGFBP-1. A polar amino acid in IGFBP-5, Gln56, corresponds to the basic Arg in IGFBP-1 (Fig. 5A).

To test whether any of these residues unique to human IGFBP-5 are critical for its transactivation activity, we mutated each to the corresponding human IGFBP-1 sequence. The effects of these mutations on transactivation activity are shown in Fig. 5B. Mutation of Pro22 to Ser did not decrease transactivation activity, suggesting that this proline residue play little role in the transactivation activity. In contrast, mutation of Glu8 to Ala significantly decreased transactivation to 71.0 ± 7.1% of that of the wild-type IGFBP-5 N-domain (n = 5, p < 0.01). The transactivation activities of mutants D11S, E12A, E43L, and E52A were also significantly reduced relative to wild-type IGFBP-5 N-domain (all p < 0.01, Fig. 5B). Mutation of the non-conserved Glu9 to Ser caused a modest reduction in transactivation activity, but this reduction did not reach statistical significance. Mutation of the polar amino acid Gln56 to Arg strongly reduced transactivation activity (46.4 ± 3.3%, n = 5, p < 0.01). These data indicate that several acidic residues and Gln56 are important for transactivation. Western immunoblot analysis revealed that expression levels of these mutant fusion proteins were similar (Fig. 5A), thus excluding the possibility that these changes were due to different levels of protein expression or degradation.

We next generated several double and triple mutants. The E30S/P31A mutant had significantly reduced activity compared with that of the wild-type IGFBP-5 N-domain (79.4 ± 11.3%, n = 5, p < 0.01, Fig. 5B). The transactivation activities of the E8A/D11S/E12A triple mutant and E52A/Q56R double mutant were 19.3 ± 0.9% and 19.8 ± 1.4% of that of the wild type IGFBP-5 N-domain (both p < 0.01, n = 3, Fig. 6A). These values are significantly lower than those of the corresponding single mutations (p < 0.05). These results suggest that residues Glu9, Asp11, Glu30, Glu43/Pro31, Glu43, Glu56, and Gln56 are important for transactivation activity. The combinatorial effects of mutating the acidic

FIGURE 3. IGFBP-5 L- and C-domains have transcriptional repression activity. A, full-length (hBPS5), L- (hBPSL), and C-domain (hBPCS) of human IGFBP-5 were fused to the Gal4DBD and introduced into HEK293 cells together with a Gal4 reporter plasmid by transient transfection. Transcriptional activity was determined and the transcriptional repressor activity is expressed as fold decrease over the pBind control group. Values are expressed as means ± S.E. (n = 3), * p < 0.01 compared with the pBind control group. Expression of the FLAG-tagged IGFBP-5 constructs was confirmed by immunoblotting and shown in B.

FIGURE 4. Nuclear expression of IGFBP-5:FLAG or IGFBP-5L + C:FLAG does not affect the transcriptional activity of the IGFBP-5 N-domain. A, nuclear localization of IGFBP-5:FLAG and IGFBP-5L:FLAG. Cells were transfected with pCMV-tag1, IGFBP5:FLAG, or IGFBP5LC:FLAG plasmids, respectively. One day after transfection, these cells were subjected to immunocytochemical staining using an anti-FLAG antibody. B, cells were co-transfected with the Gal4 reporter plasmid and the plasmids indicated. The transcriptional activity was determined as described under “Experimental Procedures.” Values are means ± S.E. (n = 3), * p < 0.01 compared with the pBind control group. Expression of the FLAG-tagged IGFBP-5 constructs was confirmed by immunoblotting and shown in C.
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A. Sequence alignment of the N-terminal region of human IGFBP-5 (hBP5), zebrafish IGFBP-5 (zBP5), and human IGFBP-1 (hBP1). The unique amino acids that were studied by mutagenic analysis are indicated.  

B. Relative transactivation activities of various single or double mutants compared with that of the wild-type IGFBP-5 N-domain. 

C. Expression levels of these mutant proteins (Fig. 6B). These mutants acted like the IGFBP-1 fusion protein in that they had essentially no transactivation activity (E8A/D11S/E12A/E43L: 7.8%/H110061.5%; E43L/E52A/Q56R: 9.5%/H110062.0%; E8A/D11S/E12A/E43L/E52A/Q56R: 5.2%/H110060.9%, all n = 5). Again, no marked differences were found in the expression levels of these mutant fusion proteins were analyzed by immunoblotting using an anti-Gal4DBD antibody and are shown in C.

Transactivation Activity of Other IGFBPs—Because nuclear localization has also been reported for IGFBP-3 and -2 (13, 14, 25–27), we speculated that IGFBPs other than IGFBP-5 may possess similar transactivation activity. We therefore investigated the activity of the other five members of the IGFBP gene family. As shown in Fig. 7A, the bovine IGFBP-3 N-domain caused a significant 17.39 ± 0.62-fold (p < 0.01, n = 3) increase over the pBind control. This activity was essentially the same as that of the human IGFBP-5 N-domain (17.44 ± 2.31, n = 5). Like IGFBP-5, the transactivation activity of IGFBP-3 appeared to be conserved, because zebrafish IGFBP-3 had strong activity (data not shown). Human IGFBP-2 N-domain also showed significant activity (10.90 ± 1.77-fold, n = 5, p < 0.01 compared with pBind control). In contrast, no activity was observed for human IGFBP-1 N-domain (1.70 ± 0.38-fold, n = 5) or IGFBP-6 N-domain (1.12 ± 0.26-fold, n = 5). The N-domain of human IGFBP-4 had modest transactivation activity (6.04 ± 0.98-fold, n = 5, p < 0.05). These results indicate that IGFBP-5, -3, -2, and possibly -4, but not IGFBP-1 or -6, possess a transactivation domain in their N-domains.

DISCUSSION

Ligand-independent effects of IGFBP-5 on cell growth, migration, and apoptosis/survival have been documented in a number of cell types (2–11). Recently we have shown that the conserved IGFBP-5 N-domain contains a functional transactivation domain, and this transactivation activity is IGF-independent (18). The present study has provided new experimental evidence for the nuclear presence of endogenous IGFBP-5 in mouse embryos in vivo. To our knowledge, this is the first time that nuclear localization of IGFBP-5 has been demonstrated under a physiological context. Our ChIP assay results further indicate that nuclear IGFBP-5 is associated with the histone-DNA complex. In U2OS cells stably transfected with FLAG:IGFBP-5, FLAG:IGFBP-5 can be co-precipitated with histone H3 and vice versa. Because dimethyl-3, 3′-dithiobispropionimidate-2HCl and formaldehyde treatments cross-link both protein-DNA and protein-protein, IGFBP-5 may interact with histone H3 directly or indirectly through other proteins or DNA. These new observations, together with the fact that IGFBP-5 has a functional transactivation domain, argue that IGFBP-5 may be involved in transcription regulation in the nucleus. This proposition is in good agreement with recent reports showing that IGFBP-5 interacts with several transcriptional regulators such as FHL2, retinoid X receptor, and retinoic acid receptor (16, 17).

We have mapped the transactivation activity of IGFBP-5 to its N-terminal region. Because proline-rich sequences have been associated with transactivation domains (23, 24), we examined the potential role of the conserved Pro22 in IGFBP-5. Substitution of this residue did not change the transactivation activity. Likewise, mutation of Pro22 had little impact (data not shown). Many well defined transcriptional activators are rich in acidic residues (28–30). Our study suggests that the IGFBP-5 N-
domain has several acidic residues that are important for transactivation. These include Glu, Asp, Glu, Glu, and Glu. Changing one or more of these residues into corresponding sequence in IGFBP-1 significantly decreased the transactivation activity of the IGFBP-5 N-domain. The E265 mutant also had reduced activity, although the reduction did not reach statistical significance. Mutation of Glu/Pro significantly decreased transactivation activity; it is unclear which of these two residues is responsible for the effect, because we did not mutate them individually. In addition to these acidic residues, changing the neutral, polar Gln to basic Arg also reduced the transactivation activity. The importance of these residues is supported by the abolition of transactivation activity in the combinatory mutants E8A/D11S/E12A/E43L, E43L/E52A/Q56R, and E8A/D11S/E12A/E52A/Q56R. Reduction of transactivation activity did not appear to be due to a change in overall acidity, because the IGFBP-6 N-domain is the most acidic among the six IGFBPs, but it has no transactivation activity. Although most of these critical residues are conserved between human and zebrafish IGFBP-5, three of them, i.e. Glu, Glu, and Gln, are not conserved in zebrafish. This may explain our previous observation that the zebrafish IGFBP-5 N-domain has considerably weaker activity than its human counterpart when tested in human cells (18).

The major IGF binding site in the N-domain of IGFBP-5 is located in the so-called mini-IGFBP-5 region (amino acids 40–92) (31). In particular, several hydrophobic or charged residues in this region, including Arg, Pro, Leu, and Leu, are critical for IGF binding (32). These are clearly distinct from the residues that are critical for the transactivation activity identified in this study. Indeed, mapping of Glu, Glu, and Gln onto the surface of mini-BP-5 clearly demonstrates spatial separation of these residues from the IG binding site (Fig. 8A). Although Glu, Glu, and Gln are required for transactivation activity, we believe that these residues alone are not sufficient to confer it, because miniBP-5 itself had no transactivation activity (18). All other critical residues, e.g. Glu, Asp, Glu, and Glu/Pro, are located in the N-terminal 1–39 region, which was originally thought not to be involved in IGF binding (31). However, a recent crystal structure of the complete N-domain of IGFBP-4 in complex with IGF-I shows that this region does contribute to IGF-I binding (33). Mapping of the equivalents of Glu, Asp, Glu, and Glu/Pro onto the surface of the N-domain of IGFBP-4 demonstrates the presence of a surface patch that includes the equivalent of Glu (52). Although the equivalents of Glu and Gln are not immediately adjacent to the other residues, they are nearby, and all of them are spatially removed from the IGF binding site (Fig. 8B). These structural analyses are consistent with our functional data showing that mutation of the key residues responsible for IGF binding did not alter the transactivation activity of the IGFBP-5 N-domain (18).

The finding that the N-domains of IGFBP-2 and -3 have strong transactivation activity is interesting, because these two IGFBPs have also been reported to localize to the nucleus (13, 25, 26). Within the IGFBP family, IGFBP-3 is most closely related to IGFBP-5 in structure, and IGFBP-3 interacts with transcription factors such as retinoid X receptor and retinoic acid receptor within the nucleus (34). The N-domain of IGFBP-3 is ~60% identical to that of IGFBP-5. Most all of the critical residues found in the human IGFBP-5 N-domain, including Glu, Asp, Glu, and Glu, are conserved in bovine IGFBP-3. The N-domain of IGFBP-2 is only 43% identical to that of IGFBP-5, but several of the critical amino acids that we identified, such as Glu, Glu, and Gln, are found in IGFBP-2. The N-domains of IGFBP-1 and -6 have the least sequence similarity to IGFBP-5 among the family members (37 and 32%, respectively), and these two IGFBPs have no transactivation activity. However, overall sequence identity is not a reliable indicator of transactivation activity, because the human IGFBP-4 N-domain is 48% identical to that of IGFBP-5, but only has modest activity.
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Among these critical residues that we identified in human IGFBP-5 N-domain, only Glu135 and Glu139/Pro141 are present in the N-domain human IGFBP-4, and almost none are present in the N-domain of human IGFBP-1 and -6. Our finding that the N-domains of IGFBP-5, -3, and -2, three IGFBPs capable of nuclear localization, all have transactivation activity raises the possibility that these IGFBPs may regulate gene expression directly or indirectly in the nucleus. It should be pointed out, however, that our results only show that the N-domains of these IGFBPs activate the GAL-4 reporter gene when fused to GALA-DBD. More studies are needed to determine whether endogenous IGFBPs have similar activity, and, more importantly, to identify their target genes.

Another intriguing finding made in this study is that, while the N-domain of IGFBP-5 has strong transactivation activity, full-length human IGFBP-5 does not. In fact, full-length IGFBP-5 suppresses transcription. Further analysis revealed that the L- and C-domains of IGFBP-5 each contain strong repression activity. Co-expression of FLAG-tagged full-length IGFBP-5 or the L- and C-domains of IGFBP-5 in the nucleus had no effect on the transactivation activity of the N-domain of IGFBP-5. These data indicate the presence of negative regulatory elements in the IGFBP-5 L- and C-domains and that the transactivation activity of IGFBP-5 is suppressed or masked by these negative regulatory elements, likely through intramolecular interaction(s). At present, it is not clear how the transactivation activity is regulated in IGFBP-5. One possibility is proteolytic cleavage. Stat6 and Stat5a can be cleaved in the nucleus (35–37), and several other transcription factors, such as ATF-1, Sp1, NF-YA and -B, and octamer-binding proteins Oct-1 and Oct-3, are proteolyzed by differentiation-associated nuclear protease in embryonic carcinoma cells (38). IGFBP-5 proteolysis has long been considered an important regulatory mechanism of extracellular IGFBP-5 functions. In many cellular systems, IGFBP-5 is proteolized into a 21-kDa N-terminal fragment that has low affinity for IGFs (39) and other smaller fragments. Two major cleavage sites are located at residues K138/K139 and S143/L144 in the L-domain (40–42). However, our ChIP assay results suggest that N-terminally tagged full-length IGFBP-5 is associated with the histone-DNA complex. In addition, two Gal4DBD constructs encoding the natural 21- to 22-kDa IGFBP-5 proteolytic fragments had no transactivation activity, suggesting that proteolytic cleavage at Lys138/Lys139 and Ser143/Leu144 may not play any major role in unmasking the transactivation activity of IGFBP-5.

Other type(s) of post-translational modification, such as glycosylation or phosphorylation, may also play a role in modulating the transcriptional activity. It has been reported that human vitamin D receptor is phosphorylated at Ser182 leading to an attenuation of its transactivation activity (43). Phosphorylation of IGFBP-3 has been shown to be involved in its nuclear translocation as well (44). By searching for the potential phosphorylation sites using the NetPhos 2.0 server (www.cbs.dtu.dk/services/Netphos/) (45), several putative phosphorylation sites can be identified in the L- and C-domains of IGFBP-5. It is plausible that the phosphorylation states of these residues could modulate the transactivation activity of IGFBP-5. Alternatively, molecular interactions with other nuclear proteins, DNA, or RNA, may play a regulatory role. For instance, the IRS-3 C-terminal region has transcriptional activity that can be unmasked when it binds to Bcl-2 via its N-terminal domain (46). Although our recent yeast two-hybrid screen for IGFBP-5 binding partners in vascular tissues did not yield any transcription factors (18), a recent study reported direct protein-protein interactions between IGFBP-5 and FHL2 (16) in bone cells. It is known that FHL2 can act both as a co-activator and co-repressor depending on binding partners and/or cell type (47, 48). Co-expression experiments will be needed to test whether FHL2 can act as a co-regulator of IGFBP-5 or vice versa.

In summary, this study demonstrates that IGFBP-5 is present in a complex containing histone and DNA in the nucleus. IGFBP-5 has a functional transactivation domain in its N-terminal region. Several residues in this region, including Glu18, Asp131, Glu135, Glu139/Pro141, Glu143, and Glu148, are critical for its transactivation activity. Changing these residues to the corresponding IGFBP-1 sequence results in significant reduction or complete loss of transactivation activity. We show that the N-domains of IGFBP-3 and IGFBP-2, two other IGFBPs that have been observed in the nucleus, also possess strong transactivation activity and that the IGFBP-4 N-domain has weak activity. To date, most, if not all, IGF-independent actions of IGFBPs have been mapped to their L- or C-domains, and the only role attributable to the N-domain has been IGF binding (49). The present study clearly demonstrates an additional functional domain in this region of IGFBPs. Further, we provide evidence for the presence of negative regulatory elements in the IGFBP-5 L- and C-domains, suggesting that the transactivation activity of IGFBP-5 is biologically regulated by a masking mechanism. Future studies focused on elucidating the unmasking mechanism(s) will provide novel insight into the molecular basis of IGFBP-5 nuclear actions.

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