Localization of the IGF binding domain and evaluation of the role of cysteine residues in IGF binding in IGF binding protein-4

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Abstract

Our previous findings suggest that binding of IGF binding protein-4 (IGFBP-4) to IGFs is essential for the inhibitory effect of IGFBP-4 on the activity of IGFs, both in vitro and in vivo. Therefore, understanding the structural determinants of IGF binding in IGFBP-4 is important to the general understanding of the biology of the IGF system. This study sought to further localize the IGF binding domain and to evaluate the role of Cys residues in IGF binding. Our data revealed that full-length IGFBP-4 peptides lacking the residues Leu72-Ser91 or Leu72-His74 or Gly75-Ser91 failed to bind to IGF-I or IGF-II, whereas deletion of the residue Leu 72 or residues Met80-Ser91 led to a 2- to 3-fold reduction in IGF-I and IGF-II binding activity. The IGF-I and IGF-II binding activities were dramatically reduced by the single mutation, Cys9/Arg (>25-fold), and to a lesser degree, by the single mutation, Cys12/Arg (the first N-terminal Cys residue was designated Cys1). The mutation Cys17/Ser or Cys18/Tyr or Cys20/Ser each resulted in a similar but moderate (>5-fold) reduction in IGF-II binding activity. The IGF-I binding activity was also dramatically reduced by the mutation Cys18/Tyr, and to a lesser extent, by the mutation Cys17/Ser or Cys20/Ser. These data suggest: 1) the IGF-I and IGF-II binding domain in IGFBP-4 involves a hydrophobic motif (Leu72-Met80) located in the distal part of the conserved N-terminal region, and 2) the N-terminal Cys residues (Cys9 and Cys12) are more critical than the C-terminal Cys residues (Cys17 and Cys20) in affecting the IGF-I and IGF-II binding. Based on these data, we speculate that the structural determinants of IGF-I and IGF-II binding in IGFBP-4 are very similar, if not identical.

Introduction

Insulin-like growth factors (IGFs) are important mediators of physiological growth and metabolism in vivo and promote proliferation and differentiation of a variety of cell types in vitro (Canalis 1993, Rosen et al. 1994, Clemmons 1997, Mohan & Baylink 1999). The actions of IGFs are either positively or negatively regulated by a family of structurally related IGF binding proteins (IGFBPs) (Canalis 1993, Rosen et al. 1994, Clemmons 1997, Mohan & Baylink 1999). The mechanisms by which IGFBPs stimulate or inhibit IGF actions have not been clearly defined and may vary among the IGFBPs. In this regard, recent evidence suggests that some of the IGFBPs such as IGFBP-3 may exert IGF independent actions (Valentinis et al. 1995, Mohseni-Zadeh & Binoux 1997), in addition to modulating IGF actions. Studies in our laboratory demonstrate that IGFBP-4 primarily acts to inhibit cell proliferation by an IGF dependent mechanism (Mohan et al. 1995, Qin et al. 1998, Miyakoshi et al. 1999). Therefore, elucidation of the structural determinants of various IGFBPs in IGF binding is important to the general understanding of the biology of the IGF system and may shed light on how these different IGFBPs exhibit different actions. In this regard, previous deletion analysis revealed that the IGF binding activity of IGFBP-4 is mainly determined by the conserved residues in the N-terminal region, and to a lesser extent, by the residues in the conserved C-terminal region (Qin et al. 1998). Although the three-dimensional structure of any intact IGFBPs has not been determined, disulfide bridging in IGFBPs appears to be important for maintaining the secondary structures required for IGF binding, since all six IGFBPs contain conserved Cys residues in both the N-terminal and the C-terminal regions and reduced IGFBPs exhibit little or no IGF binding activity (Landale et al. 1995, Qin et al. 1998, Neumann & Bach 1999). Except for that in IGFBP-6, the disulfide linkage in other IGFBPs has only
been partially determined (Hashimoto 1997, Forbes et al. 1998, Kalus et al. 1998, Neumann & Bach 1999, Standker et al. 2000). These studies demonstrate that some of the disulfide linkages are conserved whereas others may vary among different IGFBPs. Regarding the role of each individual Cys residue or disulfide linkage in IGF binding, the information has been very limited. It has previously been reported that mutation of Cys8 (Cys38) in IGFBP-1 led to a loss of IGF binding activity, whereas mutation of Cys6 (Cys34) had no effect on IGF binding (Brinkman et al. 1991a). In addition, mutation of the C-terminal Cys18 (Cys226) in IGFBP-1 results in dimer formation and loss of IGF binding activity (Brinkman et al. 1991b). The role of Cys residues in IGF binding in other IGFBPs has not been reported. Thus, the purpose of this study is to further localize the IGF binding domain and to evaluate the role of Cys residues in N-terminal and C-terminal domains of IGFBP-4 in determining IGF binding activity.

**Materials and Methods**

**Materials**

Recombinant human IGF-I and IGF-II peptides were from Bachem, Inc. (Torrance, CA, USA). The QuikChange Site-Directed Mutagenesis kit and ExSite kit were from Stratagene (La Jolla, CA, USA). XL-blue 1 Eschericia coli competent cells were from Promega (Madison, WI, USA). Reagents for SDS-PAGE were from BIO-RAD (Hercules, CA, USA). All other chemicals used were at least reagent grade and were from Sigma (St Louis, MO, USA).

**Preparation of human IGFBP-4 analog constructs with deletion or point mutation**

Recombinant 6xHis-tagged wild type IGFBP-4 was prepared as previously described (Qin et al. 1998). This recombinant peptide contained Gly\(^{-5}\) to the carboxyl terminus, Glu\(^{237}\) (the first residue Met in the signal peptide was designated \(-21\) and was designated 6xHis-BP-4\((\sim 5/237)\). The recombinant IGFBP-4 peptides with point mutations and deletions were prepared using the PCR-based QuikChange Site-Directed Mutagenesis kit and ExSite kit respectively. The primers used for PCR are given in Table 1. The PCR was carried out using the 6 xHis-BP-4\((\sim 5/237)\) plasmid as the template and DNA polymerase, \(pHi\), under the following conditions: one cycle of 98 °C for 3 min (hot start), 30 cycles each of 98 °C for 1 min (denaturation), 65 °C for 1 min (annealing), and 72 °C for 12 min (extension). The PCR products were treated with DpnI, phosphorylated with alkaline

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Primer sequences</th>
<th>New restriction site created</th>
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<tbody>
<tr>
<td>ΔLeu(^{-2})</td>
<td>Forward TGT GTG CAG GGG CTT TCT</td>
<td>Sacl</td>
</tr>
<tr>
<td>ΔLeu(^{-2})Hs(^{1,4})</td>
<td>Reverse ATG CAC GGG GAA GGC GTG TGG</td>
<td></td>
</tr>
<tr>
<td>ΔLeu(^{10})Ser(^{91})</td>
<td>Forward GCA CAC GCC TGG CCC GTG CAT CAG</td>
<td></td>
</tr>
<tr>
<td>ΔGly(^{75})Ser(^{91})</td>
<td>Reverse CTG CAC CCC TCT GAC AAG</td>
<td></td>
</tr>
<tr>
<td>ΔLeu(^{72})Ser(^{91})</td>
<td>Forward TGT GTG CAG GGG CTT CTC</td>
<td></td>
</tr>
<tr>
<td>Cys9/Arg</td>
<td>Reverse ATG CAC GGG GAG CAG GAG</td>
<td></td>
</tr>
<tr>
<td>Cys12/Arg</td>
<td>Forward GGT GTA AAG GCC CCC GGG GGG CAT CAC</td>
<td></td>
</tr>
<tr>
<td>Cys17/Ser</td>
<td>Reverse CTG CAC CCC TCT GAC AAG GAC GAG</td>
<td></td>
</tr>
<tr>
<td>Cys18/Tyr</td>
<td>Forward GGT GTA AAG GCC CCC GGG GGG CAT CAC</td>
<td></td>
</tr>
<tr>
<td>Cys20/Ser</td>
<td>Reverse ATG CAC GGG GAG CAG GAG</td>
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\(^{1}\)To facilitate screening for desired mutations, primers were designed such that each mutation (underlined nucleotides) would create a new restriction site in the newly synthesized plasmid DNA after PCR.
Expression and purification of recombinant IGFBP-4 peptides

Recombinant IGFBP-4 peptides were expressed in E. coli and purified as previously described (Qin et al. 1998). The peptides, 6×His-BP-4-Δ(72–74) and 6×His-BP-4-Δ(75–91), were purified sequentially by Ni-agarose and HPLC reverse phase chromatography. Other recombinant IGFBP-4 peptides were purified sequentially by Ni-agarose affinity and IGF-I affinity chromatography using procedures described previously (Qin et al. 1998).

Western 125I-IGF ligand blot and IGFBP-4 immunoblot analyses

125I-IGF Western ligand blot analysis and IGFBP-4 immunoblot analysis were performed as previously described (Scharla et al. 1991, Honda et al. 1996).

IGFBP-4/IGF-II solution binding assay

The binding activity of various IGFBP-4 mutants to IGF-II in solution was determined by incubating 125I-IGF-II tracer with IGFBP-4 at various concentrations (0 to 500 ng/ml) followed by separation of free and bound 125I-IGF-II tracer by polyethylene glycol precipitation as previously described (Mohan et al. 1989).

Results

Localization of the IGF binding domain

Our previous sequential deletion analysis revealed that residues Leu72 to Ser91 may represent the IGF binding domain in IGFBP-4 (Qin et al. 1998). To confirm this earlier finding and localize the critical residues for IGF binding in this motif, we prepared several full-length IGFBP-4 peptides with amino acid deletions in this critical region and evaluated their purity by SDS-PAGE under non-reducing (Fig. 1A) or reducing conditions (Fig. 1D). These peptides migrated in SDS-PAGE with anticipated molecular mass. The IGF binding activities of IGFBP-4 analogs were determined by the IGF ligand blotting, followed by densitometric analysis. Deletion of the residues Leu72 to Ser91 (data not shown) or Leu72–His74 or Gly75–Ser91 each led to the loss of IGF-I and IGF-II binding activity (Fig. 1B,C). Deletion of the residues Met80–Ser91 or Leu72 resulted in a slight reduction in IGF-I and IGF-II binding activity (40 to 70% of the wild type IGFBP-4) (Fig. 1B,C). None of these targeted deletions led to a dramatic preferential effect on IGF-I over IGF-II binding activity.

Evaluation of the role of Cys residues in IGF binding

To evaluate the role of Cys residues in IGF binding, we first prepared two IGFBP-4 mutants with point mutations at positions of Cys9 and Cys12 respectively in the N-terminal domain of IGFBP-4. We chose to mutate these two Cys residues because 1) the disulfide linkages Cys9–Cys11 and Cys10–Cys12 appear to be conserved among several IGFBPs (Hashimoto et al. 1997, Neumann & Bach 1999, summarized in Fig. 2), and 2) these linkages are either within or in close proximity to the established IGF binding domain in IGFBP-4. Under non-reducing conditions, both Cys9/Arg and Cys12/Arg mutant preparations yielded a high molecular weight band in addition to the band of expected masses (Fig. 3A). This high molecular weight band upon reduction with β-mercaptoethanol was not observed; meanwhile an approximately 38–40 kDa band was revealed (Fig. 3D). This 38–40 kDa band was recognized by the IGFBP-4 polyclonal antibody (Fig. 3E) and represented the reduced full-length IGFBP-4 as previously reported (Qin et al. 1998). The Cys12/Arg mutant preparation also gave a small molecular weight band of approximately 14 kDa, which was recognized by the IGFBP-4 antibody (Fig. 3D,E) and thus represented the IGFBP-4 proteolytic fragment. As determined by the IGF ligand blot analysis, 6×His-BP-4(Cys9/Arg) exhibited approximately a 25-fold reduction in IGF-I/IGF-II binding activity. The mutation Cys12/Arg also reduced IGF-I and IGF-II binding, but the extent of reduction was less compared with the Cys9/Arg mutation (Fig. 3B,C).

Next, we prepared three IGFBP-4 mutants with point mutations at the positions of Cys17, Cys18, and Cys20 respectively in the C-terminal domain of IGFBP-4. According to a recently published study (Standker et al. 2000), the mutation Cys17/Ser or Cys18/Tyr was expected to disrupt the recently determined disulfide linkage Cys17–Cys18, whereas the mutation Cys20/Ser was expected to disrupt the disulfide linkage Cys19–Cys20. Similar to those N-terminal Cys mutants, these C-terminal Cys mutant preparations contained extra high and low molecular weight proteins in SDS-PAGE (Fig. 4A,D). As confirmed by immunoblot analysis (Fig. 4E), these co-eluted proteins represent either dimerized intact IGFBP-4 or IGFBP-4 proteolytic fragments due to the action of E. coli proteases. The Cys20/Ser mutant migrated more slowly than the wild type IGFBP-4 and the two other mutants, although the expected molecular weights of these peptides should be very similar (Fig. 4A,B,C). This shift in apparent molecular mass of Cys20/Ser is likely due to the conformational change caused by this particular mutation, since all of the three mutant peptides...
Figure 1 Western $^{125}$I-IGF ligand blot and immunoblot analyses of the IGFBP-4 peptides with amino acid deletions. In A, B, and C, purified proteins were treated with SDS-PAGE loading buffer without reducing agent. Three doses of the wild type IGFBP-4 (160, 80, and 40 ng) were included. Half of each sample was separated by SDS-PAGE and stained with Coomassie blue (A). One quarter of each sample was used to perform either IGF-I (B) or IGF-II (C) ligand blot analysis. In D and E, purified peptides were treated with SDS-PAGE sample buffer containing β-mercaptoethanol. Two thirds of each sample were separated by SDS-PAGE and stained with Coomassie blue (D). The remaining one third of each sample was subjected to immunoblot analysis using polyclonal hiGFBP-4 antibody (E). The IGF binding activity of these IGFBP-4 peptides has been confirmed by multiple experiments.
demonstrated a major band, which co-migrated with the wild type IGFBP-4 and reacted with the IGFBP-4 antibody (Fig. 4D,E). As determined by IGF ligand blotting, these C-terminal Cys mutants exhibited approximately a 5-fold reduction in IGF-II binding activity (Fig. 4B). While these mutations also reduced IGF-I binding activity, the effect of each mutation seemed to vary. The reductions in IGF-I binding caused by the mutation Cys17/Ser and Cys20/Ser were very similar but appeared to be lower than the reduction caused by the Cys18/Tyr mutation (Fig. 4C).

To further confirm the observation that N-terminal Cys residues were more critical than the C-terminal Cys residues in IGF binding, we evaluated IGF-II binding to Cys9/Arg and Cys20/Ser mutants in a solution binding assay (Fig. 5). The estimated IGFBP-4 concentrations at which half maximal IGF-II binding occurred were approximately 0.8 ng/ml and 4 ng/ml for the wild-type IGFBP-4 and Cys20/Ser mutant, respectively. This result was consistent with the IGF-II ligand blot analysis, which revealed that the Cys20/Ser mutant exhibited an approximately 5-fold reduction in IGF-II binding activity compared with the wild-type IGFBP-4. The Cys 9/Arg mutant exhibited a much lower affinity compared with the Cys20/Ser mutant (>15-fold). The IGFBP-4 mutant lacking residues 72–74 did not demonstrate binding affinity with IGF-II as shown previously by the IGF ligand blot analysis (Fig. 1).

**Discussion**

One of the important structural characteristics of IGFBPs is that all six IGFBPs contain multiple conserved Cys residues in their N-terminal and C-terminal regions (Fig. 2). These structural features suggest a general role of disulfide linkage in determining the IGF binding activity of IGFBPs. In this study, we have determined, for the first time, the role of several representative Cys residues in IGFBP-4 in IGF binding. Moreover, using full-length IGFBP-4 analogs with targeted amino acid deletions, we have confirmed that the IGF binding domain in IGFBP-4 is located in the distal N-terminal conserved region.

Using sequential deletion analysis, we have previously shown that the IGFBP-4 peptide containing the residues Gly^5 to Leu^72 failed to bind to IGF-I and IGF-II, while...
the peptide containing residues Gly\(^5\) to Ser\(^91\) retained substantial amount of IGF binding activity (Qin et al. 1998). These data suggest that the IGF binding domain may reside in the region Leu\(^{72}\) to Ser\(^{91}\). This tentative conclusion has now been confirmed in this study by the observation that full-length IGFBP-4 missing residues Leu\(^{72}\) to Ser\(^{91}\) had completely lost IGF binding activity. Since IGFBP-4 peptide lacking residues Gly\(^75\) to Ser\(^{91}\) failed to bind to IGFs whereas deletion of residues Met\(^{80}\)-Ser\(^{91}\) only had a minimal effect on IGF binding, it is reasoned that residues Leu\(^{72}\) to Met\(^{80}\) may be the most critical residues for IGF binding. Sequence analysis revealed that this motif, L\(^72\)-M\(^73\)-H\(^74\)-G\(^75\)-Q\(^76\)-G\(^77\)-V\(^78\)-C\(^79\)-M\(^80\), is very hydrophobic. In this motif, His\(^74\) is the only charged residue. However, substitution of His\(^74\) for a hydrophobic residue, Ala, had no effect on either IGF-I or IGF-II binding (Qin et al. 1998). Substitution of His\(^74\) for Pro abolished IGF binding possibly because introduction of a Pro residue may have disrupted the secondary structure of this motif by creating a bending in the peptide backbone (Qin et al. 1998). In contrast, deleting the three residues, Leu\(^{72}\)-Met\(^{73}\)-His\(^{74}\), completely abolished IGF-I and IGF-II binding, and deletion of the single residue, Leu\(^{72}\), led to a 2-fold reduction in IGF binding. These data suggest that the residues Leu\(^{72}\) and Met\(^{73}\) in this hydrophobic motif in IGFBP-4 may be important in IGF binding. These findings are consistent with the results from a study on the interaction between IGFBP-5 and IGF-I by nuclear magnetic resonance (NMR), which suggest that the residues Ly\(^{68}\), Pro\(^{69}\), Leu\(^{70}\), Leu\(^{73}\), and Leu\(^{74}\) (Leu\(^{74}\) in IGFBP-5 is analogous to Leu\(^{72}\) in IGFBP-4) constitute a hydrophobic patch on the surface of IGFBP-5 and form the IGF binding domain (Kalus et al. 1998). This prediction by NMR is confirmed by the most recent finding that simultaneous mutation of Ly\(^{68}\) to Asn, and the other four residues to Gln in IGFBP-5 essentially abolished the IGF-I binding activity (Imai et al. 2000). Taken together, these new findings strongly suggest that the IGF binding domain in IGFBP-4 as well as in IGFBP-5 may involve a hydrophobic motif located in the distal part of the conserved N-terminal region.

Disulfide bond linkage is important in maintaining the structures required for IGF binding since reduced IGFBPs conferred little IGF binding activity (Qin et al. 1998, Neumann & Bach 1999). It has previously been shown that several N-terminal Cys residues and a C-terminal Cys residue in IGFBP-1 are critical for IGF binding (Brinkman et al. 1991a, b). To date, the disulfide linkage in the N-terminal domain of IGFBP-4 has not been determined. However, based on the conserved disulfide linkage in other IGFBPs (summarized in Fig. 2), in IGFBP-4, Cys\(^9\) is likely to be linked to Cys\(^11\), and Cys\(^10\) to Cys\(^12\). Although mutation of either Cys\(^9\) or Cys\(^12\) each led to a dramatic reduction of IGF binding activity, disulfide linkage involving Cys\(^9\) appeared to be more critical for IGF binding. Since the extent of reduction in IGF-I and

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**Figure 3** Western \(^{125}\)IGF ligand blot and immunoblot analyses of the IGFBP-4 peptides with point mutation at N-terminal Cys residues. Sample treatment was identical to that described in Fig. 1. (A) Coomassie blue stain of proteins separated by SDS-PAGE under non-reducing conditions; (B) IGF-I ligand blot; (C) IGF-II ligand blot; (D) Coomassie blue stain of proteins separated by SDS-PAGE under reducing conditions; (E) Western immunoblot of reduced IGFBP-4 peptides. The IGF binding activity of these IGFBP-4 peptides has been confirmed by multiple experiments using IGFBP-4 peptides prepared in different batches.
Figure 4 Western $^{125}$I-IGF ligand blot and immunoblot analyses of the IGFBP-4 peptides with point mutation at C-terminal Cys residues. Sample treatment was identical to that described in Fig. 1. (A) Coomassie blue stain of proteins separated by SDS-PAGE under non-reducing conditions; (B) IGF-I ligand blot; (C) IGF-II ligand blot; (D) Coomassie blue stain of proteins separated by SDS-PAGE under reducing conditions; (E) Western immunoblot of reduced IGFBP-4 peptides. The IGF binding activity of these IGFBP-4 peptides has been confirmed by multiple experiments using IGFBP-4 peptides prepared in different batches.
IGF-II binding is very similar, the disulfide linkages involving these two Cys residues may be equally critical for both IGF-I and IGF-II binding. A more precise interpretation of these data requires a future elucidation of the disulfide linkage profile in the N-terminal domain of IGFBP-4.

During the preparation of this manuscript, the disulfide linkage profile in the conserved C-terminal region of IGFBP-4 has been reported (Standker et al. 2000). Disruption of each of the two of these three C-terminal disulfide linkages had a similar effect on IGF-II binding, although the effect was less dramatic compared with the disruption of the N-terminal disulfide linkages. Since mutation of Cys17 (Cys<sup>194</sup>) or Cys18 (Cys<sup>205</sup>) each should disrupt the same disulfide linkage Cys17-Cys18, we would expect that both mutations should demonstrate a similar effect on IGF binding activity. While mutation of Cys17 or Cys18 led to a similar if not identical extent of reduction in IGF-II binding, mutation of Cys18, unexpectedly, had a greater effect on IGF-I binding than mutation of Cys17. Since Cys18 (Cys<sup>205</sup>) is located in the highly conserved motif, Cys<sup>205</sup>-Trp-Cys<sup>207</sup>-Val-Asp<sup>209</sup>, which has been suggested to play a role in enhancing IGF binding activity (Qin et al. 1998), we speculate that mutation of Cys18 may have affected the function of other adjacent residues which may be more critically involved in IGF-I binding than in IGF-II binding. Alternatively, Cys17 may form disulfide linkage with another critical Cys residue after its partner Cys18 is mutated. The resulting mismatch in disulfide linkage may further and preferentially reduce the IGF-I binding activity.

IGFBP-4 is unique in that it contains two extra Cys residues, Cys13 (Cys<sup>110</sup>) and Cys14 (Cys<sup>117</sup>), in the central region. Although we did not provide direct evidence for the role of these two Cys residues in IGF binding because of the difficulties of mutating these residues, Cys13 and Cys14 do not seem to be critical based on our previous finding that the IGFBP-4 analog lacking residues Pro<sup>94</sup> to Gln<sup>119</sup> exhibited similar IGF-I or IGF-II binding activity compared with the wild type (Qin et al. 1998, 1999).

In summary, results from this study suggest that the IGF-I and IGF-II binding domain in IGFBP-4 involves a hydrophobic motif located in the distal part of the conserved N-terminal region. The N-terminal Cys residues (Cys9 and Cys12) are more critical than the C-terminal Cys residues (Cys17 and Cys20) in affecting IGF-I and IGF-II binding. Cys18 is more critical in affecting IGF-I than IGF-II binding activity. Based on these new data and our previous findings, we speculate that the structural determinants for both IGF-I and IGF-II binding in hIGFBP-4 are very similar if not identical.

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