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Site-directed mutagenesis of the N-terminal region of IGF binding protein 1; analysis of IGF binding capability

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To define domains involved in IGF binding 60 N-terminal amino acid residues of IGFBP-1 were deleted. This deletion resulted in loss of IGF binding suggesting that the N-terminus may enclose an IGF binding domain However, most point mutations introduced in this region did not affect IGF binding In contrast to Cys-34, only substitution of Cys-38 for a tyrosine residue abolished IGF binding With the determination that all 18 cysteine residues are involved in disulphide bond formation our data suggest that, although not all cysteines contribute to the same extent, the ligand binding site may be spatially organized

IGF binding protein, N-terminus deletion mutant, Site-directed mutagenesis, Point mutation, IGF binding site, Disulphide bond

1 INTRODUCTION

Insulin-like growth factor (IGF) binding proteins (IGFBPs) form a family of structurally related proteins All IGFBPs are capable of binding IGF-I and -II, which are small peptide hormones closely related to insulin. So far six IGFBPs have been isolated and characterized and even more IGFBPs, albeit less well characterized, have been identified [1–10]. IGFBPs are generally accepted as modulators of IGF action although their precise function remains unclear. Both inhibition and potentiation of IGF action on cells has been reported [11–14].

The amino- and carboxyl-termini of the IGFBPs are extremely well conserved. This probably also holds for the tertiary structure of the proteins because of the positional conservation of 18 cysteine and most of the glycine residues. The middle part is less well conserved and may serve as a spacer between the conserved termini

Because of the high content of cysteine residues frequently found in ligand binding domains of hormone receptors, and the conservation, the N- and the C-terminal regions are the most likely candidates for an IGF binding domain. Evidence for both possibilities has been presented since fragments from either end capable of binding IGF have been described [15,16] In addition, we recently presented evidence that an IGF binding domain may be located in the vicinity of a putative intramolecular disulphide bond formed by cysteine residue 226 and its partner cysteine [17]

In this study we present evidence that probably all cysteine residues in IGFBP-1 are involved in the formation of disulphide bonds. We demonstrate that in contrast to Cys-34, Cys-38 is essential for appropriate folding of IGFBP-1 In addition, our data show that most of the mutations introduced in one of the most strongly conserved regions in the N-terminus of IGFBP-1 do not affect IGF binding.

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2. MATERIALS AND METHODS

2 1 *Construction of a deletion mutant*

A deletion spanning amino acid residues −1 to 60 was introduced in IGFBP-1 expression clone pSV19 pSV19 expresses IGFBP-1 using the simian virus 40 (SV40) early promoter and the β-globin polyadenylation region [1] Using standard DNA procedures a 5' *Hin*dIII-*Bss*HII cDNA fragment in pSV19 enclosing residues −25 to 60 was substituted for a 5' *Hin*dIII-*Hpa*II fragment enclosing amino acid residues −25 to −1 The deletion was confirmed by sequence analysis

2 2 *Random mutagenesis by chemical modification*

Random mutagenesis in the N-terminal region of IGFBP-1 cDNA was performed as described previously [17] Briefly, a 5' *Eco*RI-*Sma*I cDNA fragment encoding the N-terminal region of IGFBP-1 was subcloned in the vector PTZ19 (Pharmacia) Single stranded DNA was isolated from this construct and annealed to double stranded DNA of the same construct lacking a *Bst*EII-*Sma*I fragment in the N-terminal region of IGFBP-1 The resulting gapped duplexes were subjected to the mutagenic agent sodium bisulphite, causing C to U transitions in the single stranded DNA Finally, mutated gapped molecules were recovered and directly transfected into *E coli* strain POP101 DNA from single colonies was isolated and characterized by sequencing We found that approximately 20-30% of the isolated clones had single or double mutations From those clones that contained interesting mutations *Eco*RI-*Sma*I fragments were isolated and used to replace the corresponding *Eco*RI-*Sma*I region in expression plasmid pSV19

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2 3 *Expression in COS-1 cells, immunostaining and IGF binding*

Plasmids carrying the wild type IGFBP-1 sequence or mutations in the N-terminal region were transfected into monkey COS-1 cells Samples of the medium of the transfected cells were separated with SDS/PAGE, transferred onto nitrocellulose filters and analyzed for IGF binding and IGFBP-1 immunoreactivity as described previously [17]

2 4 *Determination of disulphide bonds in the IGFBP-1 protein*

The number of disulphide bonds was determined by comparing the number of free sulfhydryl groups in the unfolded protein before and after reduction essentially as described by Ellman [18] IGFBP1 was isolated from pooled mid-term amniotic fluid as described before [12] Silver staining of the preparations showed a purity of >98% 30–50 μg of this material was dissolved in 5 M guanidinium chloride (GdCl) To reduce the protein fresh dithiothreitol (DTT) was added to a final concentration of 100 mM Reduced or untreated protein was precipitated with cold 5% w/v TCA and collected by centrifugation Residual reducing agent was removed by washing five times with 5% TCA The precipitate was dissolved in 5 M GdCl After removing traces of turbidity by centrifugation, the protein concentration was determined by absorbance at 280 nm The sulfhydryl content was determined by reacting the dissolved protein with an equal volume of 0 1 M Tris-HCl, pH 9 1, 5 M GdCl and 0 2 mM 5'-dithionitrobenzoic acid (DTNB) The absorbance of the mixture and a reference sample was measured at 412 nm From the increase in absorbance caused by DTNB, the sulfhydryl content was calculated using a molar extinction coefficient of 13 600

3 RESULTS

3 1. *Deletion mutant*

To examine whether the N-terminal region of IGFBP-1 participates in IGF-binding we introduced a deletion in the cDNA insert of expression plasmid pSV19 We exchanged a 5' *Hin*dIII-*Bss*H1 cDNA fragment enclosing residues −25 to 60 for a 5' *Hin*dIII-*Hpa*II fragment enclosing amino acid residues −25 to −1 This procedure created a deletion spanning amino acid residues −1 to 60 An outline of the constructs is shown in Fig 1 Note that this deletion resulted in the loss of the site from which the leader sequence is being processed in the wild type (wt) IGFBP-1 precursor protein However, the newly created sequence may satisfy the criteria for a leader sequence processing site with Leu-62 as potential +1 residue [19].

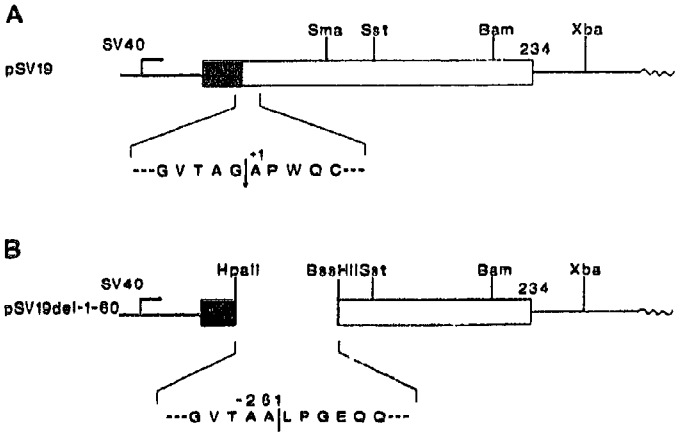

Fig 1 Diagram of expression plasmids carrying the complete IGFBP-1 cDNA fragment under control of the SV40 promotor (A) and the construct with a N-terminal deletion spanning residues −1 to 60 (B) The wavy line represents the rabbit β-globin polyadenylation region The coding region of IGFBP-1 is boxed with the signal peptide in black An arrow indicates the putative leader peptide processing site

To demonstrate the functional activity of the construct, we transfected COS-1 cells with the deletion mutant in which the coding sequence is under control of the SV40 promoter. Samples of the culture medium were separated by nonreduced and reduced SDS/PAGE and subsequently transferred onto nitrocellulose filters The filters were incubated with either [^{125}I]IGF-I (Fig. 2A) or polyclonal antibody against IGFBP-1 (Fig 2B and C)

Fig. 2A shows the results of ligand binding Culture medium from mock-transfected COS-1 cells contained proteins that bind [^{125}I]IGF-I (COS). These proteins with apparent molecular weights of 47, 45, 40 and 24 kDa are presumably binding proteins secreted by the COS-1 cells themselves An additional band of 28 kDa was observed with the construct enclosing the wild type (wt) IGFBP-1 sequence, pSV19 However, with deletion mutant pSV19del-1–60 no ligand binding different from the background bands was observed Incubation of the same filters with IGFBP-1 antibody (Fig 2B) demonstrated the expected 28 kDa band in the culture medium from pSV19 transfected COS-1 cells and a 24 kDa band with pSV19del-1–60 Under reduced conditions (Fig 2C) the latter band appears as a triplet with two major and one minor band with apparent molecular weights of 28, 27 and 26 kDa, respectively. These results demonstrate that deletion mutant pSV19del-1–60 is synthesized and secreted by the COS-1 cells. In addition, the finding that this truncated IGFBP-1 protein no longer binds IGF suggests that the deleted part of the protein may enclose an IGF binding site

3 2. *Site directed mutagenesis in the N-terminal region of IGFBP-1*

Mutations were introduced in the N-terminal part of IGFBP-1 between amino acid residues 26 and 56, using the same procedure as described previously [17] Fig 3 shows the N-terminal amino acid sequence of the selected mutants NI-VIII when compared to the wt IGFBP-1 sequence The IGFBPs encoded by the mutant genes were analysed analogous to the deletion mutant The results in Fig 4A demonstrate that COS-1 cells transfected with the mutant clones NI-VIII produce a protein capable of binding [^{125}I]IGF-I. Only mutant NI(Y38) failed to bind IGF. The latter mutant is produced by the COS-1 cells and is recognized by the antibody as is apparent from Fig. 4B However, the mutant protein migrates in two bands with apparent molecular weights of approximately 30 and 50 kDa. Note that the other mutants also show some heterogeneity in apparent molecular weight (Fig 4A and B). However, when separated under reduced conditions as shown in Fig. 4C it is obvious that all mutant proteins (NI-VIII) migrate with

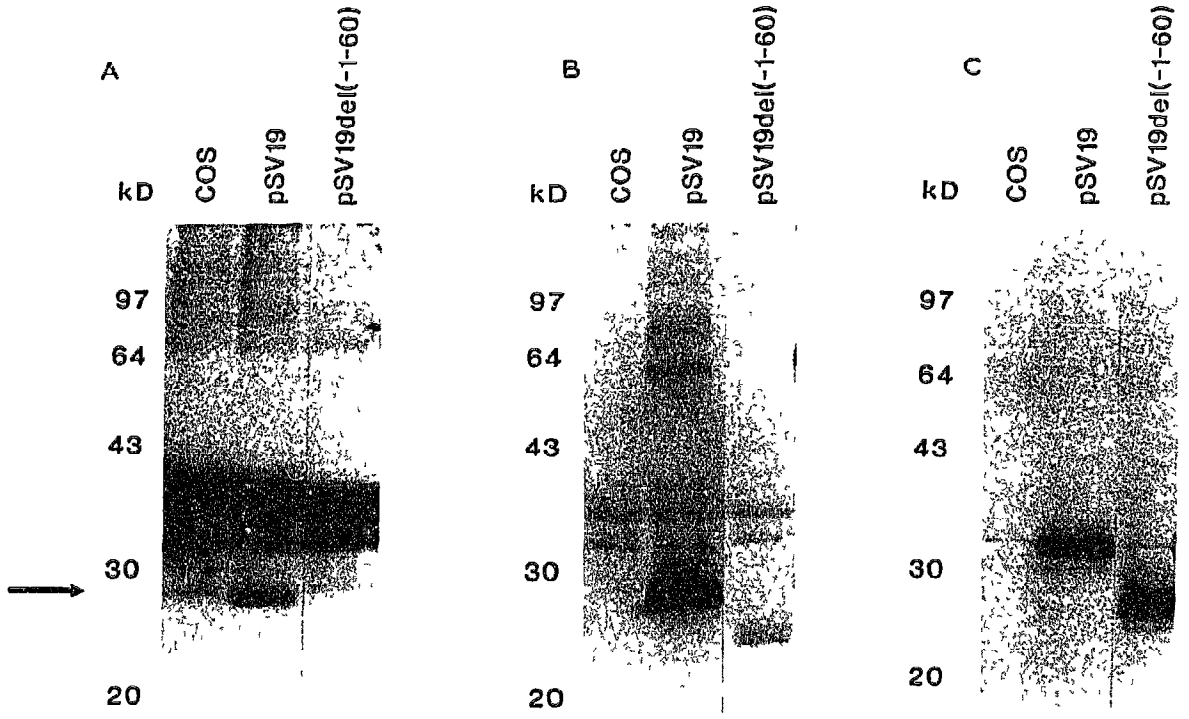

Fig 2 Analysis of mutant IGFBP-1 proteins in culture medium of COS-1 cells transfected with the expression plasmids from Fig 1 Culture medium of COS-1 cells transfected with pSV328a was used as a negative control, cells transfected with pSV19 as a positive control The binding proteins were separated by SDS/PAGE (12 5% acrylamide) and transferred onto nitrocellulose by Western blotting The filters were incubated with >200 000 cpm [^{125}I]IGF-I, washed and autoradiographed (A) After autoradiography the same filters were incubated with polyclonal anti-IGFBP-1 and made visible by immunostaining (B) C shows immunostaining of the mutant IGFBP-1 proteins separated by SDS/PAGE after reduction with β-mercaptoethanol The position of IGFBP-1 is indicated with a black arrow

the same molecular weight as the wt protein (pSV19), demonstrating that the primary structure of the mutant proteins is stil intact These apparent heterogeneity in molecular weight is most likely due to changes in the conformation of the molecule introduced by the mutations. In parallel experiments similar data were obtained when [^{125}I]IGF-II was used (results not shown).

3 3 *Determination of disulphide bonds*

Reduced and non-reduced samples of three different batches of purified IGFBP-1, with a purity of >98% were treated with DTNB. From the increase in absorbance measured at 412 nm, the sulfhydryl content was calculated. We reproducibly found 4.7±0.9 (n=4) mole -SH groups per mole reduced IGFBP-1, corresponding to 26% ±5 of the 18 mole of -SH groups as predicted from the primary structure. Increasing the denaturating agent GdCl to a final concentration of 6 M with or without 5–10 min additional heating at 100°C did not improve the results. To verify whether reduction of the IGFBP-1 protein was complete, the protein samples were precipitated in the same test tube after reaction with DTNB and measurement of the absorbance The precipitate was separated on a nonreduced SDS/PAGE and analysed with anti-IGFBP-1 according to the standard procedure. The results (not shown) indicated that the IGFBP-1 samples indeed were completely reduced. Apparently the absorbance is quenched by the IGFBP-1 protein In a parallel experiment to determine the free -SH groups in human serum albumin (HSA) we found an efficiency of 64% for this reaction suggesting that quenching of the absorbance is inherent for this method and depends on the nature of the protein In non-reduced IGFBP-1 preparations after correction for an efficiency of 26% we found 1 1±0.9 (n=4) mole free -SH groups per mole IGFBP-1. These results correspond to 0 or at most 2 free -SH groups suggesting that most if not all cysteines are involved in disulphide bonds.

4 DISCUSSION

In the present study we analyzed the effect of mutations in the N-terminal part of IGFBP-1 on IGF binding In addition, we determined the number of disulphide bonds Deletion of 60 N-terminal amino acid residues of IGFBP-1 resulted in loss of IGF binding suggesting a possible IGF binding site in this region This finding is consistent with the report that N-terminal fragments are capable of binding IGF [15] Therefore we focussed our attention on the most conserved stretch of amino acids in this region, spanning residues 26–50.

```
               26                            55
pSV19   ---VTRSAGCGCCPMCALPLGAACGVATARCAR---
NI      ---------------Y--------------------
NII     --------------------D---------------
NIII    --------------I---------------------
NIV     -----------Y--I-----------T---------
NV      -------------------------IT---------
NVI     ------Q-----------------------------
NVII    ----------D-------------------------
NVIII   --------------------------T---------
```

Fig 3 The amino acid sequence of the N-terminal region of IGFBP-1 mutants Changes in amino acid residues are indicated for the different IGFBP-1 point mutants (NI-VIII) On top the amino acid residues of the wild type sequence are shown

However, our results show that most of the point mutations introduced in this part of the N-terminus did not affect IGF binding but merely resulted in minor changes in the conformation of the IGFBP-1 molecule This effect, shown as a shift in apparent molecular weight, is most clearly seen with mutant NV(I48,T49) (Fig. 4A and B) Only substitution of Cys-38 for an tyrosine residue in mutant NI(Y38) resulted in loss of IGF binding and was accompanied by a dramatic change in conformation as suggested by the shift in migration on the gel (Fig. 4A and B). Such a structural change may influence the IGF binding site in a way that ligand binding is impossible It has been reported by others [20] that substitution of Cys-16 and Cys-35 for serine residues also abolished IGF binding It seems obvious that these three cysteine residues in the N-terminal region are essential for the integrity of the IGFBP-1 molecule due to their participation in disulphide bond formation

Therefore, we also determined the number of free -SH groups in IGFBP-1 Our data suggest that at least 16 but most likely all cysteine residues form disulphide bonds, implying that IGFBP-1 indeed has a tightly fold-

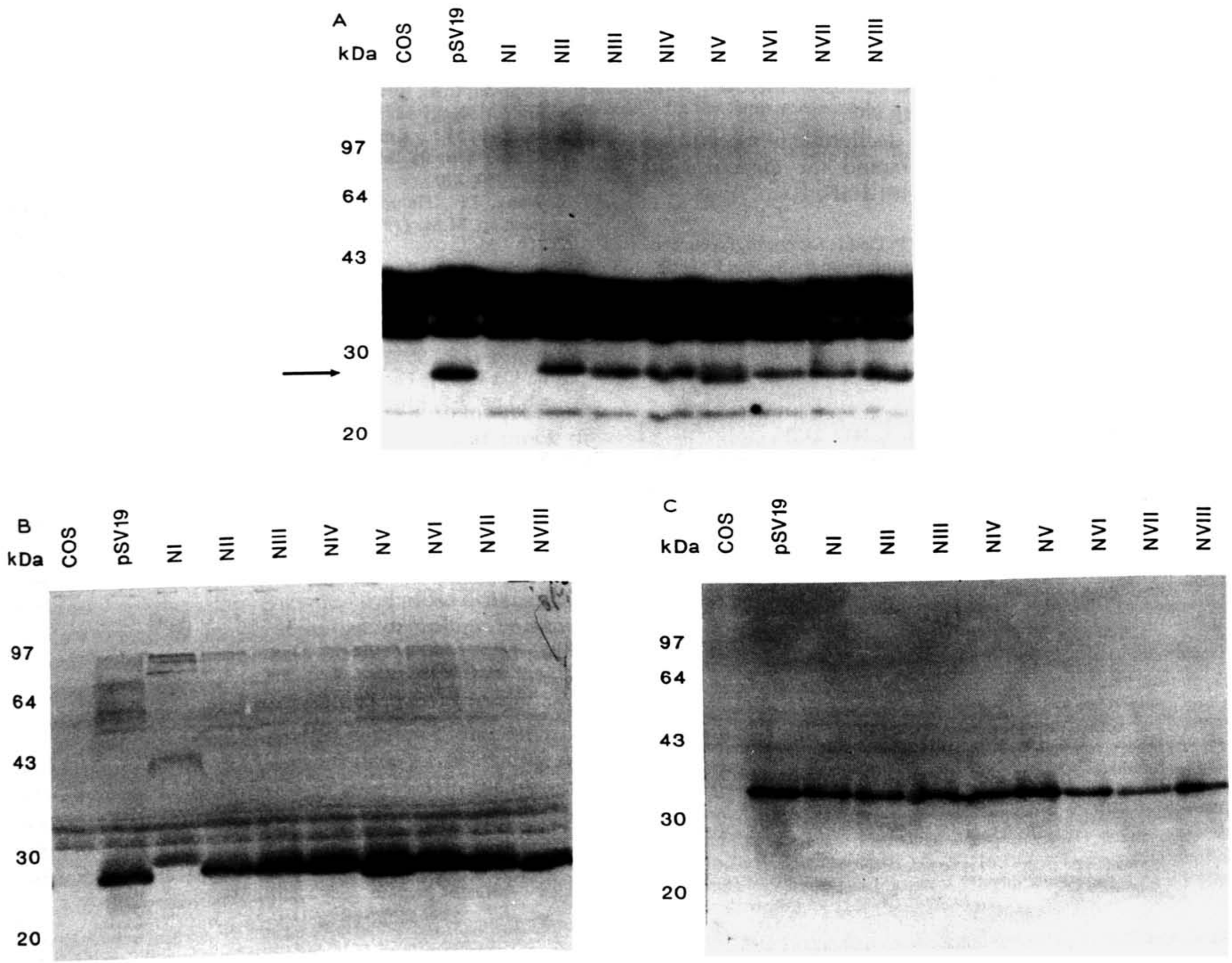

Fig 4 Analysis of mutant IGFBP-1 proteins in culture medium of COS-1 cells transfected with the expression plasmids from Fig 3 For further details see legend to Fig 2

ed conformation Considering the conservation of the cysteine residues this is most likely true for all IGFBPs

Although our data suggest that all cysteine residues are involved in disulphide bond formation apparently not all cysteine residues contribute to the structure to the same extent The results obtained with the mutants NIII(I37), NVIII(T49) and NIV(Y34,I37,T49) indicate that substitution of Cys-34 for a tyrosine residue has no effect on IGF binding nor on the apparent molecular weight (Fig 4A and B) Thus Cys-34 in contrast to its neighbouring cysteine residues (Cys-35 and Cys-38) is not essential for maintaining IGFBP-1 structure Loss of ligand binding observed in some of the mutant IGFBP-1 proteins is most probably the result of major structural changes introduced by deleting significant parts of N-terminal region or by mutations presumably preventing cysteine residues to form disulphide bonds The same seems to be true for the C-terminal region of IGFBP-1 in which we observed that Cys-226 is also essential for IGF binding [17]. Therefore, it may be possible that neither the N- nor the C-terminal ends separately comprise the IGF binding site but rather that both ends linked by disulphide bonds contribute to a spatially organized IGF binding site

Future research to assign the disulphide bonds will be of great importance to understand the structure of IGFBP-1 and its interaction with IGF.

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