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Mapping of the insulin-like growth factor II binding site of the Type I insulin-like growth factor receptor by alanine scanning mutagenesis

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Abstract The Type I insulin-like growth factor receptor is a physiological receptor for insulin-like growth factor II (IGF-II). To characterize the molecular basis of the receptor's ligand binding properties, we have examined the effects of alanine mutations of residues in the ligand binding site of the receptor on its affinity for IGF-II. The functional epitope for IGF-II comprises residues in the N-terminal L1 domain and residues at the C-terminus of the α subunit. Cysteine rich domain residues do not appear to be critical for IGF-II binding.

Keywords: Growth factor; Receptor; Affinity; Mutagenesis

1. Introduction

Insulin-like growth factors I and II (IGF-I and II) are homologous peptide growth factors that are major regulators of growth in vertebrates [1]. Transgenic experiments indicate that both peptides exert their physiological effects through binding to the Type I insulin-like growth factor receptor [1]. This receptor binds both peptides with high affinity although its affinity for IGF-I has been reported to be 2–5 times higher than its affinity for IGF-II [2–5].

The Type I IGF receptor is a member of the insulin receptor sub-class of receptor tyrosine kinases [6]. Recently, the structure of an N-terminal fragment of the receptor, comprising the L1, cysteine rich and L2 domains (amino acids 1–460), has been reported [7]. While this fragment is devoid of ligand binding activity, a mini-receptor formed by the fusion of this fragment to a peptide (amino acids 692–702) from the C-terminus of the receptor α subunit binds IGF-I with an affinity near to that of the recombinant secreted extra-cellular domain [8]. This indicates that these fragments form the minimal elements of a major IGF-I binding site of the receptor. We have recently defined the functional epitope of this IGF-I binding site using alanine scanning mutagenesis [9]. In the present study, in order to gain further insight into the molecular basis for the differences in affinities of this receptor for IGF-I and II, we have used alanine scanning mutagenesis to determine its functional epitope for IGF-II.

2. Materials and methods

2.1. Materials

All oligonucleotides were purchased from DNA technology (Aarhus, Denmark). Restriction and modifying enzymes were from New England Biolabs (Beverly, MA). Recombinant IGF-I and IGF-II (receptor grade) were from Gro Pep (Adelaide, Australia). High performance liquid chromatography purified mono-iodinated insulin-like growth factor I radio-iodinated on Tyrosine 31 (^{125}I-[Tyr^{31}] IGF-I) was from Novo Nordisk A/S [10]. Protease inhibitors were from Roche Molecular Biochemicals (Mannheim, Germany). Medium and serum for tissue culture were from Life Technologies A/S (Tåstrup, Denmark). Peak Rapid cells (293 cells constitutively expressing SV40 large T antigen) were purchased from Edge Biosystems (Gaithersburg, MD). The mammalian expression vector pcDNA3-zeo(+) was from Invitrogen (San Diego, CA). The hybridoma secreting monoclonal antibody 24–31 directed toward the IGF-I receptor α-subunit was a generous gift of Dr. M.Soos and Dr. K. Siddle (University of Cambridge, UK). Protein A-purified IgG from the hybridoma medium was kindly provided by Dr. P. Jørgensen (Novo Nordisk A/S, Bagsværd, Denmark). The construction and transient expression of alanine mutants of Type I insulin-like growth factor receptor cDNAs in 293 Peak Rapid cells has been described in detail [1].

2.2. IGF-II binding assays

IGF-II binding assays were performed by a modification of methods previously used for equilibrium binding assays of the insulin and IGF-I receptors [9,11]. Secreted recombinant IGF-I receptor was immobilized from the conditioned media of transiently transfected Peak Rapid cells in antibody coated 96 well microtiter plates as previously described [9,11]. ^{125}I-[Tyr^{31}] IGF-I (12 pmol/L) and unlabeled IGF-II (0–100 nmol/L) were incubated with immobilized receptor for 16 h at 25 °C in a total volume of 100 μL. Bound radioactivity was determined after the plates had been washed three times with ice cold wash buffer.

Dissociation constants for IGF-II were determined by curve-fitting using a single site heterologous competition model as described by Wang [12], written in Excel. Dissociation constants for IGF-I used for the fitting of data for wild type and mutant receptors were taken from our previously published study [9].

3. Results and discussion

Wild type secreted IGF-I receptor cDNAs were expressed by transient transfection in 293 Peak Rapid cells. Initial attempts to evaluate IGF-II binding were made with either commercial mono-iodinated IGF-II (Amersham) or HPLC purified mono-iodinated IGF-II produced at Novo Nordisk A/S. Neither were of sufficient quality for use in homologous competitive binding assays with unlabeled IGF-II (data not shown). Thus,

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Abbreviations: IGF-I, insulin-like growth factor I; IGF-II, insulin-like growth factor II; ^{125}I-[Tyr^{31}] IGF-I, insulin-like growth factor I radio-iodinated on Tyrosine 31

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IGF-II binding was evaluated by heterologous competition of ^{125}I-[Tyr^{31}] IGF-I by unlabeled IGF-II. The K_d of wild type IGF-II determined in this assay was 2.9 ± 0.2 nM (means $\pm$ S.E.M., $n = 8$); K_d for IGF-I determined in parallel experiments was 0.7 ± 0.06 nM (means $\pm$ S.E.M., $n = 8$). This 4- to 5-fold difference in affinity is similar to that previously reported for the full length receptor [2–5]. In the experiments described below, alanine mutations resulting in a K_d for IGF-II of greater than 5.8 nM were considered to significantly disrupt ligand receptor interactions [13].

cDNAs encoding alanine mutations of all ligand accessible residues located in candidate IGF-I binding regions of the Type I IGF receptor (L1, cysteine rich and L2 domains) and amino acids of 692–702 have been previously described [9]. In the N-terminal fragment (L1, cysteine rich and L2 domains), these are all solvent accessible residues located in the putative binding pocket of the receptor [7] and are limited to the L1 domain and the cysteine rich domain (CRD) (amino acids 1–284) on the basis of the reported size of the IGF-I molecule [14–16] and previous mutational analyses of IGF-I–Type I IGF receptor interactions [9,17]; in the crystal. structure of the N-terminal fragment of the receptor [7], IGF-I is not large enough to simultaneously contact the part of the IGF-I functional epitope [9] most distant from the L2 domain (amino acids Asn^{11}, Leu^{33}, Arg^{59} and Phe^{90}) and residues of the L2 domain even with bridging by water molecules.

Alanine mutant cDNAs were expressed by transient transfection; cDNAs encoding alanine mutations of Tyr^{54} and Thr^{93} were not transfected as we have previously demonstrated that these mutations are not expressed due to perturbation of protein folding [9]. Expression of all mutants was confirmed by the binding of ^{125}I-IGF-II with the exception of the alanine mutant of Phe^{701} which failed to exhibit significant specific tracer binding, even after 20-fold concentration of conditioned medium from transfected cells (data not shown). Expression of this mutant was confirmed by Western blotting (data not shown).

The results of the K_ds of the mutant receptors are shown in Table 1. In L1 alanine mutations of Asp^{8}, Asn^{11}, Tyr^{28}, His^{30}, Leu^{32}, Leu^{33}, Leu^{56}, Phe^{58}, Arg^{59} and Phe^{90} produce significant decrease in affinity for IGF-II. Only mutations of Leu^{33}, Leu^{56} and Arg^{59} produced increases in K_d of 5-fold or greater. As shown in Fig. 2A, all residues, whose mutation compromises affinity for IGF-II, form a continuous footprint on the base of the L1 domain of the receptor, consistent with participating in a ligand binding site for a small protein.

In the cysteine rich domain, none of the alanine mutations expressed caused any significant compromise in affinity for IGF-II (Table 1).

At the C-terminus of the α subunit, alanine mutations of Phe^{692}, Glu^{693}, Asn^{694}, Phe^{695}, His^{697}, Asn^{698}, and Ile^{700} produced 4- to 27-fold increases in K_d (Table 1). As discussed above the alanine mutant of Phe^{701} failed to bind IGF-II despite normal expression of this mutant protein, indicating that its affinity for IGF-II is too low to be measured by the methodology employed in this study. This would suggest that its K_d for IGF-II is at least a 100-fold greater than that of the wild

Table 1
Effects of alanine mutations on the affinity of the Type I insulin-like growth factor receptor for IGF-II

L1 domain			Cysteine rich domain			α subunit C-terminus		
Mutant[a]	K_d (nM)[b]	K_d Mut/K_d WT[c]	Mutant[a]	K_d (nM)[b]	K_d Mut/K_d WT[c]	Mutant[a]	K_d (nM)[b]	K_d Mut/K_d WT[c]
D8	7.5 ± 0.4	2.6 ± 0.2	R240	1.4 ± 0.1	1.4 ± 0.1	F692	77.7 ± 7.0	26.7 ± 2.4
R10	2.9 ± 0.1	1.0 ± 0.02	F241	0.5 ± 0.04	0.5 ± 0.04	E693	39.0 ± 10.1	13.4 ± 3.5
N11	9.5 ± 1.1	3.3 ± 0.4	E242	1.2 ± 0.1	1.2 ± 0.1	N694	78.8 ± 7.8	27.1 ± 2.7
D12	1.2 ± 0.1	0.4 ± 0.02	F251	0.8 ± 0.2	0.8 ± 0.2	F695	42.9 ± 8.3	14.7 ± 2.9
Y28	10.3 ± 1.1	3.6 ± 0.4	I255	1.6 ± 0.2	0.5 ± 0.06	L696	33.2 ± 5.9	11.4 ± 2.0
H30	12.2 ± 1.8	4.2 ± 0.6	L256	3.4 ± 0.2	1.2 ± 0.1	H697	28.0 ± 2.0	9.6 ± 0.7
L32	7.3 ± 0.5	2.5 ± 0.2	S257	2.6 ± 0.4	0.9 ± 0.2	N698	12.5 ± 1.7	4.3 ± 0.6
L33	29.3 ± 3.9	10.1 ± 1.3	E259	3.1 ± 0.2	1.0 ± 0.1	S699	2.0 ± 0.1	0.7 ± 0.04
S35	2.5 ± 0.2	0.9 ± 0.1	S260	2.0 ± 0.4	0.7 ± 0.1	I700	17.0 ± 1.5	5.8 ± 0.5
Y54	ND	ND	S261	3.8 ± 0.2	1.3 ± 0.1	F701	*	*
L56	15.5 ± 1.5	5.3 ± 0.5	D262	1.9 ± 0.3	0.7 ± 0.1	V702	4.8 ± 0.5	1.6 ± 0.2
F58	10.9 ± 0.7	3.7 ± 0.2	S263	3.3 ± 0.3	1.1 ± 0.1			
R59	23.6 ± 2.0	8.1 ± 0.7	E264	3.1 ± 0.3	1.0 ± 0.1			
K60	3.5 ± 0.8	1.2 ± 0.3	F266	2.6 ± 0.2	0.9 ± 0.1			
W79	2.2 ± 0.3	0.8 ± 0.1	H269	2.1 ± 0.1	0.7 ± 0.04			
L81	2.9 ± 0.1	1.0 ± 0.05	D270	2.3 ± 0.4	0.8 ± 0.1			
F82	3.5 ± 0.3	1.2 ± 0.1	E272	3.1 ± 0.3	1.1 ± 0.1			
Y83	2.0 ± 0.2	0.7 ± 0.1	M274	1.7 ± 0.3	0.6 ± 0.1			
N84	0.8 ± 0.2	0.3 ± 0.06	Q275	4.2 ± 0.4	1.4 ± 0.1			
Y85	1.0 ± 0.1	0.3 ± 0.04	E276	3.3 ± 0.6	1.1 ± 0.2			
V88	2.6 ± 0.1	0.9 ± 0.02	S279	2.2 ± 0.5	0.8 ± 0.2			
F90	8.7 ± 1.4	3.0 ± 0.5	F281	3.2 ± 1	1.1 ± 0.3			
E91	2.6 ± 0.3	0.9 ± 0.1	I282	3.3 ± 0.4	1.1 ± 0.1			
T93	ND	ND	R283	1.6 ± 0.4	0.5 ± 0.1			
R112	2.5 ± 0.1	0.9 ± 0.05	N284	$3.1 \pm 0.$	1.1 ± 0.1			
E114	4.5 ± 0.2	1.6 ± 0.06						
K115	1.8 ± 0.3	0.6 ± 0.1						
Y138	2.9 ± 0.1	1.0 ± 0.03						
V140	4.5 ± 0.2	1.5 ± 0.1						

ND: mutants not secreted.
* Affinity too low to be accurately determined, see text.
[a] Amino acids mutated are designated by the single letter code.
[b] Results are expressed as means $\pm$ S.E.M. of 3–4 independent determinations.
[c] Results are expressed as the ratio of the K_d of the mutant to the K_d of the wild type receptor (K_d Mut/K_d WT).

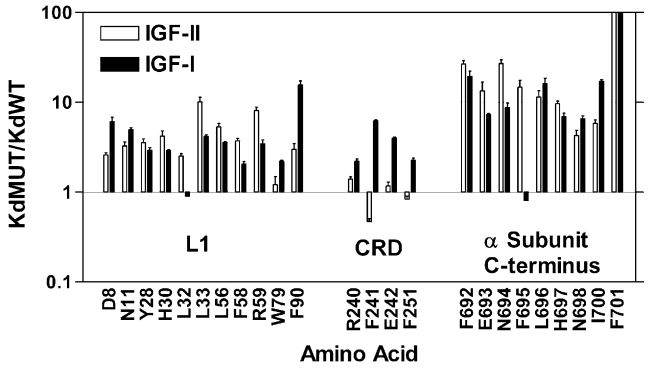

Fig. 1. Comparison of the functional epitopes for IGF-II and IGF-I. The effects of alanine mutations of amino acids, which form the Type I IGF-receptor ligand binding site, on affinity for IGF-II and IGF-I are compared. Results are presented as ratios of the dissociation constant of the mutant receptor to that of the wild type receptor. Data for IGF-I binding are taken from [9]. Results for both ligands represent means ± S.E.M. of 3–4 independent determinations. The amino acids mutated to alanine are designated by the single letter code. L1 designates amino acids located in the cysteine rich domain and CRD amino acids in the cysteine rich domain. For the alanine mutant of Phe[701], the K_d ratios for both IGF-II and IGF-I have been arbitrarily assigned values of 100 (see text for detailed discussion).

type receptor. These results indicate that the majority of the free energy of the ligand receptor interaction is provided by this sub-domain of the receptor ligand binding site.

We have previously characterized the functional epitope of the Type I insulin-like growth factor receptor IGF-I binding site by alanine scanning mutagenesis [9]. It is of interest to compare the functional epitopes for both ligands. The results of this comparison for the L1, cysteine rich and C-terminal α subunit domains are shown in Fig. 1. Both functional epitopes are qualitatively very similar but certain residues appear to participate selectively in the binding of one or other ligand. The most striking difference between them is that the cysteine rich domain residues Arg[240], Phe[241], Glu[242] and Phe[251], which together with Trp[79] from the L1 domain form a hydrophobic patch at the base of the IGF-I functional epitope (Fig. 2B and [9]), make no significant energetic contribution to the binding of IGF-II. In the L1 domain Trp[79], which compromises affinity for IGF-I, is not involved in IGF-II binding (Fig. 1). In contrast, alanine mutation of Leu[32], which contributes to IGF-II binding, has no impact on IGF-I receptor interaction (Fig. 1).

When the L1 residues contributing to the functional epitopes are compared in the context of the topology of this region of the receptor (Fig. 1), interesting differences emerge for the two ligands. Alanine mutations of residues in the first (Asp[8] and Asn[11]) and fourth turns (Phe[90]) of the L1 domain β helix are more disruptive of IGF-I binding than IGF-II binding (Figs. 1 and 2). In contrast, alanine mutations of residues in the second (Tyr[28], His[30], Leu[32] and Leu[33]) and third turns (Leu[56], Phe[58] and Arg[59]) are more deleterious to IGF-II binding (Figs. 1 and 2).

Differences in effects of alanine substitutions of residues at the C-terminus of the α subunit on binding of IGF-I and IGF-II are also observed. Alanine mutation of Phe[695] resulted in 15-fold reduction in affinity for IGF-II but was without effect on IGF-I binding (Fig. 1). Alanine mutations of Phe[692], Glu[693], Asn[694] and His[697] have more pronounced effects on affinity for IGF-II than for IGF-I (Fig. 1). The reverse is seen for mutations of Leu[696], Asn[698] and Ile[700] (Fig. 1). The effect of alanine mutation of Phe[701] was so disruptive that it was impossible to quantitate K_ds for either IGF-I or IGF-II.

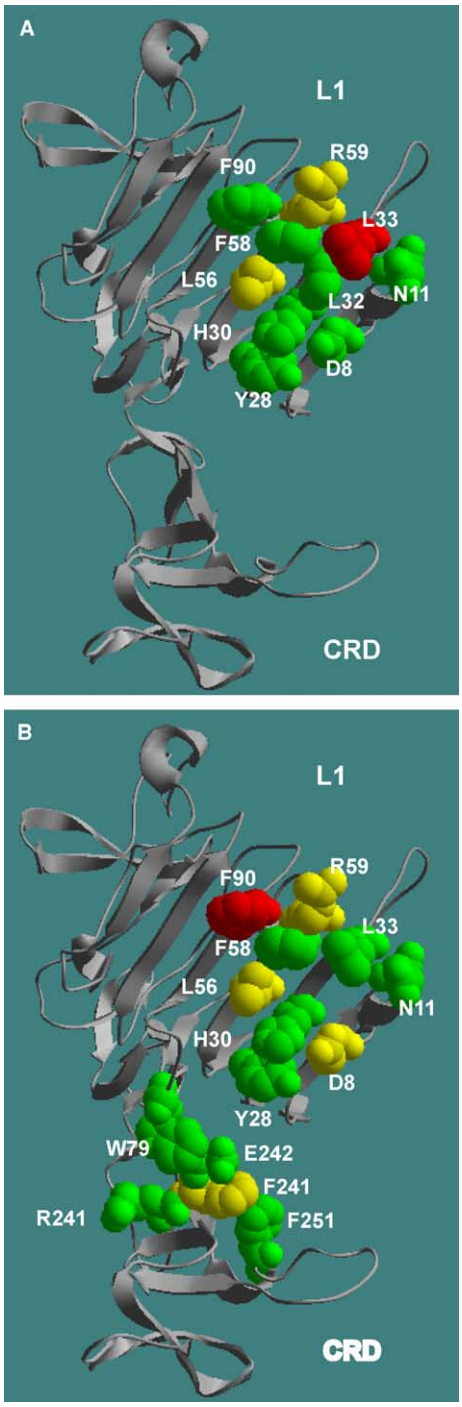

Fig. 2. Comparison of the structures of the functional epitopes of the L1 and cysteine rich domains for IGF-II and IGF-I binding. The Cα backbone of the L1 and CRDs is shown as a ribbon representation. The amino acids forming the functional epitopes for IGF-II (A) and IGF-I (B) are shown in space-filling representation. Alanine mutations of amino acids colored green produced a 2- to 5-fold reduction in affinity, those colored yellow produced a 5- to 10-fold reduction and those colored red produced a greater than 10-fold reduction. Amino acids are designated by the single letter code. This figure was prepared with the Swiss PDB Viewer [18].

In summary, we have defined the functional epitope of an IGF-II binding site of the Type I IGF-I receptor which is composed of elements of the L1 domain, and the C-terminus of the α subunit of the receptor. This contrasts with the functional epitope of receptor for IGF-I, which contains elements of the cysteine rich domain in addition to those involved in IGF-II binding. The differences between the two functional epitopes indicate that the two peptides utilize different molecular mechanisms to bind to the same ligand binding site of the receptor. It is tempting to conclude that this difference in the functional epitopes, particularly the absence of interaction of IGF-II with the receptor cysteine rich domain, accounts for the difference in affinities of this receptor for the two ligands. However, definitive confirmation of this conclusion will require elucidation of the structures of the peptide–receptor complexes and detailed analyses of the energetic contributions of the residues forming the functional epitopes of both ligands and also of the receptor.

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