Engineering the C-region of human insulin-like growth factor-1: implications for receptor binding

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Recombinant wild-type human IGF-1 and a C-region mutant in which residues 28–37 have been replaced by a 4-glycine bridge (4-Gly IGF-1) were secreted and purified from yeast. An IGF-1 analogue in which residues 29–41 of the C-region have been deleted (mini IGF-1) was created by site-directed mutagenesis and also expressed. All three proteins adopted the insulin-fold as determined by circular dichroism. The significantly raised expression levels of mini IGF-1 allowed the recording of two-dimensional NMR spectra. The affinity of 4-Gly IGF-1 for the IGF-1 receptor was ~100-fold lower than that of wild-type IGF-1 and the affinity for the insulin receptor was ~10-fold lower. Mini IGF-1 showed no affinity for either receptor. Not only does the C-region of IGF-1 contribute directly to the free energy of binding to the IGF-1 receptor, but also the absence of flexibility in this region eliminates binding altogether. As postulated for the binding of insulin to its own receptor, it is proposed that binding of IGF-1 to the IGF-1 receptor also involves a conformational change in which the C-terminal B-region residues detach from the body of the molecule to expose the underlying A-region residues.

Keywords: insulin/insulin-like growth factor-1/receptor binding/site-directed mutagenesis/structure–function relationships

Introduction

Insulin-like growth factor 1 (IGF-1) is a 70 amino acid serum protein which has many growth-promoting and metabolic activities (for a review, see Froesch et al., 1985). The first 29 residues of IGF-1 are homologous with the B-chain of insulin (B-region; 1–29), there follow 12 residues which are analogous to the C-peptide of proinsulin (C-region; 30–41) and a 21 residue region which is homologous to the A-chain of insulin (A-region; 42–62). The C-terminal octapeptide (D-region; 63–70) has no counterpart in the insulin molecule. IGF-1 is a member of the insulin superfamily whose members include insulin, IGF-2, relaxin, bombxin and the molluscan insect peptides (for a review, see Murray-Rust et al., 1992). The IGFs are the only members of the insulin superfamily in which the C-region is not proteolytically removed after translation. In the absence of crystals suitable for X-ray analysis, the tertiary structures of the IGFs have been modelled on that of porcine insulin (Blundell et al., 1978, 1983) and 2-D NMR studies of IGF-1 have confirmed that the solution structure is consistent with the model (Cooke et al., 1991; Sato et al., 1993).

The IGFs are able to bind to more than one receptor; IGF-1 binds with highest affinity to the IGF-1 receptor, a receptor structurally homologous to the insulin receptor (Massague and Czech, 1982; Ullrich et al., 1986) with which IGF-1 cross-reacts with lower affinity. The growth-promoting effects of insulin and the metabolic activity of IGF-1 are thought to arise from cross-binding to each other’s receptors. Ligand–receptor specificity in the insulin and IGF-1 systems is determined by the sequence differences between the two ligands and within the common binding sites of the two receptors (Kjeldsen et al., 1991). Studies with site-directed mutants of IGF-1 (reviewed by Cascieri and Bayne, 1990) indicate that the IGF-1 receptor recognizes tyrosines 24 (in the B-region β-strand) and 31 (in the C-region) and that binding is unaffected by the presence of the D-region. The insulin receptor also recognizes Tyr24 but the presence of the D-region impedes binding of IGF-1 to this receptor. Thus the presence of the D-region reduces the affinity of IGF-1 for the insulin receptor whereas the additional contribution of Tyr31 is thought to account for its higher affinity for the IGF-1 receptor, which may have a binding site for this residue without a counterpart in the insulin receptor (De Meyts, 1994).

The distance between the ε-amino group at B29 Lys and the α-amino group at A1 Gly in insulin varies from 7.5 to 13.5 Å, depending on the crystal type (Cutfield et al., 1981a). Mini-proinsulins cross-linked between these two residues by bivalent reagents have been important for investigating the structure–function relationships of insulin (Brandenburg et al., 1972, 1973, 1975, 1977; Brandenburg and Wollmer, 1973; Freychet et al., 1974; Gliemann and Gammeltoft, 1974). Circular dichroic (Brandenburg et al., 1972, 1973) and crystallographic (Cutfield et al., 1981b) studies have demonstrated that such cross-links do not alter the molecule’s structure significantly. There exists a linear relationship between the length of the cross-link and the binding affinity for the insulin receptor, with the lowest affinities for the shorter cross-links, and it has been estimated that motions with regard to the relative positions of A1 and B29 are important in receptor binding although they only account for a small proportion of the binding energy (Nakagawa and Tager, 1989). A single-chain (des B30) insulin in which B29 Lys forms a peptide bond with A1 Gly has an unmeasurably low in vitro potency (Markussen et al., 1985) despite having a crystal structure isomorphous with that of insulin (Derewenda et al., 1991).

In order to make sense of the crystallographic and biological data, it has been proposed that unfolding of the B-chain C-terminus and exposure of the A-chain N-terminal region of insulin is required for activity (Mirmira and Tager, 1989; McLeod et al., 1990; Derewenda et al., 1991; Hua et al., 1991; Liang et al., 1992; Murray-Rust et al., 1992). Consistent
with this hypothesis, NMR, molecular dynamics and equilibrium-folding studies (Weiss et al., 1990; Bryant et al., 1992; Krüger et al., 1994) all indicate that the B-chain C-terminus of insulin is highly flexible. The proposed conformational change would expose the evolutionarily conserved A-chain N-terminal residues and allow them to interact with the receptor (Cutfield et al., 1981a) and would certainly be restricted by an A1–B29 cross-link (Wollmer et al., 1989). The proposed receptor binding mechanism would also account for the full receptor binding potency of des (B26–B30) pentapeptide insulin (DP) amide (Fischer et al., 1985) and the near native receptor binding potency of B24 Phe → Gly insulin (Mirmira and Tager, 1989). In DP amide the B-chain C-terminal residues are absent, and the NMR structure of B24 Gly insulin shows that the loss of the B24 Phe side-chain tertiary contacts destabilizes these residues so that they no longer pack against the body of the molecule (Hua et al., 1991).

Studies on the binding of IGF-1 to the insulin receptor demonstrate that substitution of residues Phe48, Arg49 and Ser50 of the A-region turn of IGF-1 by their insulin equivalents do not affect receptor binding (Joshi et al., 1990); however, replacement of A-region residues which are conserved in both IGF-1 and insulin do prevent high affinity binding (Cara et al., 1990). The interaction of A-region residues of IGF-1 with the insulin receptor is difficult to reconcile with the solution structure of IGF-1 unless a conformational change similar to that postulated for insulin is involved. Furthermore, in view of the similarity of the IGF-1 receptor to the insulin receptor, it is also of considerable interest whether the postulated receptor-binding mechanism also applies to the interaction of IGF-1 with the IGF-1 receptor.

In this paper we characterize a ‘mini IGF-1’ in which the C-region has been removed by connecting Pro28 to Gly42 via a peptide bond. This molecule is an IGF-1 analogue of the single-chain insulin described above. The properties of mini IGF-1 are compared and contrasted with those of wild-type IGF-1 and another C-region mutant in which a 4-Gly bridge spans the 8 Å distance between Lys27 and Ala38 (4-Gly IGF-1; Bayne et al., 1989) (Figure 1).

Materials and methods

Cloning, expression and purification of IGF-1 mutants in yeast

All DNA manipulations were carried out in Escherichia coli strains TG1 and M147 (dam-) using standard procedures (Sambrook et al., 1989). Deletion of the unique BamHI site of yeast expression vector pMA91 (Mellor et al., 1985) to create pMA91ΔBam has been described (Gill, 1994). The gene for wild-type IGF-1 preceded by the MF61 pre-pro leader sequence (Bayne et al., 1988) was cloned into the unique BcII cloning site of pMA91ΔBam. The gene for 4-Gly IGF-1 was excised from E. coli vector pJY2 (Bayne et al., 1989) using BamHI and also cloned into pMA91ΔBam after removing the wild-type IGF-1 BamHI fragment from pMA91ΔBam/IGF-1. The wild-type and 4-Gly IGF-1 BamHI fragments were also subcloned into BamHI digested M13mp18 phage and sequenced by the dideoxy method. Mini IGF-1 was constructed by site-directed mutagenesis, using the deoxyoligonucleotide 5’-CTCTCATCACAACCGGATATCGATGAACTG-3’. The mini IGF-1 gene was excised from M13/mini IGF-1 DNA using BamHI and also cloned into pMA91ΔBam. Saccharomyces cerevisiae F107 (leu2-3, pep4-3) cells were transformed with the three expression constructs using the spheroplast method and grown to saturation in three 50 I fermentations in Leu–medium. Secreted IGF-1 was purified by batch binding to Bio-Rex 70, desalting on Sephadex G10 and gel filtration on Bio-Gel P10 (Gill, 1994). The extinction coefficients of wild-type, 4-Gly and mini IGF-1 in 10% acetic acid were 0.69, 0.54 and 0.45 OD_{280} units/cm, respectively.

Western blotting of monomeric IGF-1 molecules

Wild-type, 4-Gly and mini IGF-1 were denatured in Phast-gel loading buffer and electrophoresed on a 20% homogeneous Phast-gel using a Phast System apparatus (Pharmacia). The proteins were transferred onto nitrocellulose and blotted with rabbit anti-hIGF-1 antibody from an Amersham IGF-1 reagent pack for RIA (Amersham IM.1721). Bound IGF-1 was visualized by blotting with anti-rabbit IgG alkaline phosphatase conjugate (Sigma A-8025) and staining with nitroblue tetrazolium (Sigma N6876).

Purification of monomeric IGF-1 molecules by reversed-phase (RP) HPLC

Disulphide isomers of IGF-1 (Elliot et al., 1990) were separated by RP-HPLC using an isocratic acetonitrile–phosphate buffer system on a Vydac 218 TP 54 (300 Å) (Separations Group, Hesperia, CA) column (25×0.46 cm i.d.) of butyl-derivatized end-capped 5 μm silica. The correctly folded IGF-1 species were desalted on the same column using the gradient of Miller et al. (1993). The column was fitted with a pre-filter and used on a Varian 5000 liquid chromatograph. The flow-rate was 1 ml/min, the detector wavelength was 280 nm, the cell pathlength was 2 mm and the output was 10 mV/AU.

Amino acid analysis

The composition and correct processing of each IGF-1 species were verified by amino acid analysis (except for cysteine/cystine), which was carried out after 24 h of hydrolysis in 6 M HCl (containing 1% phenol) at 110°C in vacuo. Samples were analysed on an LKB Biochrom 4151 alpha plus amino acid analyser utilizing ninhydrin post-column detection.

Circular dichroism spectroscopy

CD measurements were carried out in 5% acetic acid on an AVIV (Lakewood, NJ) CD 62 DS spectrometer calibrated with d_{20} camphorsulphonic acid (Chen and Yang, 1977). One preparation of wild-type, 4-Gly and mini IGF-1 species was measured (0.93, 1.13 and 0.87 mg/ml, respectively, in a 0.2 mm pathlength cell in the far-UV and a 7.5 mm pathlength cell in the near-UV region), the number of scans varying between three and 10 depending on the signal-to-noise ratio. The spectral bandwidth was 1.5 nm and the temperature was 27°C. Data were collected at 0.1 nm intervals in the near-UV and at 0.2 nm intervals in the far-UV region. Splines were fitted through the averaged data points and the CD set to zero at 250 nm. For comparison purposes, protein concentrations were normalized for tyrosine content, determined from the second derivative of the near-UV absorption spectrum in the 290–295 nm region (Balestrieri et al., 1978). In order to obtain CD

Fig. 1. The amino acid sequences of wild-type, 4-Gly and mini IGF-1 in the C-region.
difference spectra extending to 185 nm in the far-UV region, the spectra of 4-Gly and mini IGF-1 were subtracted from that of non-glycosylated IGF-1 produced in E.coli.

NMR spectroscopy

Large quantities of mini IGF-1 for NMR analysis were desalted on a Vydac 218 TP 1010 (300 Å, 10 μm) preparative-scale column (25×1.00 cm i.d.) at 2 ml/min. Two-dimensional NMR spectra of mini IGF-1 were recorded on a Varian Unity 500 MHz spectrometer. The sample was prepared by dissolving lyophilized protein in 80% H2O-10% D2O-10% deuteric acid. The protein solution (2–3 mM) was filtered through a 0.45 μm low protein binding disposable filter (Millipore) before placing the sample in the NMR sample tube. Nuclear Overhauser effect spectroscopy (NOESY) was performed at 30°C and pH 2.5 in the phase-sensitive mode using the time-proportional phase incrementation method in ω1 and quadrature detection in ω2 (Drobny et al., 1979; Bodenhausen et al., 1980). The two-dimensional spectra were recorded with 512×2048 data points and with spectral widths of 6000 and 7000 Hz, respectively. The water resonance was presaturated with a weak radiofrequency field using a 1 s preparation period, and the induction decays from the NOESY spectra (with mixing times 50, 100, 150, 200, 250 and 300 ms) and total correlation spectroscopy (TOCSY) spectra (with mixing times 22 and 33 ms) were multiplied by a 60° phase-shifted sine-bell in both dimensions. Both NOESY and TOCSY spectra were zero-filled in ω1 and Fourier transformed to obtain final spectra with 1024×1024 data points. Spectra were baseline corrected using a polynomial function. All data were processed and analysed with the program SNARF, written by Frans van Hoesel (Groningen, The Netherlands).

Computer modelling

The structural effects of the C-region modifications were examined using the IGF-1 model (Blundell et al., 1983) as the starting geometry (entry IGF1 in the Brookhaven database) and the CHARMm22 extended atom potential (Brooks et al., 1983) which includes an explicit representation of all non-hydrogen atoms and the polar hydrogens. The positions of the polar hydrogens were built using the HBUILD (Brünger and Karplus, 1983)
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Fig. 5. Far-UV CD spectra of yeast-produced and E.coli-produced IGF-1 superposed for comparison (all spectra measured in 5% acetic acid). For comparison, concentrations were normalized for tyrosine content.

Fig. 6. Far-UV CD spectra of wild-type, 4-Gly and mini IGF-1 superposed for comparison.

1988) function of CHARMM and charges were assigned using the standard CHARMM parameter sets (Momany and Rone, 1992). The insertion of the 4-Gly bridge between residues Lys27 and Ala38 was carried out using QUANTA (MSI, Burlington, MA). The connection of Pro28 to Gly42 via a peptide bond to give a starting model of mini IGF-1 was carried out similarly. Solvent was implicitly represented by a distance-dependent dielectric (ε = R) and non-bonded interactions were truncated by a shifted potential (Brooks et al., 1983) with an 11 Å cut-off (Loncharich and Brooks, 1989). Initially, the conformations of the amino acids at the sites of the mutations were allowed to relax to a local energy minimum (while the rest of the molecule was kept fixed) with the steepest descents and adapted basis Newton–Raphson algorithms (Brooks et al., 1983). This was followed by energy minimization with harmonic restraints on all the non-hydrogen atoms of the rest of the molecule. The force constants used to restrain the atoms were scaled down periodically from 1 to $10^{-3}$ kcal/mol Å$^2$. Energy minimizations were carried out until the change in the average force on the atoms between subsequent steps was $\leq 10^{-3}$ kcal/mol Å. Each optimized structure was then solvated using a modified TIP3P model (Jorgensen et al., 1983) with the dielectric constant set to 1. Each structure was embedded in a 31 Å radius sphere of pre-equilibrated water molecules; any water molecule whose oxygen atom was within 2.8 Å of any protein atom was then removed, thus ensuring that the protein was surrounded by a shell of water molecules at least 6 Å thick. The hydrogen atoms of the added waters were rebuilt in the field of the static proteins using HBUILD. The systems were then subjected to full energy minimizations, with the outer 3 Å shell of water molecules restrained harmonically with a force constant of 0.1 kcal/mol Å$^2$. The starting geometry of the wild-type IGF-1 model was subjected to the same energy minimization protocols as the 4-Gly and mini IGF-1 models.

Fig. 7. Far-UV CD difference spectra between IGF-1 (non-glycosylated from E.coli) and 4-Gly IGF-1 and between IGF-1 and mini IGF-1.

Fig. 8. Near-UV CD spectra of wild-type, 4-Gly and mini IGF-1 superposed for comparison.
Fig. 9. The dNN connectivities in the 200 ms NOESY spectrum of mini IGF-1 at 30°C. The cross-peaks for the segments Ala8-Cys18, Ile43-Arg50 and Leu54-Cys61 are connected by lines (amino acid residues are numbered as in wild-type IGF-1). The chemical shifts are given relative to DSS.

Equilibrium binding to the IGF-1 receptor
Tracer binding to membrane-bound IGF-1 receptors overexpressed on the surface of stably transfected baby hamster kidney cells (Andersen et al., 1992) was carried out in the presence of increasing concentrations of wild-type, 4-Gly and mini IGF-1. Transfected BHK cells were washed with Hanks balanced salt solution and the cell concentration determined using a haemocytometer (Scherf Pritzison, Germany). Approximately 40 000 cells in 200 µl were incubated (in duplicate) with 25 µl of [125I]IGF-1 (~15 000 c.p.m.) and increasing concentrations of unlabelled ligand in 25 µl of binding buffer, pH 7.6 [100 mM Hepes, 100 mM NaCl, 5 mM KCl, 1.3 mM MgSO4, 1 mM EDTA, 15 mM sodium acetate, 10 mM glucose and 1% bovine serum albumin (BSA)]. The number of cells used was chosen to give ~20% binding in the absence of competing ligand. After 3 h at 4°C the cells were centrifuged at 10 000 g for 5 min, the unbound ligand was aspirated and the pellet was counted for 10 min. [125I]IGF-1 (recombinant from E.coli; Groprep, Adelaide, Australia) was iodinated exclusively on Tyr31 at Novo Nordisk, Denmark (Schäffer et al., 1993). Non-linear least-squares analysis of the competition data was performed assuming one high-affinity bound ligand per receptor dimer (Christoffersen et al., 1994).

Thymidine incorporation assay
Subconfluent monolayers of NIH 3T3 cells (American Type Culture Collection) expressing ~10^6 IGF-1 receptors per cell (Steele-Perkins et al., 1989) in 96-well microtitre plates were starved in Dulbecco’s modification of Eagle’s medium (DMEM) with 2% neonatal calf-serum (NCS) for 2 days to achieve quiescence. Wild-type, 4-Gly and mini IGF-1 were added in different concentrations; after 17 h, the medium was aspirated and the cells pulse-labelled at 37°C with 0.2 µCi/well [methyl-1',2',3']thymidine (Amersham) in fresh medium for 3 h. Finally, the cells were solubilized in 0.2 M NaOH, harvested on Whatman glass microfiber filters using a Betaplate™ 96-well harvester (Pharmacia) and counted for radioactivity (5 min).

Fat cell assay
Isolated fat cells were prepared by the method of Gliemann (1965) from the epididymal fat pads of male Sprague–Dawley rats (~150 g fed ad libitum). Tissue was digested in collagenase buffer (Krebs-Ringer Hepes containing 3.5% w/v human serum albumin and 1 mM glucose) with 0.5 mg/ml of bacterial collagenase (Worthington type 1; ~276 U/mg) for 45 min at 37°C. The adipocytes were washed three times in washing buffer (KRH containing 3.5% w/v HSA and 0.27 mM glucose) and resuspended in incubation buffer (KRH containing 1% HSA, 0.1 mM glucose and 0.5 mg/ml bacitracin). Cell numbers were determined using a haemocytometer (Scherf Pritzison, Germany). Hormone stimulated lipogenesis from glucose was determined by the method of Moody et al. (1974) after fat cells (0.5% v/v = 400 000 cells/ml) had been incubated in triplicate in plastic scintillation vials for 2 h at 37°C in 0.5 ml of incubation buffer containing 180 mCi [3-3H]-d-glucose (Amersham) and 50 µl of the insulin or IGF-1 analogue. Incubation was terminated by the addition of 3 ml of toluene scintillator [0.3 g of 1,4-bis(5-phenyl-2-oxazolyl)benzene-2,2’-p-phenylenebis(5-phenyloxazole) (POPOP) and 5 g of 2,5-diphenyloxazole (PPO) in 1 l of toluene] and total lipids were extracted by allowing the sample to stand for 24 h at 4°C. Under these conditions, unmetabolized labelled glucose is not extracted into the organic phase and is not counted. Controls
with no hormone added were prepared by adding cells to aliquots of the isotope and stopping the incubation immediately. The samples were counted in a β-counter (Tri-carb 460-C Liquid Scintillation System) for 7 min and the counts corrected for scintillator background.

**Results**

Upon gel filtration of the secreted IGF-1 on Bio-Gel P10 to separate aggregated from monomeric material, the elution order wild-type > 4-Gly > mini is consistent with the reduced Stoke's volume expected upon progressive removal of the hydrophilic C-region (Figure 2). Approximately half of the recombinant IGF-1 molecules produced in yeast have been shown to be glycosylated with two mannose residues on Thr29 (Gellerfors et al., 1989) and this residue is eliminated in both C-region mutants. Glycosylation of wild-type IGF-1 which is identified by a shoulder on the monomer peak is eliminated in 4-Gly and mini IGF-1. The purity and identity of each IGF-1 species were confirmed by denaturing SDS–PAGE followed by Western blotting (Figure 3) and amino acid analysis. All three IGF-1 species bound the antibody, whereas glycosylated wild-type IGF-1 and proteins in the ladder did not. Desalting of the correctly-folded IGF-1 species by gradient RP-HPLC demonstrated similar retention times for wild-type and 4-Gly IGF-1 and a slightly longer retention time for mini IGF-1, which is less hydrophilic owing to complete removal of the C-region (Figure 4).

The far-UV CD spectrum of yeast-produced IGF-1 is presented in Figure 5, superposed upon the spectrum of E.coli-produced IGF-1. The spectra are similar to that of insulin, but unlike that of E.coli-produced IGF-1, the spectrum of yeast-produced IGF-1 does not extend down to 185 nm, probably owing to glycosylation (polysaccharides absorb at wavelengths shorter than 190 nm; Beychok, 1966). The far-UV CD spectra of wild-type, 4-Gly and mini IGF-1 are superposed in Figure 6, demonstrating that all three molecules have similar secondary structure. Far-UV CD difference spectra between IGF-1 and 4-Gly IGF-1 and between IGF-1 and mini IGF-1 resemble the classic spectrum of a random coil (Johnson, 1990), indicating that the C-region of IGF-1 contains little if any regular secondary structure (Figure 7). A similar spectrum has been reported for the difference of proinsulin and insulin far-UV CD spectra (Frank et al., 1972) and for the proinsulin C-peptide (Wollmer et al., 1975; Vogt et al., 1976). In the near-UV region (Figure 8) where the CD is due to transitions of the aromatic and disulphide side chains, the spectra are qualitatively similar to that of insulin, with a positive band at ~255 nm and a broad negative band at ~265 nm, arising from a superposition of the longer wavelength disulphide and tyrosine transitions (Wollmer et al., 1977, 1980). The shallow nature of the negative band is characteristic of monomeric insulin; this extreme shows a sharp increase in dimeric and single-chain insulin (white) superposed for comparison (entries 4ins and 6ins, respectively, in the Brookhaven database). The side chains of the insulin receptor binding determinant (B25 Phe) are also shown.

Computer modelling shows overall root mean square deviations (Cα backbone) from the wild-type IGF-1 structure of ~1.1 Å for both 4-Gly and mini IGF-1, however, the perturbations are larger at the start of the B-region and in the D-region. The loss of the hydrogen bond between Asn26 and Arg37 and the salt-bridge between Arg36 and Asp45 in both C-region mutants results in large perturbations of the the first A-region helix. The perturbations in the C-region appear to be transmitted to the B-region helix through changes in the conformations of Phe25 and Val31 side chains. Owing to the loss of side-chain contacts, large changes (~140°) take place in the side-chain dihedral angles of residues that border the mutated loop region (the potential for hydrogen bonding with C-region residues is eliminated) as the changes are absorbed. The superposed IGF-1 models are shown in ribbon form in Figure 10a. For comparison, the superposed X-ray structures of insulin and single-chain insulin are shown in Figure 10b.

Competition data for the inhibition of [125I]IGF-1 binding to BHK cells overexpressing the IGF-1 receptor by the different IGF-1 species are presented in Figure 11. The concentration
of wild-type IGF-1 required for half-maximum inhibition of tracer binding was ~1 nM. The corresponding value for 4-Gly IGF-1 was ~100 nM, a reduction of ~100-fold in affinity. The data for mini IGF-1 showed too much scatter to be fitted but are consistent with no binding.

In order to assess the mitogenicity of the IGF-1 analogues, stimulation of DNA synthesis in NIH 3T3 cells (murine cells transfected with human IGF-1 receptor DNA) was determined from the quantity of [\textsuperscript{3}H]thymidine incorporated into DNA after exposure of the serum starved cells to hormone or serum only [10% neonatal calf serum (NCS)] for 17 h. The averaged results of three experiments are presented in Figure 12, expressed as the percentage of [\textsuperscript{3}H]thymidine incorporation relative to stimulation with 10% NCS only. Half-maximum stimulation occurs at ~0.4 nM wild-type IGF-1. 4-Gly IGF-1 appears to be capable of eliciting the same maximum response as wild-type IGF-1 at the higher concentrations; half-maximum stimulation occurs at ~7 nM, a reduction of ~20-fold in potency. If mini IGF-1 is capable of stimulating DNA synthesis to the same maximum level as wild-type IGF-1, then its ED\textsubscript{50} value is at least 1 \mu M. It is not clear whether mini IGF-1 is capable of eliciting the maximum response.

In the presence of insulin, adipose tissue incubated in vitro incorporates more glucose carbon into fatty acids and glycerol (Cahill et al., 1959). Data from two lipogenesis experiments are presented in Figure 13. Half-maximum stimulation of lipogenesis occurs at ~0.1 nM insulin concentration and at ~40 nM wild-type IGF-1 concentration, a reduction of ~400-fold in potency. The potency of 4-Gly IGF-1 is reduced by ~10-fold compared with that of wild-type IGF-1, and mini IGF-1 shows no lipogenesis stimulating effect, even at 1 \mu M concentration.

**Discussion**

Both 4-Gly and mini IGF-1 were correctly-folded as demonstrated by the far-UV CD spectra and 2-D NMR spectra in the case of mini IGF-1. Furthermore, the binding affinity of both C-region mutants for IGF binding protein-3 has been shown to be essentially the same as that of wild-type IGF-1 (Heding et al., 1996). The reduced affinity of 4-Gly IGF-1 for the IGF-1 receptor and its lowered mitogenicity compared with wild-type IGF-1 can be ascribed in large part to the loss of Tyr31 which is known to be important for IGF-1 receptor binding (Bayne et al., 1989). The lack of affinity of mini IGF-1 for the same receptor and the absence of mitogenicity suggest that binding and mitogenicity in IGF-1 depend not only on the presence of Tyr31 but also on properties which 4-Gly and mini IGF-1 do not share. One such property is the longer...
bridge connecting the A- and B-regions in 4-Gly IGF-1, which would facilitate a greater conformational change than would be possible in mini IGF-1.

The in vitro potency of insulin analogues in the lipogenesis assay has been shown to be proportional to their relative binding affinities at the insulin receptor (Glimmern and Gammeltoft, 1974). Previously, the affinity of 4-Gly IGF-1 for the insulin receptor has been shown to be the same as that of wild-type IGF-1 (Bayne et al., 1989), suggesting that Tyr31 is not involved in this interaction. In the present lipogenesis assay, 4-Gly IGF-1 was slightly less potent than wild-type IGF-1 (~10-fold reduction), indicating that the loss of Tyr31 and a reduction of flexibility arising from the 4-Gly bridge slightly reduce the affinity of this analogue for the insulin receptor. The complete absence of lipogenicity in mini IGF-1 suggests that this is not due to the absence of Tyr31 but is mainly a result of the decreased flexibility in this region of the molecule.

We hypothesize that the loss of activity in mini IGF-1 arises from the inability of the B-region β-strand to separate from the body of the molecule and expose conserved core residues for interaction with the IGF-1 and insulin receptors. We propose to test this hypothesis by expressing a mini IGF-1 molecule incorporating an Asn–Gly site at the A-B-region junction and assessing whether cleavage with hydroxylamine recovers activity.

In conclusion, receptor binding in insulin has been demonstrated to require not only the presence of the correct interaction surfaces but also the propensity for conformational change. The properties of the C-region IGF-1 mutants described here suggest that receptor binding in IGF-1 may require a similar conformational change.

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