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Kinetics of Insulin-like Growth Factor II (IGF-II) Interaction with Domain 11 of the Human IGF-II/Mannose 6-phosphate Receptor: Function of CD and AB Loop Solvent-exposed Residues

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Ligands of the IGF-II/mannose 6-phosphate receptor (IGF2R) include IGF-II and mannose 6-phosphate modified proteins. Disruption of the negative regulatory effects of IGF2R on IGF-II-induced growth can lead to embryonic lethality and cancer promotion. Of the 15 IGF2R extracellular domains, domains 1–3 and 11 are known to have a conserved β-barrel structure similar to that of avidin and the cation-dependent mannose 6-phosphate receptor, yet only domain 11 binds IGF-II with high specificity and affinity. In order to define the functional basis of this critical biological interaction, we performed alanine mutagenesis of structurally determined solvent-exposed loop residues of the IGF-II-binding site of human domain 11, expressed these mutant forms in *Pichia pastoris*, and determined binding kinetics with human IGF-II using isothermal calorimetry and surface plasmon resonance with transition state thermodynamics. Two hydrophobic residues in the CD loop (F1567 and I1572) were essential for binding, with a further non-hydrophobic residue (T1570) that slows the dissociation rate. Aside from alanine mutations of AB loop residues that decrease affinity by modifying dissociation rates (e.g. Y1542), a novel mutation (E1544A) of the AB loop enhanced affinity by threefold compared to wild-type. Conversion from an acidic to a basic residue at this site (E1544K) results in a sixfold enhancement of affinity *via* modification principally of the association rate, with enhanced salt-dependence, decreased entropic barrier and retained specificity. These data suggest that a functional hydrophobic binding site core is formed by I1572 and F1567 located in the CD loop, which initially anchors IGF-II. Within the AB loop, residues normally act to either stabilise or function as negative regulators of the interaction. These findings have implications for the molecular architecture and evolution of the domain 11 IGF-II-binding site, and the potential interactions with other domains of IGF2R.

Keywords: insulin-like growth factor-II; IGF-II/mannose 6-phosphate receptor; surface plasmon resonance; ligand–receptor binding kinetics; transition state thermodynamics

Abbreviations used: IGF-II, insulin-like growth factor; IGF2R, insulin-like growth factor II/mannose 6-phosphate receptor; CD-MPR, cation-dependent mannose 6-phosphate receptor; LOI, loss of imprinting; SPR, surface plasmon resonance; ITC, isothermal titration calorimetry; hGH, human growth hormone; RU, response units.

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Introduction

The mammalian cation-independent mannose 6-phosphate/insulin-like growth factor II receptor (IGF2R) is a type I integral membrane protein and P-type lectin, with multiple functions attributable to its wide variety of known ligands.[1–3] The ~270 kDa glycosylated protein consists of an N-terminal signal sequence (amino acid residues 1–44), 15 homologous extracytoplasmic repeating domains (residues 45–2313), a transmembrane region (residues 2314–2336) and a C-terminal cytoplasmic domain (residues 2337–2499).[4,5] Its 15 repeated domains are each ~147 residues in length and display significant similarity in amino acid sequence and disulphide distribution to each other (16–38% identity) and the single extracytoplasmic domain of the cation-dependent mannose 6-phosphate receptor (CD-MPR) (14–28% identity).[5] Crystal structures have now been solved for domains 1, 2, 3[6] and 11,[7] and show that each domain has a similar topology consisting of a flattened nine-strand β-barrel, shared with the CD-MPR and avidin,[8] suggesting that the 15 extracytoplasmic domains represent 15 homologous structural units. The main function of the IGF2R and the CD-MPR are the delivery of newly formed acid hydrolases, of which there are ~50, to the lysosome through binding to their mannose 6-phosphate (M6P)-labelled residues.[3] IGF2R also processes a number of other M6P and non-M6P-labelled ligands. Domains 3 and 9, and recently 5,[9] have been identified as the binding sites for the mannosylated proteins such as latent TGF-β,[10] proliferin and granzyme B and the protease cathepsin. Site-directed mutagenesis studies have since identified the core mannose 6-phosphate-binding site residues of domains 3 and 9 to consist of a glutamine, a glutamate, a tyrosine and an arginine residue,[11,12] an arrangement also shared with the CD-MPR.[13] Of the currently identified non-mannosylated ligands (IGF-II, retinoic acid, urokinase-type plasminogen activator receptor and plasminogen) IGF-II has been by far the best studied with the binding site being localised to domain 11.[14–16]

IGF-II (7.5 kDa) is a small mitogenic peptide hormone that functions principally during embryonic growth, where its activity is tightly regulated, but is also frequently deregulated in tumours.[17–19] Like IGF-I, IGF-II exerts its mitogenic affect predominantly by signalling through the IGF1R, leading to tyrosine kinase activation and stimulation of both the mitogen-activated protein (MAP) kinase and PKB/AKT signalling cascades. Downstream targets include the FOXO transcription factors, GSK3β, MDM2 and mTOR leading to up-regulation of pro-growth and anti-apoptotic signals.[20] In mammals, tight regulation of IGF-II activity is achieved by high-affinity binding to six IGF binding proteins (IGFBP 1-6) and by binding to the IGF2R at the cell surface, leading to internalisation of IGF-II and subsequent degradation within the lysosome.[21–23] Previous NMR studies have established the structure of mature IGF-II[24,25] and site directed mutagenesis has been used to identify residues F48, R49, S50, A54, L55 as being critical to the interaction with IGF2R.[26,27] Although IGF-II is relatively structurally conserved, the IGF-II-binding site of IGF2R is present only in mammalian species, where embryonic and placental growth regulation of IGF-II by the IGF2R also involves reciprocal imprinting of the genes coding these proteins.[28] Disruption of *Igf2* in the mouse results in reduced growth (60% of wild-type) from embryonic days 9–11,[29,30] whereas mice with disruption of *Igf2r* exhibit foetal overgrowth and fatal cardiac hyperplasia.[31,32] The growth and perinatal lethality phenotype is rescued when *Igf2* is also disrupted, suggesting the principal critical function of IGF2R is the regulation of IGF-II.[33] The specific functional interaction between IGF-II and IGF2R and its critical role in development has been highlighted more recently, as parthenogenetic embryos with maternal allele *Igf2* expression can lead to normal development of live mice with two maternal genomes, and epigenetic suppression of *Igf2r* may account for large offspring syndrome following somatic cell cloning in some species.[34,35]

Aberrant regulation of IGF-II activity has been implicated repeatedly as a common feature of tumours in both mouse and human.[36] For example, increased expression of IGF-II by loss of imprinting (LOI) has been described in a plethora of tumour types, including Wilms' tumour,[37] colorectal carcinoma,[38] rhabdomyosarcoma,[39] Ewing's sarcoma,[40] cervical carcinoma,[41] lung carcinoma[42] and phaeochromocytoma.[43] *IGF2* LOI is particularly associated with increased relative risk of developing colorectal carcinoma.[44,45] *IGF2R* also acts as a tumour suppressor, as loss of heterozygosity of the receptor has been detected in a number of tumour types, including liver, lung, and head and neck tumours.[46–49] Moreover, loss of function mutations of the receptor have been characterised,[50] and over-expression of IGF2R causes decreased growth and increased apoptosis in tumour models.[51–55]

The crystal structure of IGF2R domain 11 has been solved at 1.4 Å resolution using anomalous scattering of sulphur,[7] and has been confirmed by others.[56] The domain 11 IGF2R structure reveals two hydrophobic sites on the surface of domain 11, the first that identifies the putative IGF-II-binding site within the cleft of the β-barrel structure, spatially analogous to the hydrophilic sugar-binding site of the CD-MPR, and a second that is implicated in domain–domain interactions.[7,57] The IGF-II-binding pocket (400 $Å^3$) is formed by the β-strands A, B, C and D and the loops AB, CD and FG, with shorter loops conferring a shallower binding cavity than that of the CD-MPR. Residues Y1542, S1543 and G1546 (AB loop), F1567, G1568, T1570 and I1572 (CD loop), S1596, P1597 and P1599 (FG loop) have been identified as being significantly solvent-exposed and therefore potentially involved in the IGF-II interaction.[7]