Binding affinities of insulin-like growth factor-I (IGF-I) fusion proteins to IGF binding protein 1 and IGF-I receptor are not correlated with mitogenic activity

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Abstract In this report, comparisons between molecular affinities and cellular proliferation activities have been made for insulin-like growth factor-I (IGF-I) and two IGF-I fusion proteins in order to evaluate fusion proteins as tools for receptor binding studies. Binding affinities and growth promoting effects of the N-terminal fusion Z-IGF-I and the C-terminal fusion IGF-I-Z, and native recombinant human IGF-I, were analyzed. Binding kinetic properties of the three IGF-I variants were analyzed using BIAcore kinetic interaction analysis testing for binding to both human IGF binding protein 1 (IGFBP-1) and a soluble form of the human IGF type I receptor extracellular domains (sIGF-I^R). The growth promoting effects on SaOS-2 human osteosarcoma cells of the different fusion proteins were analyzed. A comparison of receptor binding affinities and growth promoting effects shows that the fusion protein receptor affinity does not correlate with proliferative potential. The IGF-I-Z fusion, with the lowest receptor affinity, shows similar proliferative potential to native IGF-I. However, the Z-IGF-I fusion protein, with twice the receptor affinity of IGF-I-Z, displays only about 70% of the IGF-I-Z growth promoting activity. Both IGF-I fusion proteins possess similar affinity to IGFBP-1. These results indicate that determinants other than the receptor affinity could be involved in the regulation of IGF-I proliferative action. This study demonstrates that ligand fusion proteins may be useful to study mechanisms of ligand induced receptor activation.

Key words: Insulin-like growth factor-I; Insulin-like growth factor-I receptor; Insulin-like growth factor binding protein-1; Protein-protein interaction; Biosensor

1. Introduction

Insulin-like growth factor-I (IGF-I) is a small, 7.6 kDa protein which stimulates a variety of growth promoting and metabolic effects. The bioactivities of the IGF molecules in circulation are modulated by a group of specific high affinity binding proteins (IGFBPs), which share no sequence homology with IGF receptors. Seven distinct IGFBPs (IGFBP-1–7) have to date been characterized in mammalian systems [1–3]. The cellular response to IGF-I is primarily mediated through the specific interaction between IGF-I and the cellular IGF-I receptor (IGF-I^R) [4]. Both IGF-I^R and the homologous insulin receptor are suggested to be activated through a ligand induced conformational change and subsequent trans-phosphorylation of the intracellular tyrosine kinase domains leading to mitogenic and metabolic signaling [5–8]. In this study, we explore the use of IGF-I fusion proteins to study IGF-I specific protein-protein interactions. We decided to test IGF-I binding properties as fusions to the IgG binding Z domain, a 63 amino acid protein frequently used as an affinity handle [9]. Z domains have been extensively used as fusion partners in recombinant production systems to increase the production of the desired protein, to solubilize the protein product and as an important tool for purification and detection of a variety of recombinant proteins [9,10]. The Z protein is well characterized, and a NMR solution structure has recently been published [11]. The fusion protein production approach was chosen to serve several purposes. The use of fusion proteins in the analysis of specific protein interactions has some major advantages, the predominant one being ease of production and purification of proteins with altered biochemical characteristics, i e. mutated variants. The production of IGF molecules meets certain difficulties and makes specific demands on production and purification, such as the need to refold the molecule and specifically purify only the correctly folded variant with native disulfide pairing, generally resulting in low yields. The successful use of the Z domain in facilitating IGF-I production has been described previously. The use of a fusion protein is further likely to affect the binding kinetic properties and therefore modulate the binding equilibria for the different interactions. N- and C-terminal fusions may differentially inhibit binding to either receptor or binding protein, thereby possibly providing tools to rationalize the signaling properties of the IGF molecules. A comparison was made between N- and C-terminal fusions to IGF-I and the native unfused molecule for their ability to bind to IGFBP-1, and to IGF-I^R as well as cell proliferative activities. We demonstrate that the cell proliferative potency is not correlated with receptor affinity in this system. The different properties of the fusion proteins used in terms of both receptor and IGFBP binding could be potentially useful to create tools for dissecting IGF signaling mechanisms such as receptor conformational rearrangements and intracellular phosphorylation pathways.

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Abbreviations: CD, circular dichroism; EDC, *N'*-ethyl-*N'*-(dimethylaminopropyl)-carbodiimide; NHS, *N*-hydroxysuccinimide; IGF-I, insulin-like growth factor-I; IGFBP-1, insulin-like growth factor binding protein-1; IGF-I^R, insulin-like growth factor-I receptor; sIGF-I^R, soluble insulin-like growth factor-I receptor extracellular portion; RU, resonance units; Gdn-Hcl, guanidine hydrochloride; FCS, fetal calf serum; PFPA, pentafluoropropionic acid

2. Materials and methods

2.1. Production and purification of IGF-I proteins

IGF-I was produced as fusion to the 63 amino acid synthetic immunoglobulin G (IgG) binding domain Z, derived from the B-domain

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of staphylococcal protein A [12]. The Z-IGF-I protein was produced using an *Escherichia coli* intracellular production system. *E. coli* RV 308 transformed with the production vector pKP522 [13] was grown in a 5 l bioreactor in minimal medium supplemented with 1% yeast extract. Proteins were released by dissolving harvested cells in 6 M Gdn-HCl. The suspension was diluted to a final Gdn-HCl concentration of 1 M, using 1×TST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) and centrifuged before passing the supernatant over an IgG Sepharose 6FF affinity chromatography column (Pharmacia Biotech, Uppsala, Sweden). The column was washed with 10 column volumes of 1×TST buffer. The pH was lowered by washing the column with two column volumes of 5 mM ammonium acetate pH 5. The fusion protein was eluted with 0.2 M acetic acid pH 2.8. The protein eluate was subsequently concentrated to approximately 2 mg/ml by ultrafiltration using a Filtron Omegacell (Filtron Corp., Northborough, MA, USA) with 3 kDa cut-off. Final purification was performed by reverse phase HPLC with a linear gradient of 35–42% acetonitrile/water, 0.25% PFPA, at 1 ml/min over 50 min at a temperature of 50°C using a Kromasil KR-100-10 C8 (4.6×250 mm) column (Eka Nobel, Surte, Sweden). The protein was finally lyophilized and stored at −80°C. IGF-I-Z protein was produced using an *E. coli* secretion production system where the desired product is fused to a synthetic signal peptide and secreted to the periplasmic space. The *E. coli* strain UL 632 (L. Isaksson, personal communication) transformed with plasmid pKP1026 was grown in a 5 l bioreactor. Harvested cells were treated by cold osmotic shock [14] and the released periplasmic protein fraction was purified using IgG affinity chromatography. The affinity chromatography was performed as described above except that the column was equilibrated in 150 mM ammonium acetate pH 7.5. Final purification was by RP-HPLC using a gradient of 33–40% acetonitrile, 0.25% PFPA using the same column as before. Native recombinant IGF-I was prepared as described elsewhere [15,16].

2.2. IGF-I receptor

Soluble recombinant human IGF-I^R was produced in human 293 kidney cells [7]. The transmembrane and intracellular tyrosine kinase parts of the C-terminal β-domain have been replaced by the IgG binding Z domain (8.1 kDa). The secreted soluble form of the receptor forms an $(\alpha\beta'Z)_2$ receptor ectodomain moiety. The receptor was purified from conditioned cell medium using IgG Sepharose affinity chromatography as described [7]. The acid eluted material was collected in tubes containing one-third of the eluted volume of 1 M HEPES pH 8 in order to neutralize the eluted material and retain the purified receptor at a pH where activity is preserved. The receptor material was concentrated by ultrafiltration and stored in aliquots at −80°C.

2.3. Production and purification of IGFBP-1

Recombinant human IGFBP-1 was produced in DON cells transfected with a papilloma viral vector containing an expression cassette with the cloned human IGFBP-1 gene under the control of a BPV promoter [17]. The medium was clarified from cells by centrifugation and the supernatant was applied to an IGF-I Sepharose column, prepared from CNBr activated Sepharose CL-4B (Pharmacia Biotech), equilibrated with 1×TST. After sample application, the column was washed with five column volumes of TST followed by five volumes of 5 mM ammonium acetate at a linear flow rate of 5 cm/min. Bound IGFBP-1 was eluted using 1 M acetic acid pH 2.8 at the same flow rate. Eluted material was further applied to an S-Sepharose cation exchange column (Pharmacia Biotech) equilibrated with a buffer containing 90% 20 mM ammonium acetate pH 4.5 and 10% 500 mM ammonium acetate pH 7.0. After washing with equilibration buffer, the IGFBP-1 was eluted with a linear gradient of 10–70% 500 mM ammonium acetate pH 7.0 in 20 mM ammonium acetate pH 4.5 over 30 min, at a flow rate of 1 ml/min. Thereafter, purified IGFBP-1 protein was lyophilized and stored at −20°C.

2.4. Z protein

Pure Z protein was used as control in the BIAcore measurements. The Z protein was produced intracellularly in *E. coli* and IgG affinity purified as described elsewhere [18].

2.5. Protein analysis

Quantitative amino acid composition analyses were determined by acid hydrolysis followed by analysis using a Beckman 6300 amino acid analyzer, equipped with a System Gold data handling system (Beckman, Fullerton, CA, USA). Protein homogeneity was evaluated by SDS-PAGE or by RP-HPLC.

2.6. Circular dichroism

CD spectra were recorded using a Jasco J720 spectropolarimeter (Jasco Inc., Japan). Protein samples were dissolved in 10 mM potassium phosphate buffer pH 7.0, to a final concentration of 0.1 mg/ml. Spectra were recorded at 250–184 nm at a step resolution of 0.1 nm and a scanning speed of 5 nm/min, using 1 mm cuvettes. Each spectrum is the average of five accumulated scans. Actual concentration of each protein sample was determined, after recording spectra, by quantitative amino acid composition analysis.

2.7. Biosensor analysis

The BIAcore Sensorchip CM5 (certified grade), surfactant P20 and amine coupling reagents *N'*-ethyl-*N'*-(dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) and ethanolamine hydrochloride were obtained from Biacore AB (Uppsala, Sweden). All other buffer chemicals were obtained from Sigma (St. Louis, MO, USA) or Fluka (Buchs, Switzerland). All kinetic measurements were performed with the larger molecule IGFBP-1 or IGF-I^R, respectively, as the immobilized acceptor molecule. Immobilization of IGFBP-1 was performed at 5 μl/min in 1×HBS (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.05% P20) as driving buffer. IGFBP-1 was dissolved in 50 mM sodium acetate pH 4.7, at a concentration of 50 mg/ml. The protein was immobilized via primary amine groups as previously described [19] utilizing EDC/NHS carbodiimide coupling reagents, to a final resonance value between 700 and 2000 resonance units (RU). IGF-I receptor immobilization was performed by a similar procedure at pH 4. Final levels of IGF-I^R immobilization were 6500–7500 RU. All experiments were performed using 1×HBS as driving buffer at a flow rate of 8 μl/min. The injection of analyte was controlled using the *kinject* command in the Bialogue control software. Each sample was injected twice at five different concentrations in random order over the same surface in each measurement series. The concentrations used in IGFBP-1 kinetics were: IGF-I: 327, 109, 54.5, 27.3, 13.6 nM; Z-IGF-I and IGF-I-Z: 300, 100, 50, 25, 12.5 nM. Each protein was analyzed using at least two separate experiments, with independent acceptor molecule immobilizations. The calculated rates are the mean values of each measurement series. Kinetic measurements were performed by injection of each analyte for 300 s followed by disassociation in buffer flow for 400 s. The immobilized IGFBP-1 was regenerated after each cycle using a 12 μl injection of 100 mM HCl. IGF-I ligand concentration used in receptor measurements were for native IGF-I: 262, 131, 66, 33, 16.5 nM; Z-IGF-I: 350, 177, 88.6, 44.3, 22.1, 11 nM; IGF-I-Z: 381, 191, 95.5, 47.8, 23.9, 11.9 nM. The immobilized IGF-I^R surface was regenerated after each cycle by injection of 12 μl acidic regeneration solution containing 0.3 M sodium citrate pH 5 and 0.4 M NaCl. Ligand samples were injected twice at six different concentrations in random order over the same surface in each measurement series. The temperature in all kinetic experiments was 298 K. Unfused Z at 300 nM was used as control for non-specific Z-derived interactions. Kinetic parameters were calculated using the kinetics evaluation software package, BIAevaluation 2 (Biacore AB). The theory of calculating kinetic binding parameters from BIAcore measurement techniques has been extensively described, for a review see [20].

2.8. Cell proliferation assay

The relative proliferative activity of the different proteins was tested on human osteosarcoma SaOS-2 cells. Cells, at a density of approximately 30 000 cells/ml, were seeded in 96 well plates in 100 ml of McCoy medium supplemented with 15% fetal calf serum (FCS) (HyClone, Logan, UT, USA) and 1000 units/ml PEST (penicillin/streptomycin) (Sigma). Cells were incubated for 24 h and the media were replaced with McCoy containing 0.1% HSA. Following an additional 24 h incubation the test proteins were added in concentrations from 0.06 nM to 2.5 nM. Aliquots of lyophilized test protein were dissolved and diluted directly in HSA containing serum free growth medium, immediately before addition. Cells were incubated for 72 h before determining the metabolic response using EZ4U colorimetric assay quantification according to the manufacturer's instructions (Biomedica, Austria).

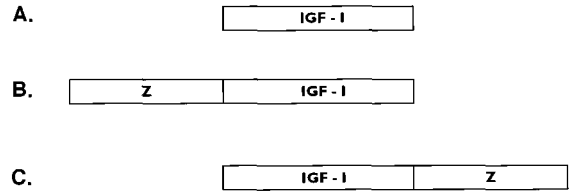

Fig. 1. Schematic representation of the used fusion proteins, Z-IGF-I and IGF-I-Z. The molecular mass of the proteins is (A) 7649 Da (70 residues), (B) 16927 Da (152 residues) and (C) 15726 Da (142 residues).

3. Results

3.1. Protein production and purification

All the protein components IGF-I, IGF-I-Z, Z-IGF-I, sIGF-I^R and IGFBP-1 were produced and purified to obtain highly characterized proteins to be assayed in binding and biological assays. The Z-IGF-I and IGF-I-Z fusion proteins, as outlined in Fig. 1, were produced in *E. coli* and purified to homogeneity. Both preparations were estimated to be more than 95% pure by SDS-PAGE (Fig. 2), or by RP-HPLC analysis. The IGF-I receptor was essentially homogeneous after the one step IgG affinity purification. The receptor was produced and purified as a homogeneous $(\alpha\beta'Z)_2$ moiety. The receptor was glycosylated to 26.1% by mass [7]. IGFBP-1 was purified to homogeneity and determined to be more than 90% pure by SDS-PAGE. The amino acid composition contents were according to what was expected from the respective deduced amino acid sequences (data not shown).

3.2. Circular dichroism

Far UV circular dichroism was applied to monitor the secondary structure content and possible differences between the IGF-I fusions. The far UV spectra of Z-IGF-I and IGF-I-Z were normalized according to quantitative amino acid analysis of the actual samples (Fig. 3). The spectra are very similar and mostly overlap over the entire wavelength range, indicative that the overall secondary structure content was the same in both fusion proteins.

3.3. BIAcore measurements

The determination of the kinetic parameters and the association equilibrium constants was performed using the label free, real time BIAcore biosensor technology. The larger acceptor molecules IGF-I^R and IGFBP-1 were immobilized in two separate sets of experiments and the IGF-I analytes passed over the acceptor molecule surface. A representative Sensorgram showing relative response vs. time of IGF-I binding to IGF-I^R is shown in Fig. 4. The binding kinetic parameters derived from the BIAcore data of the IGF-I variants binding to IGFBP-1 and IGF-I^R are summarized in Table 1. The cumulative error in the determined association constants from all runs was estimated to be less than 12%, when calculated as the square root of the sum of the squares of the errors in amino acid analysis, pipetting and data fitting. The association rates of the IGF-I fusions are slowed down for both IGFBP-1 and IGF-I^R binding to about the same extent. The disassociation rates shows that Z-IGF-I has a similar k_{off} as that of native IGF-I for IGFBP-1 binding and about 1.5 times slower k_{off} for IGF-I^R binding. The reverse is seen for IGF-I-Z with somewhat higher k_{off} for IGFBP-1 binding and close to native k_{off} for IGF-I^R. The derived association equilibrium constant K_A shows the IGFBP-1 binding to be reduced to a similar extent for both fusions, approximately 1.8 times, whereas receptor binding is reduced by 2.4 times for Z-IGF-I and by 4.7 times for IGF-I-Z, respectively.

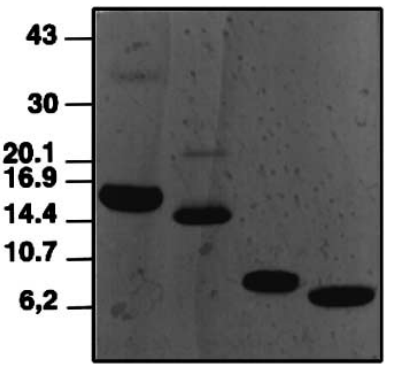

Fig. 2. SDS-PAGE of the purified fusion proteins. Lane 1, Z-IGF-I; lane 2, IGF-I-Z; lane 3, IGF-I; lane 4, Z domain.

3.4. Proliferation assay

The IGF-I fusion proteins' proliferative response was tested in cell proliferation assays using human SaOS-2 cells, and the

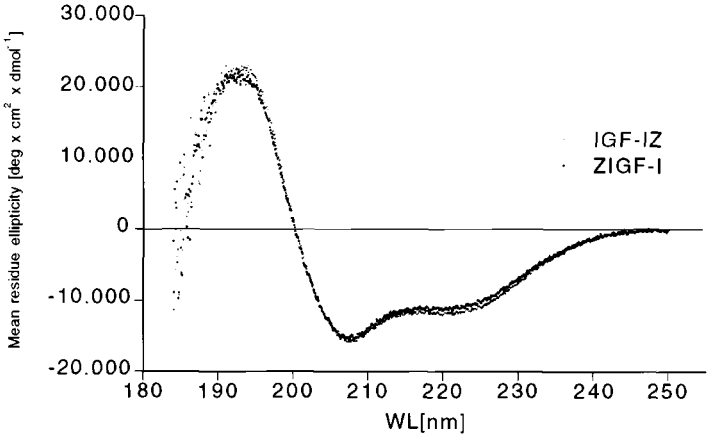

Fig. 3. Superposition of the far UV CD spectra, between 185 and 250 nm, of Z-IGF-I and IGF-I-Z.

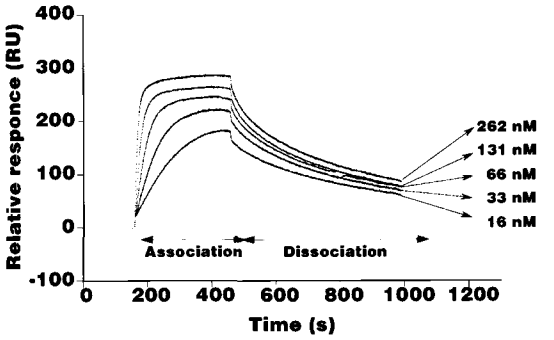

Fig. 4. BIAcore analysis of IGF-I binding to immobilized IGF-I^R-Z. The sensorgram shows the relative response in RU after background subtraction versus time in seconds. The concentrations of IGF-I is indicated by numbers in the graph. The ligand association phase injection time was 300 s followed by dissociation in buffer flow for 900 s at a low rate of 8 μl/min.

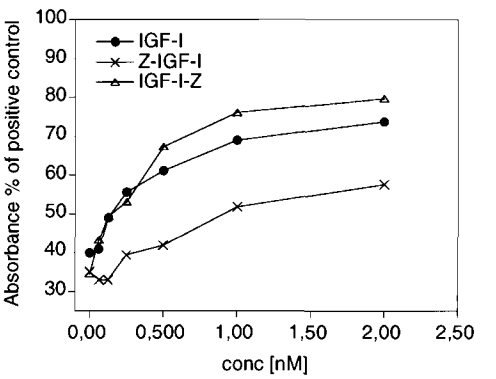

Fig. 5. Representation of the growth stimulating effects of the IGF-I fusion proteins. The stimulatory effect, monitored with the EZ4U assay, is expressed as percent of FCS internal control and plotted against concentration of ligand. The symbols used are: ●, IGF-I; ×, Z-IGF-I; △, IGF-I-Z. The values shown are the mean of two separate experiments performed in eight separate samples at each concentration.

EZ4U colorimetric based system. The growth promoting effects of the different proteins in the used concentration range is shown in Fig. 5. The data are expressed as percent stimulation of internal standard containing 10% FCS. IGF-I and IGF-I-Z reach about 75% of maximal FCS stimulation whereas Z-IGF-I reaches about 55%.

4. Discussion

In this paper, we have analyzed in vitro binding characteristics, as well as in vivo biological proliferation activities, of fusion proteins based on IGF-I. The IGF-I variants represent C- and N-terminal fusions to a one domain protein A analogue (designated Z) of 58 amino acid residues. This domain was chosen on the basis of being a highly stabile domain with a known fold and its well characterized advantages as an affinity fusion partner for the production of IGF molecules. Affinities to sIGF-I^R and IGFBP-1 were studied and both types of fusions showed a similar weakened affinity to IGFBP-1. However, the relative affinities to sIGF-I^R differed significantly between the Z-IGF-I and IGF-I-Z proteins. The structural properties of the fusion proteins were tested by circular dichroism. The data suggest that the overall secondary structure content is similar for both fusion proteins. The relative locations of the two domains do not drastically change the packing of the fusion proteins, which could possibly be the case since IGF-I has been shown to be sensitive to structural aberrations [24]. Recombinant IGF-I accumulates as two distinct folding forms, native and mismatched, differing in their disulfide bond arrangement, the mismatched form having structural aberrations, decreased α-helicity, and diminished or even absent IGFBP and IGF-I^R binding [15,25]. The produced IGF-I fusion proteins both display high affinity binding to IGFBP-1 and IGF-I^R, which is only the case for correctly folded material. The preparations are homogeneous as determined by HPLC and SDS-PAGE analysis. Since the mismatched form possesses distinctly different elution profiles on reverse phase compared to native form, the purified fusion proteins are concluded to be in the correct disulfide conformation.

Table 1
Summary of kinetic data of IGF-I fusion proteins binding to IGFBP-I and IGF-I^{R}Z

Ligand	IGF-I	Z-IGF-I		IGF-I-Z	
			rel. rate		rel. rate
Binding to IGF-I^R					
k_{on}/ (M^{-1} $s^{-1}\times10^{-5}$)	4.7	1.3	0.28	1.1	0.23
k_{off}/ ($s^{-1}\times10^{4}$)	16.7	10.7	0.64	17.9	1.07
K_A/ ($M^{-1}\times10^{-9}$)	0.28	0.12	0.43	0.06	0.21
ΔΔG/ (kcal mol^{-1})	0	0.50		0.91	
Binding to IGFBP-I					
k_{on}/ (M^{-1} $s^{-1}\times10^{-5}$)	6.1	3.5	0.57	4.3	0.70
k_{off}/ ($s^{-1}\times10^{4}$)	3.5	3.5	1	4.5	1.29
K_A/ ($M^{-1}\times10^{-9}$)	1.7	1.0	0.59	0.96	0.56
ΔΔG/ (kcal mol^{-1})	0	0.31		0.34	

The relative rates are expressed as the fraction of unfused IGF-I rates to each acceptor molecule.

BIA analysis enables the determination of both association rate and dissociation rate, hence distinguishing between on-rate and off-rate in the contribution to the equilibrium binding constant. The on-rate for the fusion proteins is slowed down compared to native IGF-I, both for IGFBP-1 and for IGF-I^R association (Table 1). The association with the receptor is more affected than IGFBP-1 binding which could reflect a more complicated binding mechanism for IGF-I to its receptor where the receptor association is accompanied by a conformational change of the receptor [7]. Association of IGF-I with the receptor involves determinants from both α-subunits and covers a large part of the ligand. The basic IGF-I (p*I* 8.3) fused to the acidic Z (p*I* 5.7) could lead to an electrostatic attraction between the two fusion halves at neutral pH. Some of the initial electrostatic attraction between IGF-I and the receptor might be diminished by a close contact between IGF-I and its fusion partner. This should be seen as a lowered on-rate since medium range electrostatic attraction is an early step in the formation of protein-protein complexes. The fusion protein may also simply hinder the structural rearrangement resulting in slower on-rates. The off-rates show changes in both receptor and IGFBP-1 binding, differing between the fusions. Predominant, though, is the decreased disassociation of Z-IGF-I from IGF-I^R. A decreased disassociation rate leading to prolonged signaling might have consequences for both cellular responses and the rate of receptor turnover. The influence on off-rates is smaller than on the on-rates, indicating that the sites of specific binding are

not, or to a lesser extent, affected by the presence of the fusion partner.

The relative locations of the domains in the fusion proteins have not been established. The N- and C-termini are separated by 21 Å in the model built structure of IGF-I [26], compared to only 5 Å in the NMR structure [27]. However, the NMR structure lacks distance restraints (NOEs) for both N- and C-termini. This indicates a large flexibility which makes modeling of the relative locations of the IGF-I and Z domains of the two fusion proteins difficult. The integrity of the IGF-I N-terminus is necessary to maintain IGFBP binding. The naturally occurring variant of IGF-I, des1–3 IGF-I, lacking the first three amino acids, drastically lowered IGFBP binding compared to native IGF-I [28]. Previous reports on the IGF-I interaction with IGFBP-1 have claimed that N-terminal extensions of the molecule hinder the IGF-I binding to IGFBP-1 (Long-R3-IGF-I/long IGF-I) [29]. IGF-I receptor specificity has been studied previously using fusion proteins [30], using the flag epitope purification system. No difference in receptor specificity was reported, although the absolute value of binding was lowered for the fusion compared to native IGF-I. The predominant difference between Z fusions and the other described IGF-I fusions is that the Z fusion partner is a characterized molecule with a well defined specific fold of known three-dimensional structure. A shorter fusion partner such as the flag epitope, a His-tag or subdomains of other proteins, e.g. Long-R3 with a 13 amino acid extension, may not form an independently folding unit and possibly fold back on the major domain and interfere with some specific binding determinants, thus altering binding strength. Thus, the use of intact domain fusions seems favorable if retained binding is desired.

Most interestingly, the proliferative effects measured using the colorimetric EZ4U assay demonstrate that the sIGF-I^R affinity and the growth promoting effects are not correlated: IGF-I-Z has only 21% of the native IGF-I receptor affinity but is still as potent as a proliferative agent. Z-IGF-I has 41% of the receptor affinity but a much reduced proliferative effect. Thus, fusion proteins with high affinity for the receptor may not necessarily be a potent mitogen because more than one interaction to the receptor is likely to be involved in receptor signaling [5–7]. The IGF-I fusion's IGFBP-1 affinities are within error identical, about 60% of native, suggesting that IGFBP binding is not an explanation. However, the IGFBP expression pattern for the SaOS-2 cells was not studied. There are at least seven different IGFBP molecules. If the relative affinities to other IGFBPs differed between the two IGF-I fusions, this might contribute to the observed difference in proliferative potential. Therefore, an additional experiment using [^{3}H]thymidine incorporation with CHO-DG44 cells was performed (data not shown). The results were similar, as defined by the same ranking of the fusion proteins in their proliferative potential. This shows that the relative order of the three IGF-I variants is not sensitive to the choice of cell type in the analysis or the method of analysis of the proliferative response. Another explanation is that the fusion partners affect the receptor signal transduction mechanism differently by steric hindrance. The IGF-I receptor activation involves several steps, the binding of a ligand using determinants from both α-subunits, inducing a conformational change and thus transferring the signal to the cytoplasmic tyrosine kinase domains. The signaling also involves a timing event and the duration of the signal, i.e. the ligand dissociation rate, is believed to be the efficiency determining step in receptor activation potential [5,6,21]. Recent studies from our laboratory have also demonstrated that binding of IGF-I to IGF-I^R is accompanied by a large decrease in entropy, compatible with a structural rearrangement of the extracellular receptor domains [7]. Differences in the fusion proteins' ability to allow receptor conformational changes, imposed by steric hindrance, or to allow the formation of correct receptor signaling conformation might change the signaling properties of the fusion proteins. Different structural rearrangements could lead to different receptor kinase activity or different phosphorylation patterns affecting both intracellular signaling pathways and receptor turnover. As recently demonstrated by Reddy et al. [22], receptor affinity per se is not correlated with mitogenic potency in the EGF/EGFR system. A single-site EGF mutant (Y13G) with 50-fold lowered receptor affinity was more potent than the wild type due to decreased ligand depletion and less induction of receptor down-regulation. It has further been demonstrated that the insulin receptor β-domain tyrosine kinases are asymmetrically trans-phosphorylated upon ligand binding and activation [23]. The Z-IGF-I and IGF-I-Z bind IGF-I^R with high affinity but could possibly alter the receptor phosphorylation pattern due to simple steric hindrance of the α-subunit conformational change. Taken together, this demonstrates that affinity and efficiency are not directly linked in the events of IGF-I receptor activation since other factors such as receptor trafficking and signaling dynamics complicate the picture.

In conclusion, this work shows that kinetic binding properties of the used IGF-I fusion proteins do not correlate with biological activity in the IGF signaling system. This study exemplifies a characterization of a system where the order of the fusion protein components not only changes kinetic binding properties but also differentially changes the mitogenic activity properties. The exact nature of the influence of the different IGF-I fusions on the receptor trafficking is subject to future investigations. The use of fusion proteins to alter the kinetic and dynamic properties of a given ligand seems promising as a tool in the dissection of intracellular signaling events.

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References

[1] Jones, J.I. and Clemmons, D.R. (1995) Endocr. Rev. 16, 3–34.
[2] Oh, Y., Nagalla, S.R., Yamanaka, Y., Kim, H.-S., Wilson, E. and Rosenfeld, R.G. (1996) J. Biol. Chem. 271, 30322–30325.
[3] Rechler, M.M. (1993) in: Vitamins and Hormones, Vol. 47, pp. 1–114, Academic Press, New York.
[4] Siddle, K. (1992) Prog. Growth Factor Res. 4, 301–320.
[5] De Meyts, P. (1994) Diabetologia 37, 135–148.
[6] De Meyts, P. et al. (1994) Horm. Res. 42, 152–169.
[7] Jansson, M., Uhlen, M. and Nilsson, B. (1997) Biochemistry 36, 4108–4117.
[8] Schaefer, E.M., Erickson, H.P., Federwisch, M., Wollmer, A. and Ellis, L. (1992) J. Biol. Chem. 267, 23393–23402.
[9] Nilsson, B. and Abrahmsén, L. (1990) in: Methods in Enzymology. Gene Expression Technology (Goeddel, D.V., Ed.), Vol. 185, pp. 144–161, Academic Press, San Diego, CA.

[10] Nilsson, B., Forsberg, G. and Hartmanis, F. (1990) Methods Enzymol. 198, 3–16.
[11] Jendeberg, L., Tashiro, M., Tejero, R., Lyons, B.A., Uhlen, M., Montelione, G.T. and Nilsson, B. (1996) Biochemistry 35, 22–31.
[12] Nilsson, B. et al. (1987) Protein Eng. 1, 107–113.
[13] Jansson, M., Li, Y.-C., Jendeberg, L., Andersson, S., Montelione, G.T. and Nilsson, B. (1996) J. Biomol. NMR 7, 131–141.
[14] Ausubel, F.M. (1995) Wiley Interscience, New York.
[15] Forsberg, G., Palm, G., Ekebacke, A., Josephson, S. and Hartmanis, M. (1990) Biochem. J. 271, 357–363.
[16] Moks, T. et al. (1987) Biochemistry 26, 5239–5244.
[17] Luthman, H. et al. (1989) Eur. J. Biochem. 180, 259–265.
[18] Cedergren, L., Andersson, R., Jansson, B., Uhlen, M. and Nilsson, B. (1993) Protein Eng. 6, 441–448.
[19] Löfås, S. and Johnsson, B. (1990) J. Chem. Soc. Chem. Commun. 21, 1526–1528.
[20] Karlsson, R., Roos, H., Fägerstam, L. and Persson, B. (1994) Methods 6, 99–110.
[21] Hubbard, S.R., Wei, L., Ellis, L. and Hendrickson, W.A. (1994) Nature 372, 746–754.
[22] Reddy, C.C., Niyogi, S.K., Wells, A., Wiley, H.S. and Laffenburger, D.A. (1996) Nature Biotechnol. 14, 1696–1699.
[23] Lee, J., O'Hare, T., Pilch, P.F. and Shoelson, S.E. (1993) J. Biol. Chem. 268, 4092–4098.
[24] Jansson, M. et al. (1997) J. Biol. Chem. 272, 8189–8197.
[25] Hober, S., Forsberg, G., Palm, G., Hartmanis, M. and Nilsson, B. (1992) Biochemistry 31, 1749–1756.
[26] Blundell, T.L., Bedarkar, S., Rinderknecht, E. and Humbel, R.E. (1978) Proc. Natl. Acad. Sci. USA 75, 180–184.
[27] Cooke, R.M., Harvey, T.S. and Cambell, I.D. (1991) Biochemistry 30, 5484–5491.
[28] Bagley, C.J., May, B.L., Szabo, L., McNamara, P.J., Ross, M., Francis, G.L., Ballard, F.J. and Wallace, J.C. (1989) Biochem. J. 259, 665–671.
[29] Francis, G.L. et al. (1992) J. Mol. Endocrinol. 8, 213–223.
[30] Zhang, W., Gustafson, T.A., Rutter, W.J. and Johnson, J.D. (1994) J. Biol. Chem. 269, 10609–10613.