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Rescue of ligand binding of a mutant IGF-I receptor by complementation

Anders Chakravarty [a], Jane Hinrichsen [a], Linda Whittaker [b], Jonathan Whittaker [b,c,*]

[a] Receptor Biology Laboratory, Novo Nordisk A/S, Hagedorn Research Institute, DK-2820 Gentofte, Denmark
[b] Department of Nutrition, Case Western Reserve University, Cleveland, OH 44106-4906, USA
[c] Department of Biochemistry, Case Western Reserve University, Cleveland, OH 44106-4906, USA

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Abstract

The IGF-I receptor binds IGF-I with complex kinetics characterized by a curvilinear Scatchard plot, suggesting receptor heterogeneity and apparent negative cooperativity. To explore the molecular mechanisms underlying these properties, we have characterized the binding of a hybrid receptor formed from a wild-type receptor monomer and a mutant receptor monomer devoid of binding activity. Receptor hybrids were generated by transient co-transfection of cDNAs encoding wild-type and mutant receptors with unique epitope tags. Hybrid receptors were purified from transfected cells by sequential immuno-affinity chromatography and their ligand-binding properties were determined. Complementation produced a hybrid with near wild-type affinity. Dissociation studies demonstrated that the hybrid did not exhibit negative cooperativity.

Keywords: IGF-I; Mutant; Hybrid receptor; Complementation; Affinity; Dissociation; Negative cooperativity

The Type I insulin-like growth factor (IGF-I) receptor mediates the physiological actions of the mammalian growth factors IGF-I and -II [1,2]. It is a member of the insulin receptor subclass of tyrosine kinase receptors. This subclass of receptors is characterized by a dimeric structure; the receptor dimers are formed from two disulfide linked monomers which are in turn composed of disulfide linked α and β subunits [3].

The Type I IGF receptor binds both IGF-I and IGF-II with high affinity [3]. Recent studies have revealed significant similarities between IGF-I interactions with the Type I IGF receptor and those of insulin with the insulin receptor [4]. Both ligand–receptor interactions exhibit complex kinetics characterized by equilibrium-binding properties suggestive of multiple classes of binding sites with differing affinities and dissociation kinetics suggestive of negative cooperativity. Both high affinity ligand-binding and complex-binding kinetics have been shown to be dependent on the integrity of the dimeric structure for both receptors [5–9].

Recent alanine scanning studies of the Type I IGF receptors have identified a residue, F701, at the C-terminus of the α-subunit, which is critical for IGF-I binding [10]. Alanine mutation of this residue leads to a reduction in affinity of 2–3 orders of magnitude without any apparent compromise in receptor structure. Thus, in order to further investigate the molecular basis of ligand–receptor interactions, we examined the effect of complementing the mutant F701A receptor monomer by a wild-type receptor monomer in a hybrid receptor dimer. Evaluation of the binding properties of the hybrid indicates that complementation restores high affinity binding but not negative cooperativity.

* Corresponding author. Fax: +1 216 368 6644.
E-mail address: jonathan.whittaker@case.edu (J. Whittaker).

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Materials and methods

Materials. All oligonucleotides were purchased from DNA technology (Aarhus, Denmark). Restriction and modifying enzymes were from New England Biolabs. Recombinant IGF-I (receptor grade) was from Gro Pep (Adelaide, Australia). High performance liquid chromatography-purified mono-iodinated [^{125}I-Tyr^{31}]IGF-I was from Novo Nordisk A/S. Protease inhibitors were from Roche Molecular Biochemicals (Mannheim, Germany). Medium and serum for tissue culture were from InVitrogen. 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. Monoclonal antibodies against the FLAG and AU5 epitopes were from Sigma and Covance, respectively. All molecular biological procedures were performed by standard methods [11].

Construction of cDNAs encoding epitope tagged receptors. Epitope tagged cDNAs were prepared by a combination of oligonucleotide-directed and cassette mutagenesis. Oligonucleotide-directed mutagenesis was used as previously described to insert an in-frame *Bam*HI site, GGATCC, encoding a Gly-Ser linker, at the 3′ end of the coding region of the wild-type and mutant IGF-I receptors. Oligonucleotide cassettes encoding an in-frame N-terminal *Bam*HI site, triple repeats of the FLAG (DYKDDDDK) or AU-5 (TDFYLK) epitopes followed by a stop codon and an *Xba*I site were ligated into the *Bam*HI and *Xba*I sites of pcDNA3.1Zeo+ (InVitrogen). *Hin*dIII–*Bam*HI fragments containing the modified IGF-I coding regions were then ligated into the *Hin*dIII and *Bam*HI sites of the plasmids containing the cassettes encoding the epitope tags to generate cDNAs encoding IGF-I receptors fused to C-terminal FLAG or AU-5 tags via Gly-Ser linkers.

Cell transfection. Log phase PEAK Rapid cells (EDGE Biosystems) were transiently transfected using Fugene 6 as previously described. In order to express hybrid receptors, cDNAs encoding each component of the hybrid receptor were co-transfected in equimolar amounts; three combinations of cDNAs were co-transfected—FLAG tagged wild-type and AU-5 tagged wild-type (WT-WT), FLAG tagged F701A and AU-5 tagged wild-type (F701A-WT), and FLAG tagged F701A and AU-5 tagged F701A (F701A-F701A). Detergent lysates of transfected cells were prepared 72 h post-transfection.

Isolation of hybrid receptors. Enriched glycoprotein fractions of detergent lysates of transfected cells were prepared by wheat germ agglutinin chromatography. FLAG tagged receptors were purified from the glycoprotein fractions by immuno-affinity chromatography on anti-FLAG agarose (Sigma) according to the manufacturer's directions. Tagged receptors were eluted with FLAG peptide 0.1 mg/ml and then subjected to a further round of wheat germ agglutinin chromatography to remove peptide. Finally hybrid receptors were isolated from the FLAG eluates by capture in a microtiter plate coated with anti-AU-5 IgG. In control experiments, in which mixtures of lysates from cells transfected with FLAG tagged or AU5 tagged wild-type IGF-I receptor cDNAS were processed, there was no detectable tracer [^{125}I-Tyr^{31}]IGF-I binding to AU5 coated plates.

IGF-I-binding assays. Equilibrium-binding assays were performed on immobilized hybrid receptors as previously described [10]. Dissociation assays were performed as described by Brandt [12].

Results

Equilibrium-binding studies

We generated hybrid IGF-I receptors by transient co-transfection of PEAK cells by combinations of cDNAS encoding wild-type FLAG and wild-type AU-5 (WT-WT), F701A FLAG and F701A AU-5 (F701A-F701A), and F701A FLAG and wild-type AU-5 (F701A-WT) receptors followed by sequential immuno-affinity purification. To assess the feasibility of performing detailed ligand-binding studies, we measured tracer [^{125}I-Tyr^{31}]-IGF-I binding to the isolated hybrid receptors. The F701A–F701A hybrid did not exhibit any detectable tracer binding (data not shown). This was consistent with our previous findings with this mutation of the recombinant secreted receptor extracellular domain [13]. In contrast, both WT-WT and F701A-WT hybrids bound significant amounts of [^{125}I-Tyr^{31}]IGF-I (data not shown). This indicates that complementation of the inactive F701A monomer by a wild-type receptor monomer can rescue its ligand-binding function.

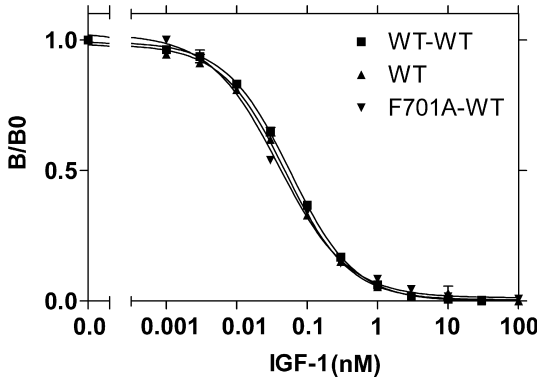

Fig. 1. Equilibrium-binding properties of hybrid receptors. IGF-I receptor cDNAS were transiently expressed in 293 PEAK cells. Receptors were harvested by detergent lysis and hybrids were isolated as described under Materials and methods. Equilibrium-binding studies were performed with [^{125}I-Tyr^{31}]IGF-I (12 pM) and varying concentrations of unlabeled IGF-I at 25 °C. Results are means and SEM of four experiments.

Competitive-binding assays with labeled and unlabeled IGF-I were performed on the WT-WT and the F701A-WT hybrid receptors and with wild-type IGF-I receptor. The equilibrium-binding properties of the hybrids and the wild-type receptor were nearly indistinguishable (Fig. 1). Computer fitting to a two site sequential model [4,14], which has been shown to give the best fit for IGF-I receptor equilibrium-binding data, indicated two populations of binding sites for both hybrid and wild-type receptors. The K_ds for the two populations of binding sites were not significantly different for any of the receptors (Table 1).

Table 1
Dissociation constants of hybrid receptors

Receptor	Dissociation constant[a] (nM)	
	K_{d1}	K_{d2}
WT	0.016 ± 0.001	0.11 ± 0.02
WT-WT	0.017 ± 0.001	0.25 ± 0.08
F701A-WT	0.016 ± 0.001	0.31 ± 0.08

[a] Data from Fig. 1 were fitted to a two site sequential binding model [4,14] to obtain dissociation constants.

Dissociation studies

In order to study the dissociation properties of the WT-WT and the F701A-WT hybrid receptors, they were incubated overnight at 4 °C with [^{125}I-Tyr^{31}]IGF-I after immobilization on anti-AU-5 coated microtiter plates. After extensive washing to remove unbound IGF-I, dissociation at room temperature was initiated by the addition of buffer or buffer containing 10^{-7} M IGF-I. Dissociation in buffer alone followed an almost identical pattern for both hybrids (Fig. 2). In contrast, 10^{-7} M IGF-I produced a clear acceleration of the dissociation of [^{125}I]IGF-I bound to the wild-type hybrid compared to buffer alone. This is similar to the behavior of IGF-I dissociation reported for intact cells. However, IGF-I was without significant effect on dissociation from the F701A-WT hybrid when compared to buffer alone (Fig. 2).

Discussion

We have characterized the ligand-binding properties of an isolated hybrid receptor composed of wild-type receptor monomer and a mutant monomer that we have previously demonstrated to be structurally intact and devoid of ligand-binding activity [10]. Equilibrium-binding data for this hybrid were virtually indistinguishable from those of wild-type receptor and could be fit to a two site sequential model [4,14] with dissociation constants that were not significantly different from those of the wild-type receptor. However unlike wild-type receptor, this hybrid receptor did not exhibit negative cooperativity, i.e., dissociation of prebound [^{125}I-Tyr^{31}] IGF-I was not accelerated by the presence of 10^{-7} M IGF-I in the dissociating buffer. Our studies thus extend a previous observation made with an active/inactive hybrid of the related insulin receptor by Taouis et al. [15] and in addition provide a more detailed description of the ligand-binding properties of such hybrid receptors.

High affinity ligand binding and negative cooperativity of the insulin and IGF-I receptors are known to require both α-subunits [5–9], but how the two subunits interact to form the high affinity-binding site is unknown. Theoretical models of insulin and IGF-I binding to their respective receptors have been proposed to explain the complex nature of these ligand–receptor interactions [14,16–18]. In the model of De Meyts [14], which best explains the binding properties of these receptors, it is suggested that the high affinity binding of either ligand to its cognate receptor and its negatively cooperative behavior result from an alternative cross-linking of receptor monomers by a single ligand molecule. The ligand is assumed to contain two distinct binding sites located on opposite sides of the molecule that sequentially and asymmetrically cross-link two distinct cognate sites on the receptor monomers. This generates the high affinity interactions which are not observed with binding to receptor monomers. Binding to the unoccupied sites represents the low affinity component of binding. Also, in this model, structural constraints prevent a second simultaneous cross-link. Thus, binding of a second ligand molecule and formation of a second cross-link lead to disruption of the first. This would have the effect of accelerating the dissociation of the first ligand molecule bound.

Our experimental findings with the F701A wild-type hybrid receptor provide support for this model. The ability to bind ligand with high affinity indicates that the ability to form cross-links is conserved, i.e., there must be a second ligand-binding site on the mutant receptor. We have recently found direct evidence for the existence of a second ligand-binding site of the homologous insulin receptor (J. Whittaker, submitted for publication). Also, as the equilibrium-

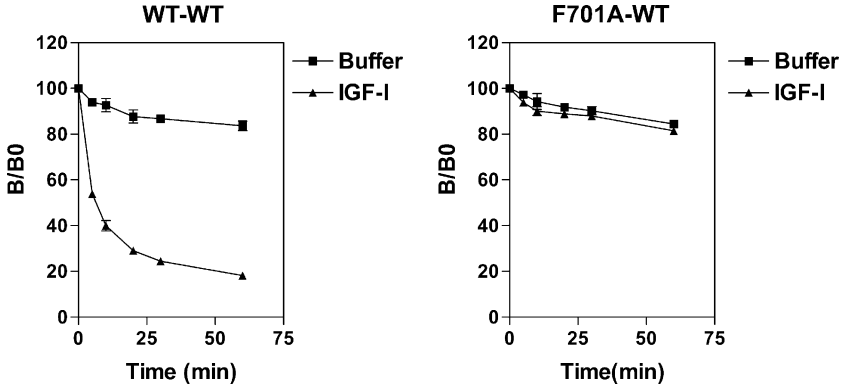

Fig. 2. Dissociation properties of hybrid receptors. Hybrid receptors, immobilized in microtiter plates, were incubated overnight at 4 °C with [^{125}I-Tyr^{31}]IGF-I (50 pM). Unbound [^{125}I-Tyr^{31}]IGF-I was removed by washing with ice-cold PBS 0.05% Tween 20. Dissociation at 25 °C was initiated by the addition of buffer or buffer containing 10^{-7} M IGF-I. Bound [^{125}I]IGF-I was determined at the indicated time points. Results are shown as means and SEM of four replicates from a representative experiment.

binding properties of the hybrid differ little from those of the wild-type IGF-I receptor, this second binding site must represent the low affinity component of binding.

According to the model [14], the inactivation of the site containing F701 would also be predicted to prevent a second ligand molecule from forming a cross-link with the receptor monomers and thus abrogate the negative cooperativity of the hybrid receptor. The failure to observe acceleration of the dissociation of prebound [^{125}I-Tyr^{31}]IGF-I from the F701A-wild-type hybrid by the presence of 10^{-7} M IGF-I in the dissociation buffer is consistent with this prediction.

While our findings appear to be consistent with De Meyts' model [14], it is possible that the deleterious effects of the F701A mutation on negative cooperativity are unique to this mutation. Further experiments will be necessary to answer this question.

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