

## Characterization of Insulin-like Growth Factor-free Interaction between Insulin-like Growth Factor Binding Protein 3 and Acid Labile Subunit Expressed from *Xenopus* Oocytes

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The acid-labile subunit (ALS) is known to interact with the IGF binding protein (IGFBP) in the presence of insulin-like growth factors (IGFs). Studies, however, indicate that ALS forms a doublet with IGFBP3, independent of IGFs. To characterize the structural domain required for the IGF-free ALS-IGFBP3 interaction, seven recombinant human IGFBP3 mutants were generated: three deletion mutants and four site-specific mutants that had altering N-terminal regions of IGFBP3. ALS and IGFBP3 mRNAs were co-injected into *Xenopus* oocytes, and their products were cross-linked and immunoprecipitated using antisera against ALS or IGFBP3. Among the deletion mutants, the mutant of D40 (deleted in 11-40th amino acids) exerted no effect in the interaction with ALS, while D60 ( $\Delta$ 11-60) demonstrated a moderate reduction. D88 ( $\Delta$ 11-88), however, showed a significant decrease. In the case of site-specific mutants, the mutation that altered the IGF binding site (codons 56 or 80) exerted a significant reduction in the interaction, whereas codons 72 or 87 showed no significant change in the interaction with ALS. The stability of the ALS-IGFBP3 interaction was analyzed according to a time-dependent mode. Consistent with the binding study, mutants on the IGF binding sites (56 or 80) consistently show a weakness in the ALS-IGFBP3 interaction when compared to the mutants that covered the non-IGF binding sites (72 or 87). This study suggests that the N-terminal of IGFBP3, especially the IGF binding site, plays an important role in interacting with ALS as well as in stabilizing the dual complex, independent of IGFs.

**Keywords:** Acid-labile subunit, IGF Binding Protein 3, Insulin-like growth factor, Protein interaction, *Xenopus* oocyte

### Introduction

The insulin-like growth factors (IGFs) and their binding proteins (IGFBPs) circulate in the vascular system as ternary complexes with the acid-labile subunit (ALS), a glycosylated 85-kDa member of the leucine-rich repeat superfamily. ALS associates with the insulin-like growth factor (IGF)-I or -II and with the IGF binding protein-3 (IGFBP-3) to form a 150-kilodalton complex in the circulation. As a component within the high molecular complex, IGFs can remain in the pool of growth factors for tissues without their potential hypoglycemic effects (Baxter and Dai, 1994). This complex may regulate the serum IGFs by restricting IGFs in the vascular system and prolonging their endocrine actions (Firth *et al.*, 1998).

ALS is known to interact with the IGF binding protein (IGFBP) in the presence of IGFs. Recent reports, however, showed that IGFBP and ALS form a dual complex that is free of IGFs (Lee and Rechler, 1995; Hashimoto *et al.*, 1997; Choi and Lee, 2002) and that IGF-free ALS-IGFBP complexes are plentiful in adult rat serum (Lee and Rechler, 1996). Although the IGF-dependent interaction between ALS and IGFBP has been extensively studied, little is known about how IGFBP directly interacts with ALS to form a dual complex, regardless of IGFs. The human IGFBP group consists of six members named IGFBP1 to IGFBP6, among which IGFBP-3 is the most abundant. IGFBP-3 is composed of N- and C-terminal conserved regions and a highly variable central region (Ueki *et al.*, 2000). The C-terminal conserved region is believed to harbor major structural determinants that are required for binding to ALS (Firth *et al.*, 1998). Studies using truncation variants, however, indicate that the C-terminal deleted IGFBP3 still binds to ALS. This implies that additional binding points may exist in other regions.

In the circulatory system, a significant fraction (more than 10%) of IGFBP3-ALS complexes is found as IGFs-free (Firth *et al.*, 1998). These doublets probably recruit IGFs poorly,

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therefore, the storage function of each binding protein would be greatly hampered (Lee and Rechler, 1996). Increasing the fraction of the IGF-free binary complex might even represent pathological ramifications. Under these circumstances, this study is to focus upon the mechanism concerning the interaction between IGFBP3 and ALS that are independent of IGFs.

This study's focus is the significance of the N-terminal consensus region in binding with ALS under IGF-free conditions. The central domain of IGFBPs exhibits low homology among members and contains cleavage sites for specific proteases. IGFBP-3 was mutagenized to generate three deletion and four site-specific variants. For this study, we utilized *Xenopus* oocyte since they have demonstrated efficiency and fidelity in the translation, processing, and functional expression of foreign proteins (Melton *et al.*, 1984). *Xenopus* oocytes resemble plant seeds, considering a very inert system not translating internal messages until the embryonic stage of midblastula (Shih *et al.*, 1979). This translational inertness makes it feasible to exclusively express injected messages, which enables the study of the interaction between foreign proteins. Using the *Xenopus* oocyte system, this study investigates whether the N-terminal consensus region in IGFBP3 plays an important role in the intermolecular interaction with ALS to form dual complexes under IGF-free conditions.

## Materials and Methods

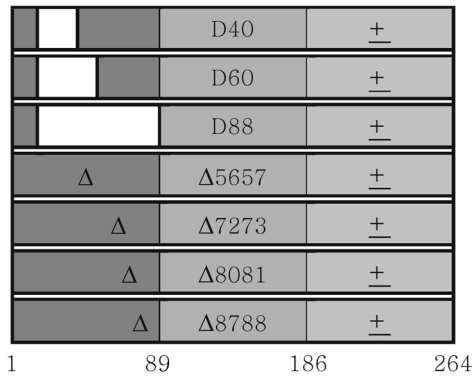
**Production of Synthetic mRNAs** The synthetic mRNAs of IGFBP3 and ALS were prepared from pSP6 harboring respective cDNA according to Ceriotti *et al.* (1991). To prepare a mutant IGFBP3, human recombinant IGFBP3 cDNA was inserted into the EcoRI site of pAlter (Promega, Madison, USA) for mutagenesis. For N-terminal deletion mutants, 5 primers containing *accagaactctcc*, *aagaaggattttat*, and *gacgtgcactgctac* were used to delete codons 11-40, 11-60, and 11-88, respectively. The resulting mutants were named D40, D60, and D88. To generate a site-specific mutant, the 56-57(I-Y), 72-73(D-E), 80-81(L-L), and 87-88(C-V) codons were altered to *S-A*, *N-G*, *S-S*, and *W-A*, respectively. The altered sequences were confirmed by sequencing and the mutagenized cDNA was inserted into pSP6. Each construct was accordingly named pSPA56, pSPA72, pSPA80, and pSPA87. To abolish the known ALS binding site in the C-terminus, codons 228-232 (KGRKR), a 15mer of AtgGcGgGgAGgcG, was used to generate a MDGEA amino acid sequence in the C-terminus. The binding study utilized double mutant altering, both at the N-terminus region and the C-terminus ALS binding site (Fig. 1). For the stabilization experiment, single mutants with an altered N-terminus were employed. For transcription *in vitro*, CAP (m7GpppG) was supplemented in the 10X molar excess of GTP to ensure the effective capping of the RNA transcript during the initial 45-min incubation. GTP was added to the concentration equivalent of CAP. The DNA templates were removed by incubating for 15 min at 37°C with RNase-free DNase I. The RNA transcript was purified by phenol extraction and ethanol precipitation.

**Microinjection** An ovary was manually excised from an adult *Xenopus*. The ovary was copiously washed in OR2 (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, 3.8 mM NaOH, 1.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 5.0 mM HEPES, pH 7.8) and incubated in fresh OR2 at 14°C until injection. Stage V-VI oocytes were manually defolliculated with a pair of fine forceps and injected with 50 nl of an injection mixture containing mRNA and labeling isotope, [<sup>35</sup>S]methionine/cysteine by Eppendorf Micromanipulation System. The injected oocytes were incubated in a modified OR2 (MOR2; 25 mM NaCl, 1.0 mM KCl, 1.0 mM CaCl<sub>2</sub>, 2.0 mM NaOH, 1.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 5.0 mM HEPES, pH 7.8) at 20°C.

**Assay on protein synthesis and accumulation** Synthesis of IGFBP3 or ALS was analyzed according to the accumulation of each labeled protein in the medium. The oocytes were injected with synthetic mRNA (25 ng) and [<sup>35</sup>S]met/cys (0.5 mCi; 1170 Ci/mmol) in a 50 nl injection mixture. The injected oocytes were incubated in MOR2. The IGFBP3 accumulation in the medium was measured at 0, 1, 2, 3, 6, and 12 h following injection. The oocytes were chased in MOR2 containing 9.0 mM cold met/cys after 1 h of pulse labeling. The secretion was monitored up to 12 h following chase. Following injection, the oocytes were incubated at 20°C in OR2, followed by immunoprecipitation using antisera against human IGFBP3 (Upstate Biotech, Lake Placid, USA) or an affinity-purified polyclonal antibody against recombinant ALS (IDS Ltd, Boldon, UK), as previously described (Lee *et al.*, 2001). Immunoprecipitation was performed at 15°C for 4 h in a BIPIP buffer (150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 mM Tris-HCl, pH 7.4). The precipitants were separated on a reducing 5% SDS/PAGE and visualized by autoradiography (Brennan *et al.*, 2002). The intensity of the proteins was measured by scintillation, counting each band that was excised from the gel.

**Analysis of IGFBP3-ALS binding in solution** The association between the IGFBP3 and ALS was analyzed in MOR2 media following secretion of IGFBP3 and ALS. For the experiment, injected mRNA included double mutations: the putative C-terminal ALS binding site along with each N-terminal mutation (Fig. 1). The mRNA (25 ng) for ALS or IGFBP3 was injected with [<sup>35</sup>S]met/cys (0.5 mCi) in a 50 nl injection per oocyte at the equator. Typically, 2 oocytes were injected and incubated in 200 µl MOR2 per experiment. As a negative control for the specific interaction, the oocytes were injected with 10 ng ovalbumin (Oval) mRNA along with that of ALS. Following incubation and cross-linking with 0.25 mM disuccinimidyl suberate (DSS, Pierce, Rockford, USA), the IGFBP3-ALS complex was immunoprecipitated by adding antisera against IGFBP3 (Upstate Biotech) in 1:500 dilution. Ovalbumin was immunoprecipitated using affinity-purified polyclonal antibodies (Sigma, St. Louis, USA) against ovalbumin (Kim *et al.*, 2001).

**Stability of ALS-IGFBP3 complex** The stability of the IGFBP3-ALS doublet was analyzed on the basis of time-dependent mass of the dual complex in solution. In contrast to the previous binding assay, this study utilized mutations with an alteration only on the N-terminal region. Thus, the C-terminal consensus region was intact in the mutations that were employed. Following injection and incubation for 3 h in 100 µl MOR2 per oocyte, the oocytes were



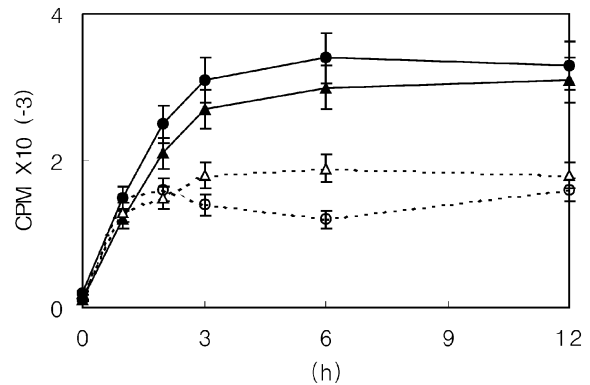
**Fig. 1.** Diagram for mutant constructs. This study generated 3 deletion and 4 site-specific mutations in N-terminus. Each N-terminus mutant is labeled in the central domains. In the three N-terminus deletion mutants (D40, D60, D88), light blocks represent the deleted portions that spanned codons 11-40, 11-60, and 11-88, respectively. Deltas in the N-terminus indicate the sites of the specific mutations while the numbers in the label refer to the two mutagenized codons. The binding experiments employed mutants with the binding sites abolished (-) in the C-terminus; whereas, the stabilization assay utilized the mutants with intact C-terminus (+).

removed and the aliquot of the media was subjected to cross-linking with 0.25 mM DSS. The IGFBP3-ALS complex was then immunoprecipitated using antisera against IGFBP3. The remainder of the media was incubated at 20°C. The Aliquots of the media were sampled at 3 and 6 h time points following a 3-h incubation in MOR2 media. The doublets were probed as previously described.

## Results

This study investigated whether the N-terminus of IGFBP3 plays a role in the IGF-free interaction between IGFBP3 and ALS. More importantly, this study focused on the potential N-terminal structural determinants of IGFBP3 in the intermolecular association between ALS and IGFBP3. Following the secretion of IGFBP3 and ALS that was expressed in the *Xenopus* oocytes into the medium, the interaction of the ALS or IGFBP3 was analyzed according to the ALS-IGFBP3 doublet formation and the stability of the doublet.

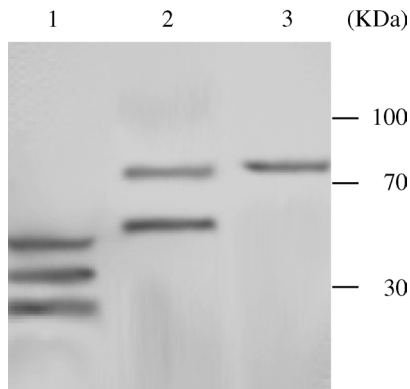
**Accumulation of IGFBP3 in a linear pattern** The rate of protein synthesis and accumulation was determined following the IGFBP3 injection. Figure 2 shows that IGFBP3 variants accumulate in media. Unless injected with foreign messages such as IGFBP3 and ALS, the oocytes do not appear to secrete any significant protein product. Despite the different translational machinery, the oocytes correctly processed IGFBP3 and its variants and secreted the protein product into the media. Following the co-injection of IGFBP3 and ALS mRNAs, the accumulation of labeled IGFBP3 or ALS was measured up to 12 h after the injection. When the rate of



**Fig. 2.** Analysis of IGFBP3 and ALS expression. The accumulation kinetics was studied following injection of IGFBP or ALS mRNA. The media was sampled at 1, 2, 3, 6, and 12 h following injection. Accumulation of labeled protein decreased from 6 hr after injection, both for IGFBP3 (-○-) and ALS (-△-). Following a 1-h incubation, the oocytes were incubated in media containing 100X excess Met/Cys. The labeled portions were significantly leveled off within 1.5 h for IGFBP3 (-●-) and ALS (-▲-).

protein accumulation in media was measured against time, IGFBP3 accumulated at a constant rate once the secretion was detected from the media. The oocytes appeared to synthesize and secrete the proteins in a linear fashion for at least 6 h. A prompt response to the dilution of the labeling [<sup>35</sup>S]met/cys was obvious by a comparison of [<sup>35</sup>S]met/cys incorporation into IGFBP3 between the pulse and chase periods. In the presence of cold met/cys, however, the incorporation of [<sup>35</sup>S]met/cys was significantly reduced within 1/2 h chase, and virtually eliminated within 1 h of chase. This period may represent the time that was taken for the absorption of the cold amino acids and secretion of proteins into the media. This clearly indicates that oocytes synthesize and secrete the injected messages in a linear and a very sensitive mode. Figure 3 shows IGFBP3 and ALS that are found in the media. No difference was apparent in the expression level among the condensed versions of the mutants. When ALS and IGFBP3 were expressed together, the expression level was unaffected by the co-expression.

**N-terminal domain interacting with ALS independent of IGFs** The oocytes were co-injected with the mRNAs of ALS and IGFBP3. ALS and IGFBP3 were expressed with each specific molecular weight and formed a binary complex with varied affinities. Following incubation for 3 h, the media were subjected to cross-linking and immunoprecipitation. Figure 4 shows the interaction between ALS and IGFBP3. In the presence of ALS, lane C shows that the N-terminal intact IGFBP3 still forms ALS-IGFBP3 doublets, despite the mutation on the known binding site for ALS (KGRKR). The level of ALS-IGFBP3 doublet formation appears to be 20% when compared to the wild-type IGFBP3 (lane W). The



**Fig. 3.** Expression of recombinant ALS and IGFBP3s. The mRNAs of IGFBP3 and ALS were injected into oocytes. Lane 1 denotes the proteins that were co-expressed from mRNAs of the three IGFBP3 deletion mutants. From the top, the three bands represent proteins of D40, D60, and D88, respectively. Lane 2 refers to the labeled proteins that were secreted from the oocytes that were co-injected with mRNAs of intact IGFBP3 and ALS. The proteins were immunoprecipitated by anti-IGFBP3, and later by anti-ALS antisera. Lane 3 represents ALS that was immunoprecipitated from the oocytes that were injected singularly with ALS mRNA.

complex is well preserved by cross-linking treatment. Ovalbumin did not bind with ALS, which was different than the IGFBP3-ALS interaction.

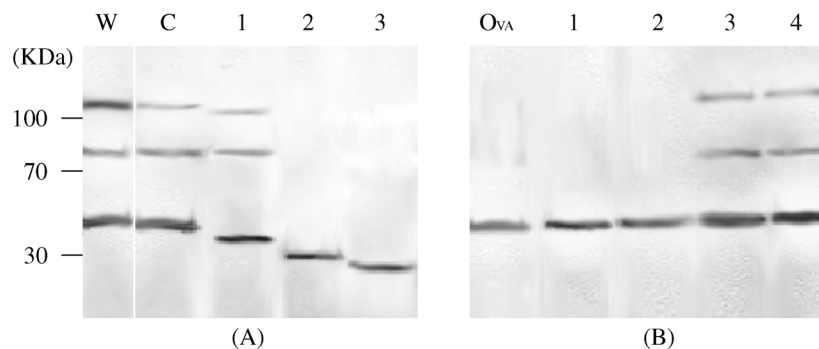
Figure 4-A shows that ALS was still included in the lanes containing the IGFBP3-ALS complex. Considering the fact that ALS is missing in the other lanes that only show IGFBP3s, then the anti-IGFBP3 antisera did not pull down the ALS, due to cross reactivity. Rather, this ALS likely originated from the IGFBP3-ALS complex, either insufficiently or else never

cross-linked. The ALS fraction was separated during the process, post to immunoprecipitation. Based on this assumption, the ratio of IGFBP3-ALS doublets vs. free IGFBP3 monomers seems less meaningful in representing the tendency for the formation of IGFBP3-ALS doublets (data not shown). Figure 4-A indicates that the minor truncated variant, D40, still forms doublets with ALS that are similar to the single mutant that is only on the C-terminal. In contrast, other truncated variants exhibited a significant reduction in the formation of doublets. This result implies that the critical determinant may reside in the rest of the N-terminal consensus sequence (i.e., codons 41-88).

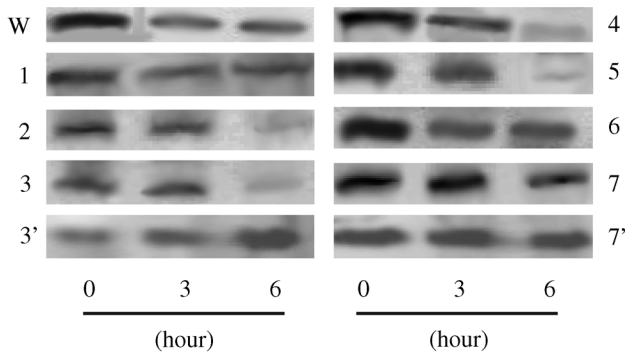
In addition to the truncated mutation, four site-specific mutants were tested for their ability to form doublets with ALS. Figure 4-B shows that, among the four mutants, the mutant on codons 72 & 73 did not appear to affect the binding with ALS (lane 3). This is consistent with the outcome from the deletion study. Similar to the mutant on 72 & 73, the mutations on 87 & 88 produced comparable results (lane 4). On the other hand, the mutants on the putative IGF binding sites showed a lack of binding with ALS. The mutant on 56 & 57 showed less interaction with ALS (lane 1). A similar level of inhibition was apparent for the mutant on 80 & 81 (lane 2). This result may be an indication that the ALS-IGFBP3 interaction occurs at multiple sites other than the C-terminal site. The N-terminal is also important in the binding. The IGF binding sites may serve as contacting sites for the ALS-IGFBP3 interaction.

#### N-terminal domains stabilize ALS-IGFBP3 interaction

This experiment employed the mRNA with the intact C-terminal consensus region and each N-terminal mutation, which differs from the interaction in the previous study. The oocytes were incubated for 3 h following the injection. When



**Fig. 4.** Assay on mutant IGFBP3s interacting with ALS. Three condensed and four site-specific mutants were assayed to discover if they were capable of forming a binary complex, despite the mutation. (A) Three condensed IGFBP3 mutants were co-expressed with ALS. The incubation media was treated with the cross-linking agent and immunoprecipitated by anti-IGFBP3 antisera. Lanes W and C show that IGFBP3, the totally intact (W) and N-terminal intact IGFBP3 (C), forms doublets with ALS (*top bands*). Lanes 1, 2, and 3 refer to precipitants of D40, D60, and D88 by anti-IGFBP3 antiserum. Lanes 2 and 3 show no ALS, which is different from lane 1 (including ALS in the precipitant). (B) The four site-specific IGFBP3 mutants were each co-expressed with ALS. Lanes 1-4 show the immunoprecipitant by anti-IGFBP3 antisera. Ova refers to a negative control lane, indicating a lack of a doublet that is formed between ALS and ovalbumin (1,  $\Delta 5657$ ; 2,  $\Delta 8081$ ; 3,  $\Delta 7273$ ; 4,  $\Delta 8788$ ).



**Fig. 5.** N-terminal sequences to stabilize IGFBP3-ALS doublets. The injected oocytes were incubated for 3 h, then the oocytes were removed from the media. The incubation media was drawn immediately. Samples were drawn at 3 and 6 h following the initial drawing. At each drawing, the samples were subjected to cross-linking and immunoprecipitation by anti-IGFBP3 antisera. (W: intact IGFBP3, 1, D40; 2, D60; 3, D88; 3' to represent free form of D88; 4,  $\Delta$ 5657; 5,  $\Delta$ 8081; 6,  $\Delta$ 7273; 7,  $\Delta$ 8788; 7' to represent free form of  $\Delta$ 8788.)

the media containing the IGFBP3 variants and ALS was incubated for 3 or 6 h more, then the difference was evident in the stability of the IGFBP3-ALS doublets (Fig. 5). A reverse relationship was evident in the relative IGFBP3 mass between the free and ALS-bound forms when compared to D88 and  $\Delta$ 8788 (lanes 3 and 7). This pattern remained consistent for the other mutants (data not shown). Among the deletion mutants, the deletion spanning codon 11-40 appeared unaffected by the alteration. In terms of site-specific mutants, panels 4 and 5 show that the mutants on the putative IGF binding sites ( $\Delta$ 5657 and  $\Delta$ 8081) appeared less stable in maintaining the direct interaction between ALS and IGFBP3.

This result strongly indicates that the N-terminal plays an important role in stabilizing the IGFBP3-ALS doublets following their formation. With the deletion of the region that spanned the putative IGF binding site in IGFBP3, IGFBP3-ALS doublets become less stable, even in the presence of intact binding by the C-terminal region (KGRKR).

## Discussion

The IGFBP3 and ALS play crucial roles in modulating the endocrine effect of IGFs by sequestering the surplus in the circulation. Previous studies claimed that the two proteins interacted with each other, primarily in the presence of IGFs. This study negates this claim and shows that the N-terminal of IGFBP3 bears a significant ramification in the IGF-free interaction with ALS. In addition, the IGF binding motifs were more significant than the non-IGF binding region in the N-terminal region.

The messages of ALS and IGFBP3 are translated correctly within the oocytes, and the protein products interact to form

doublets following secretion into the incubation media. The present study shows that the *Xenopus* oocyte expression system is an effective alternative for expression of biologically active IGFBP3 and ALS. This is the first study that reports that IGFBP and ALS can interact in solution without the purification process. Recombinant IGFBP-3 and ALS that are expressed and secreted from the *Xenopus* oocyte have comparable biological activities in the binding assays.

Wild-type IGFBP3 associates with ALS to form a binary complex in the media, despite their different physiological conditions. The interaction is highly specific, considering the fact that IGFBP3 formed no complex with ovalbumin. In the presence of the mutation on the C-terminal ALS binding site, we compared the binding affinity of various N-terminal versions of IGFBP3 with that of the wild-type protein. The slightly deleted N-terminal variant D40 showed a binding affinity that was equivalent to that of the wild-type IGFBP3. D60 and D88, however, produced little or no ALS-IGFBP3 doublet with the C-terminal domain altered. These results indicate that the intermolecular interaction between ALS and IGFBP3 involves N-terminal domains along with the C-terminal region. This N-terminal structural integrity was significantly disrupted by deletion in D60 or D88.

Site-specific mutations that altered the IGF binding sites showed a reduced affinity for the IGFBP3-ALS interaction, while those that did not mutate the IGF binding sites appeared unaffected. The results in the present study strongly imply that the structural determinants concerning the ALS-IGFBP3 interaction occur on the IGF binding sites without the mediation of IGFs. The IGF binding sites are the prime candidates for the ALS-IGFBP3 interaction, in addition to the C-terminal. Human ALS harbors a region of acidic residues of DDDADE in the N-terminal region which interact with its highly basic counterpart of KGRKR (Leong *et al.*, 1992). An unidentified motif that interacts with the N-terminal of IGFBP3 should exist elsewhere in ALS. The interaction between residues may be abolished as the result of the mutation, considering the fact that the ALS binding is known to be sensitive to increased ionic strength (Holman and Baxter, 1996).

The significance of the N-terminal region was also supported in the stabilization experiment. Following doublet formation, the ALS-IGFBP3 doublets gradually dissociated from each other in a time-dependent manner. The dissociation rate varied with the different rates, according to the N-terminal status, despite the intact C-terminal domain (KGRKR). Faster dissociation was evident among doublets that consisted of IGFBP3 mutants covering IGF binding sites.

Various expression systems were employed to produce recombinant IGFBPs and ALS. These recombinant proteins were well expressed in *E. coli* and with a considerable amount of soluble protein. The *E. coli*-expressed proteins, however, do not represent native IGFBPs and ALS, due to the lack of post-translationally modification. Mammalian systems modify recombinant IGFBPs correctly, although yields of

recombinant IGFBPs are lower than the yields from *E. coli*. The study of the IGFBP-ALS interaction, however, has been challenging since the purity of the recombinant proteins is not easily assured following overexpression.

According to the electrophoretic mobility, the media contains a distinctive mixture of proteins. All of the mobility corresponds with the three N-linked glycosylation sites in the IGFBP-3 and ALS sequences. A major protein of the molecular weight is equivalent to fully glycosylated IGFBP-3 and ALS. This study, however, did not research the significance of the glycosylation for the direct interaction between IGFBP3 and ALS. The effect of deglycosylation on the interaction should be further studied.

In summary, this study has shown that mutations on the N-terminal of IGFBP3 appear to undermine the interaction with ALS in the absence of IGFs. Thus, the significance of the N-terminal region and IGF binding site, in particular, is significant for this interaction. Together with the ALS-IGFBP3 binding study and stability study for the IGFBP3-ALS doublet, this study suggests that ALS interacts with IGFBP3 in the absence of IGFs, based on the N-terminal structural domains in the IGFBP3 as well as the C-terminal determinants. Further study is necessary to investigate the extent of the IGF-free IGFBP3-ALS doublet formation in the presence of a sufficient level of IGFs.

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