A Novel Approach to Identify Two Distinct Receptor Binding Surfaces of Insulin-like Growth Factor II*‡

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Very little is known about the residues important for the interaction of insulin-like growth factor II (IGF-II) with the type 1 IGF receptor (IGF-1R) and the insulin receptor (IR). Insulin, to which IGF-II is homologous, is proposed to cross-link opposite halves of the IR dimer through two receptor binding surfaces, site 1 and site 2. In the present study we have analyzed the contribution of IGF-II residues equivalent to insulin’s two binding surfaces toward the interaction of IGF-II with the IGF-1R and IR. Four “site 1” and six “site 2” analogues were produced and analyzed in terms of IGF-1R and IR binding and activation. The results show that Val13, Phe28, and Val14 (equivalent to site 1) are critical to IGF-1R and IR binding, whereas mutation to alanine of Gln18 affects only IGF-1R and not IR binding. Alanine substitutions at Glu12, Asp15, Phe19, Leu53, and Glu57 analogues resulted in significant (>2-fold) decreases in affinity for both the IGF-1R and IR. Furthermore, taking a novel approach using a monomeric, single-chain minimized IGF-1R we have defined a distinct second binding surface formed by Glu12, Phe19, Leu53, and Glu57 that potentially engages the IGF-1R at one or more of the FnIII domains.

Insulin-like growth factor II (IGF-II)§ is a single-chain polypeptide with homology to IGF-I and insulin. Its 67 amino acids are arranged, like those counterparts in IGF-I, in four domains in the order B, C, A, and D from the N terminus (Fig. 1). IGF-II and -I share >60% sequence identity across these domains, whereas their B and A domains are homologous to the B and A chains of insulin (1). The three-dimensional structures of the IGFs and insulin are very similar. A central B domain α-helix (Gly17–Cys21 in IGF-II) and two smaller anti-parallel α-helices in the A domain (Glu44–Arg19 and Leu53–Tyr29 in IGF-II) are the key structural features of these proteins; the α-helical segments are stabilized by three strictly conserved disulfide bonds (2–8).

The mitogenic and metabolic activities of the IGFs and insulin result from their interaction with the type 1 IGF receptor (IGF-1R) and/or the exon 11- (IR-A) and exon 11+ (IR-B) isoforms of the insulin receptor (IR). These class II receptor tyrosine kinases exist at the membrane as preformed disulfide-linked homodimers composed of two α and two β subunits in a β-α-α-β arrangement (reviewed in Refs. 9–11). The IR and IGF-1R share between 41 and 84% sequence similarity that is most pronounced in their tyrosine kinase domains. Despite the homology in sequence and structure between these receptors, each exhibits distinct ligand binding preferences. The IGF-1R and IR bind their cognate ligands with high affinity (IGF-I and insulin, respectively). Both the IGF-1R and IR-A bind IGF-II with high affinity and are capable of mediating IGF-II action (12). Interestingly, the IR-B has a low affinity for IGF-II. The discriminating factors for this isoform discrimination are largely undefined but may involve steric hindrance between the 12 amino acids encoded by exon 11 of IR-B and the IGF-II C domain.

There are currently no structures of any of these ligand-receptor complexes. The binding of insulin to the IR is certainly the best characterized of these interactions. Insulin has two IR binding surfaces: the “site 1” binding surface lies within the insulin dimerization surface, whereas “site 2” overlaps its hexamer-forming surface. Insulin is proposed to cross-link opposite halves of the insulin receptor dimer through these two receptor binding surfaces (13, 14). The stoichiometry of binding at physiological insulin concentrations is 1:1. However, each IR has two potential ligand binding pockets both also consisting of two ligand binding surfaces (13 and 14). The stoichiometry of binding at physiological insulin concentrations is 1:1. However, each IR has two potential ligand binding pockets both also consisting of two ligand binding surfaces (13 and 14). The stoichiometry of binding at physiological insulin concentrations is 1:1. However, each IR has two potential ligand binding pockets both also consisting of two ligand binding surfaces (13 and 14). The stoichiometry of binding at physiological insulin concentrations is 1:1. However, each IR has two potential ligand binding pockets both also consisting of two ligand binding surfaces (13 and 14). The stoichiometry of binding at physiological insulin concentrations is 1:1. However, each IR has two potential ligand binding pockets both also consisting of two ligand binding surfaces (13 and 14). The stoichiometry of binding at physiological insulin concentrations is 1:1. However, each IR has two potential ligand binding pockets both also consisting of two ligand binding surfaces (13 and 14). The stoichiometry of binding at physiological insulin concentrations is 1:1. However, each IR has two potential ligand binding pockets both also consisting of two ligand binding surfaces (13 and 14). The stoichiometry of binding at physiological insulin concentrations is 1:1. However, each IR has two potential ligand binding pockets both also consisting of two ligand binding surfaces (13 and 14). The stoichiometry of binding at physiological insulin concentrations is 1:1. However, each IR has two potential ligand binding pockets both also consisting of two ligand binding surfaces (13 and 14). The stoichiometry of binding at physiological insulin concentrations is 1:1. However, each IR has two potential ligand binding pockets both also consisting of two ligand binding surfaces (13 and 14). The stoichiometry of binding at physiological insulin concentrations is 1:1. However, each IR has two potential ligand binding pockets both also consisting of two ligand binding surfaces (13 and 14). The stoichiometry of binding at physiological insulin concentrations is 1:1. However, each IR has two potential ligand binding pockets both also consisting of two ligand binding surfaces (13 and 14). The stoichiometry of binding at physiological insulin concentrations is 1:1. However, each IR has two potential ligand binding pockets both also consisting of two ligand binding surfaces (13 and 14). The stoichiometry of binding at physiological insulin concentrations is 1:1. However, each IR has two potential ligand binding pockets both also consisting of two ligand binding surfaces (13 and 14).
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additional interaction of the IGF-I C domain with the cysteine rich (CR) domain of IGF-1R contributes to high affinity binding (20).

In contrast to our knowledge of IGF-I and insulin receptor interactions there is relatively little known about the interaction of IGF-II with the IGF-1R and IR. Sequence and structural similarities suggest that IGF-II shares similar IR and IGF-1R binding surfaces (and similar modes of binding) to insulin and IGF-I. Three IGF-II residues that correspond to insulin’s site 1 binding surface, i.e. Val43 (ValA3), Phe26 (PheB24), and Tyr27 (PheB25) (insulin homologues in parentheses) have previously been shown to be important for IR and IGF-1R binding (23). The IGF-II C domain is largely responsible for the differences in specificity between IGF-I and IGF-II for the IR and IGF-1R (24), although, unlike the IGF-I C domain, alanine scanning mutagenesis of the IGF-1R and IR suggests it does not contact the CR domain of either receptor (21, 25).

In the present study we have mapped by site-directed mutagenesis the IGF-1R and IR binding surfaces of IGF-II. This has allowed the first comprehensive comparison of the receptor binding surfaces of IGF-II, IGF-I, and insulin. Furthermore, we have taken a novel approach using a minimized IGF-1R (see supplemental Fig. S1) to define two receptor binding surfaces on IGF-II. This has allowed us to identify subtle differences between the ligand binding surfaces of all three ligands, which could contribute toward receptor binding specificity. In addition we have investigated the possible contribution of the IGF-1R FnIII domains to IGF-II binding.

EXPERIMENTAL PROCEDURES

Materials and Cell Lines—Oligonucleotides (supplemental Table S1) were purchased from Geneworks Pty Ltd. (Adelaide, South Australia). Restriction enzymes were from New England Biolabs (Hitchin, UK) or Geneworks Pty Ltd. LongTMR3IGF-I was purchased from Novozymes GroPep Pty Ltd. (Adelaide, South Australia). Greiner Lumitrac 600 96-well plates were from Omega Scientific (Tarzana, CA). Human insulin was purchased from Novo Nordisk ( Bagsvaerd, Denmark). The DELFIA europium-labeling kit and DELFIA enhancement solution were purchased from PerkinElmer Life Sciences. Eu-IGF-II was produced as described by Denley et al. (24) according to the manufacturer’s instructions. IGF-I mono-iodinated at Tyr31 (125I-IGF-I) (26) was a gift from Novo Nordisk A/S. P6 IGF-1R cells (BALB/c3T3 cells overexpressing the human IGF-1R) (27) were a kind gift from Prof. R. Baserga (Philadelphia, PA). IGF-1R-negative cells overexpressing the IR-A (R-IR-A) or the IR-B (R-IR-B) were generated as described (24).

Construction of Expression Plasmids Encoding Human IGF-II Analogues—The IGF expression vector was developed by King et al. (28), and IGF-II cDNA was introduced into the vector as previously described (29). The QuikChange site-directed mutagenesis kit (Stratagene) was used to incorporate the 10 different mutations into IGF-II (supplemental Table S1). Resultant constructs were transformed into Escherichia coli JM101 (lac Iq) for expression. IGF-II mutants were expressed as fusion proteins with the first 11 amino acids of porcine growth hormone ([Met I] pGH (1–11)) after isopropyl β-D-thiogalactoside induction. Inclusion bodies were isolated as previously described (24).

Purification of IGF-II Analogues—The purification of the IGF-II analogues used in this study was as described in Delaine et al. (29). Purified proteins were analyzed by mass spectrometry and N-terminal sequencing (Dr. Chris Bagley and Chris Cursaro, Adelaide Proteomics Facility) and were shown to have the correct masses and to be >95% pure. All IGF-II analogues maintained the same fold as native IGF-II as determined by far-UV CD spectral analysis as previously described (29) (see supplemental Fig. S2). Quantitation of analogues was performed by comparing analytical C4 high-performance liquid chromatography profiles with profiles of standard LongTMR3IGF-I preparations (24).

Immunocaptured IGF-1R and IR Binding Assays—Receptor binding was measured essentially as described by (24). Briefly, R-IR-A, R-IR-B, or P6 IGF-1R cells were serum-starved for 4 h before lysis in lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 mM MgCl2, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5) for 1 h at 4 °C. Lysates were centrifuged for 10 min at 2,200 × g, then 100 μl was added per well to a white Greiner Luminitrac 600 96-well plate previously coated with anti-IR antibody 83-7 (30) or anti-IGF-1R antibody 24-31 (31) as appropriate. Approximately 500,000 fluorescent counts of Eu-IGF-II (IGF-1R and IR-A binding) or Eu-insulin (for IR-B binding) were added to each well along with increasing concentrations of unlabeled competitor in a final volume of 100 μl and incubated for 16 h at 4 °C.

Wells were washed four times with 20 mM Tris, 150 mM NaCl, and 0.1% (v/v) Tween 20 (TBST), then twice with water, and then DELFIA enhancement solution (100 μl/well) was added. Time-resolved fluorescence was measured using 340 nm excitation and 612 nm emission filters with a BMG Lab Technologies Polarstar fluorometer (Mornington, Australia). Assays were performed in triplicate at least three times.

Mini-IGF-1R Binding Assays—To construct an expression vector for the mini-IGF-1R (L1, CR, and L2 domains fused to the CTa peptide, residues 692–706, see supplemental Fig. S1) an oligonucleotide cassette encoding the CTa peptide preceded by an in-frame BamHI site and followed by a stop codon and an XbaI site was ligated into the BamHI and XbaI sites of pcDNA3.1zeo(+) (Invitrogen). An inverse BamHI site was introduced into the IGF-1R cDNA after the codon for Asp463, the C terminus of the IGF-1R L2 domain, by site-directed mutagenesis (32). The resulting HindIII-BamHI fragment, encoding the N terminus of the IGF-1R, including the L1, CR, and L2 domains, was subcloned into the pcDNA3.1zeo(+) plasmid containing the CTa peptide cassette to produce pCDZ mini-IGF-1R.

The recombinant secreted mini-IGF-1R protein was expressed by transient transfection of 293PEAK cells as previously described (20). Competitive binding assays with 125I-Tyr31 IGF-I and IGF-II analogues were performed on mini-IGF-1R from conditioned media harvested from transfected cells immobilized in microtiter plates coated with anti-receptor monoclonal antibody 24–31 as previously described (20). Dissociation constants were obtained by non-linear regression analysis of assay data by the method of Wang (33).
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Whole Cell Binding Assays—R-IR-A or P6 IGF-1R cells were serum-starved for 4 h and then harvested using cell disrupting buffer (40 mM Tris, pH 7.5, 10 mM EDTA, 150 mM NaCl), washed twice in ice-cold Dulbecco’s minimal essential medium (Intravention) and then resuspended in assay buffer (100 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 8 mM glucose, and 0.5% bovine serum albumin). 10^5 cells were incubated in a 1.5-ml centrifuge tube with Eu-IGF-II and competing IGF-II in a total volume of 300 μl of assay buffer overnight at 4 °C. Cells were pelleted at 1025 × g for 1.5 min, and the supernatant was aspirated. Cells were washed with 500 μl of cold TBS and pelleted, and the supernatant was aspirated again. Pellets were incubated with Enhancement solution (100 μl) in the dark for 30 min and then transferred to a white Greiner Lumitrac 600 96-well plate. Time-resolved fluorescence was measured as described above. Assays were performed in triplicate at least three times.

We have demonstrated that the IGF-2R does not interfere in these binding assays as Thr^{16} → Ala IGF-1R and Phe^{19} → Ala IGF-II behave identically to IGF-II (Table 1) and neither bind IGF2R (29).

IGF-1R and IR-A Phosphorylation Assays—Receptor phosphorylation was detected essentially as described by (24). Briefly, P6 IGF-1R cells (2.5 × 10^5 cells/well) or R-IR-A cells (5 × 10^5 cells/well) were plated in a Falcon 96 well flat bottom plate and grown overnight at 37 °C, 5% CO_2. Cells were starved in serum-free medium for 4 h before treatment with IGF-1 or IGF-II analogue in 100 μl of Dulbecco’s minimal essential medium with 1% bovine serum albumin for 10 min at 37 °C, 5% CO_2. Cells were lysed with ice-cold lysis buffer containing 2 mM Na_3VO_4 and 100 mM NaF, and receptors were captured onto white Greiner Lumitrac 600 96-well plates pre-coated with anti-IR antibody 83-7 (500 ng/well) (30) or anti-IGF-1R antibody 24–31 (250 ng/well) (31) as appropriate and blocked with 20 mM Tris, 150 mM NaCl, and 0.1% (v/v) Tween 20 (TBST)/0.5% bovine serum albumin (30, 31). Following overnight incubation at 4 °C, the plates were washed three times with TBST. Phosphorylated receptor was detected by incubation at room temperature for 2 h with europium-labeled antiphosphotyrosine antibody PY20 (76 ng/well, PerkinElmer Life Sciences). Wells were washed four times with TBST, twice with water, and then DELFIA enhancement solution (100 μl/well) was added. Time-resolved fluorescence was detected as described above. Assays were performed in triplicate at least three times.

RESULTS

Production and Characterization of IGF-II Analogues—To define and compare the receptor binding surfaces of IGF-II with those of insulin, two groups of IGF-II analogues were produced (Fig. 1). The site 1 analogues, Val^{14} → Thr, Gln^{18} → Ala, Phe^{28} → Leu, and Val^{43} → Met IGF-II, are so called because they incorporate amino acid substitutions at residues corresponding to insulin’s site 1 binding surface (Val^{B12}, Tyr^{B16}, Tyr^{B26}, and Val^{A3} in insulin, respectively). The site 2 analogues contain alanine substitutions at the residues Glu^{12}, Asp^{15}, Phe^{19}, Asp^{52}, Leu^{53}, and Glu^{57}, which correspond to insulin’s second binding surface (His^{R10}, Glu^{B13}, Leu^{B17}, Ser^{A12}, Leu^{A13}, and Glu^{A17}, respectively). Following successful expression in E. coli, analogues were purified, and their structural integrity was analyzed by far-UV CD spectroscopy. All analogues had CD spectra indistinguishable from that of IGF-II (29) (supplemental Fig. S2), indicating correct folding and maintenance of structural integrity.

IGF-1R Binding Properties of IGF-II Analogues—Competition binding assays were used to measure the affinities of IGF-II and IGF-II analogues for IGF-1R immunocaptured from P6 IGF-1R cell lysates (Fig. 2, A and B) and for IGF-1R present on P6 IGF-1R cells (Fig. 2, C and D), because there is evidence that some analogues behave differently in the two assay formats (34, 35). The affinities of IGF-II for either immunocaptured or membrane-bound IGF-1R were consistent with the literature (24, 36) (Table 1). As has previously been reported (24, 36) the IGF-II affinity for immunocaptured receptors was lower than IGF-1R on intact cells while relative binding affinities of the different analogues generally remained the same. To allow direct comparison of IGF-II analogue affinities between the two assays we have chosen to present these as affinities relative to wild-type IGF-II (Table 1).

With the exception of Gln^{18} → Ala, which displayed only a modest decrease in affinity (41–52% of IGF-II) for the IGF-1R, the site 1 analogues bound to this receptor with drastically reduced affinities. This was most marked for Val^{43} → Met IGF-II with an affinity <1% that of wild-type IGF-II. Phe^{28} → Leu and Val^{43} → Thr IGF-II bound the IGF-1R with ~10% the affinity of IGF-II (less on the whole cell receptor). Among the
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Relative binding of IGF-II and the IGF-II analogues for the IGF-1R and IR isoforms

Binding affinities of the analogues to IGF-II were derived from the IC₅₀ values for all but the mini-IGF-1R binding data, which were derived using Kᵢ values. Relative binding is expressed as a percent of IGF-II ± S.E. Data is derived from at least 3 separate experiments performed in triplicate.

| IGF-II* | R¹ IR-A | IR-A | IR-B | P6 IGF-1R | IGF-1R | Mini-IGF-1R |
|---------|---------|------|------|-----------|--------|-------------|
|         | (2.0 ± 0.5 nM) | (4.0 ± 0.4 nM) | (29.7 ± 3.8 nM) | (1.1 ± 0.3 nM) | (3.4 ± 0.2 nM) | (28.5 ± 0.8 nM) |
| Val¹⁵ → Thr | 19.5 ± 6.3 | 9.1 ± 1.1 | 187.2 ± 20.5 | 40.9 ± 20.6 | 10.7 ± 1.4 | 19.1 ± 0.4 |
| Glu¹² → Ala | 129.5 ± 12.4 | 144.4 ± 5.4 | 16.5 ± 3.0 | 10.6 ± 5.8 | 5.19 ± 5.1 | 43.9 ± 0.0 |
| Phe⁶ → Leu | 6.2 ± 2.5 | 7.5 ± 1.7 | NA | 2.8 ± 0.8 | 10.5 ± 3.3 | 9.4 ± 0.07 |
| Val¹² → Met | NA | NA | NA | NA | NA | NA |
| Glu¹² → Ala | 11.3 ± 1.7 | 128.1 ± 17.0 | 210.6 ± 75.4 | 16.3 ± 6.7 | 40.7 ± 1.8 | 256.8 ± 6.9 |
| Asp¹⁵ → Ala | 7.4 ± 3.4 | 16.5 ± 3.0 | 15.6 ± 4.3 | 10.6 ± 5.8 | 9.2 ± 0.5 | 121.2 ± 0.2 |
| Phe⁶ → Ala | 50.9 ± 26.7 | 30.8 ± 1.7 | 51.8 ± 4.1 | 112.2 ± 45.2 | 35.8 ± 3.3 | 191.3 ± 5.1 |
| Asp¹⁵ → Ala | 113.2 ± 57.0 | 133.3 ± 14.0 | 134.4 ± 34.5 | 110.8 ± 47.2 | 86.7 ± 3.9 | 154.9 ± 2.5 |
| Leu² → Ala | 89.7 ± 17.6 | 25.2 ± 1.3 | 42.6 ± 4.3 | 45.9 ± 10.7 | 40.3 ± 1.3 | 102.3 ± 4.7 |
| Glu¹² → Ala | 32.6 ± 1.9 | 17.1 ± 2.3 | 48.2 ± 27.7 | 34.2 ± 0.7 | 51.0 ± 1.7 | 51.0 ± 1.7 |

a The IC₅₀ of IGF-II in each assay, or Kᵢ in the case of the mini-IGF-1R assay, is shown in parenthesis.

b NA = an IC₅₀ could not be derived. Binding to R¹ IR-A = binding to R¹ IR-A cells, IR-A = to immunocaptured IR-A, IR-B = to immunocaptured IR-B, P6 IGF-1R = to BALB/c3T3 cells overexpressing the IGF-1R, IGF-1R = to immunocaptured IGF-1R, Mini-IGF-1R = to immunocaptured mini-IGF-1R.

c Only two experiments were performed for this analogue.
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FIGURE 3. Competitive binding of IGF-II to immunocaptured IR-A and and R' IR-A cells. Immunocaptured IR-A (A and B) or R' IR-A cells (C and D) were incubated with Eu-IGF-II in the presence or absence of increasing concentrations of IGF-II (○), Val14→Thr IGF-II (△), Gln18→Ala IGF-II (□), Phe28→Leu IGF-II (○), or Val53→Met IGF-II (■) (A and C, top legend) or IGF-II (○), Glu12→Ala IGF-II (□), Asp15→Ala IGF-II (■), Phe19→Ala IGF-II (□), Asp52→Ala IGF-II (□), Leu53→Ala IGF-II (□), or Glu57→Ala IGF-II (□) (B and D, bottom legend). Results are expressed as a percentage of binding in the absence of competing ligand, and the data points are the mean ± S.E. of triplicate samples. Error bars are shown when greater than the size of the symbols. Representative graphs of four separate experiments are shown.

FIGURE 4. Activation of the IGF-1R and IR-A by IGF-II and IGF-II analogues. P6 IGF-1R cells and R' IR-A cells were serum starved for 4 h and then incubated with increasing concentrations of IGF-II (○), Val14→Thr IGF-II (△), Gln18→Ala IGF-II (□), Phe28→Leu IGF-II (○), or Val53→Met IGF-II (■) (A and C, top legend) or IGF-II (○), Glu12→Ala IGF-II (□), Asp15→Ala IGF-II (■), Phe19→Ala IGF-II (□), Asp52→Ala IGF-II (□), Leu53→Ala IGF-II (□), or Glu57→Ala IGF-II (□) (B and D, bottom legend) for 10 min. Solubilized IGF-1R (A and B) and IR-A (C and D) were immunocaptured, and phosphorylated tyrosines were detected with Eu-PY20. Receptor phosphorylation is expressed as a percentage of the maximal phosphorylation induced by IGF-II. The graphs shown are representative of three experiments, and data points are means ± S.E. of triplicate samples.

IR Binding Properties of IGF-II Analogues—Further competition assays were used to measure the affinities of IGF-II analogues for the IR-A present on intact R' IR-A cells (Fig. 3, C and D, and Table 1) and immunocaptured IR-A (Fig. 3, A and B, and Table 1) and IR-B (Table 1). As seen for the IGF-1R, the affinities of the wild-type ligands for the IR-A were higher in the whole cell receptor assay than those for the immunocaptured receptor (Table 1), and these are consistent with previous reports (24, 36).

The relative binding affinities for the immunocaptured IR-A are IGF-II = Glu12→Ala = Asp52→Ala = Glu57→Ala = Asp15→Ala > Val14→Thr > Phe28→Leu (Table 1). A similar order of relative affinities was seen for the IGF-1R, although Gln18→Ala, in contrast to its 2-fold decrease in affinity for the IGF-1R, bound the IR-A with wild-type affinity (129–144%). In addition, the IR-A and IR-B binding properties of the IGF-II analogues were essentially identical; no analogue exhibited >2-fold difference in its binding to the IR-A compared with the IR-B (Table 1).

The affinities of the analogues relative to IGF-II for the immunocaptured IR-A and IR-A on cells were, for the most part, comparable (Fig. 3 and Table 1). Glu12→Ala, however, was an exception as this analogue exhibited a 10-fold decrease in affinity for the IR-A in the R' IR-A whole cell receptor assay but had a very similar affinity (128%) to IGF-II for the immunocaptured receptor. The opposite was true for Leu53→Ala IGF-II (89.7% of IGF-II for R-IR-A cells and 52.5% of IGF-II for immunocaptured IR-A). Asp15→Ala and Val14→Thr also showed a slight difference (~2-fold) across the two assay formats.

Receptor Phosphorylation Properties of IGF-II Analogues—To correlate receptor binding with receptor activation, the abilities of IGF-II and the analogues to stimulate IGF-1R and IR-A phosphorylation were assessed in kinase receptor activation assays (Fig. 4). Maximal IGF-1R phosphorylation was achieved following 10-min treatment of P6 IGF-1R cells with 100 nM IGF-II; higher concentrations resulted in less than maximal phosphorylation (Fig. 4, A and B). In contrast, IGF-II was a less potent activator of the IR-A than IGF-1R and IGF-II-induced stimulation of the IR-A was still approaching its maximum at 1 μM (Fig. 4, C and D).

The ability of most of the analogues to stimulate receptor phosphorylation correlated well with their receptor binding
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Unlike insulin and IGF-I, with which it shares structural homology and a number of biological activities, IGF-II binds both the IGF-1R and IR-A with high affinity. However relatively little was previously known about the IGF-II receptor binding interfaces. The present study systematically identifies specific residues of IGF-II required for these interactions using a series of IGF-II analogues inspired by the known receptor binding surfaces of insulin.

Two distinct receptor binding surfaces have been described for insulin with each proposed to contact opposite halves of the insulin receptor dimer. Site 1 comprises (IGF-II homologues in parenthesis): GlyA1 (Gly41), IleA2 (Ile42), ValA3 (Val43), GluA5 (Glu45), ThrA8 (Phe48), TyrA9 (Tyr49), AsnA21 (Ala61), ValB12 (Val14), TyrB16 (Gln18), GlyB23 (Glu25), PheB24 (Phe26), PheB25 (Tyr27), and TyrB26 (Phe28) and, as the first identified of insulin’s receptor binding surfaces, is often referred to as the “classic” binding surface. A further six residues, SerA12 (Asp52), LeuA13 (Leu53), GluA17 (Glu57), HisB10 (Glu12), GluB13 (Asp15), and LeuB17 (Phe19), collectively known as site 2, were later shown by alanine scanning mutagenesis to contribute to IR binding (9, 14, 35, 38). We recombiantly expressed and purified a series of IGF-II analogues with amino acid substitutions at positions corresponding to the two receptor binding surfaces of insulin and determined their receptor binding affinities. This allowed a direct comparison between the binding surfaces of insulin and IGF-II for the IR, and between IGF-I and IGF-II binding surfaces for the IGF-1R.

Given evidence in the literature that some analogues may behave differently in the two assay formats (22, 35), the affinities of all IGF-II analogues were measured using both immunocaptured receptors (IGF-1R, IR-A, or IR-B) and whole cell receptor binding assays with cells expressing IGF-1R or IR-A. This observation was affirmed in the present study where receptor binding by the Glu12 → Ala analogue, for instance, was more affected when binding whole cells than immunocaptured receptor, an observation also made for the equivalent analogue of IGF-1 (Glu9 → Ala IGF-I) (22). The opposite was true for Phe19 → Ala on the IGF-1R and Leu53 → Ala on the IR-A where a greater effect was seen in immunocapture assays than whole cell binding assays. The interaction of the IGFs and insulin with these receptors is a dynamic process that almost certainly involves flexible regions of the receptor and structural change, which is apparently not fully recapitulated in the absence of membrane anchorage. It has been shown that steric interactions between the two αβ receptor halves (apart from specific ligand binding determinants) are necessary to produce high affinity binding of ligand to the IR (39). It is possible that detergent lysis of receptors prior to immunocapture could disturb these interactions and thus disrupt the receptor dimer interface and in turn ligand binding. Alternatively, although the antibodies used to immunocapture the receptor do not directly compete with ligand binding (30, 31), immunocapture of the receptor may impose restrictions on how the receptor is able to change its conformation upon ligand binding. Consistent with either mechanism, and in agreement with previous reports (24, 36), in our hands IGF-II had a higher affinity for the IGF-1R and IR-A on whole cells compared with immunocaptured receptor.

Among the site 1 residues analyzed in the present study Val14, Phe28, and Val43 all proved critical to the binding of IGF-II to both the IGF-1R and IR isoforms, with ≥10-fold decreases in affinity resulting from their mutation. These residues form a continuous hydrophobic patch together with the aromatic sidechains of Phe26 and Tyr27 previously shown to be important for IR and IGF-1R binding (23). This hydrophobic cluster appears to provide the major contribution to the free energy of binding to both receptors; the corresponding residues are also important for the binding of both insulin to the IR and IGF-I to the IGF-1R. Indeed, mutation at ValA3 (Val43 in IGF-II) in insulin is associated with diabetes mellitus (Wakayama insulin) (40), whereas Val mutation to Met at the equivalent residue 44 in IGF-I was identified in a patient with severe growth retardation (41, 42). In the case of insulin, Val43 at the N terminus of the A chain is largely buried within a cleft between the A and B chains. It has been proposed that displacement of the C terminus of the B-chain upon insulin binding exposes ValA3 and IleA2 and thus allows it to directly contact the IR (43). Photoaffinity cross-linking studies at ValA3 point to its interaction with the FnIII-2 insert domain of the IR (40, 43), which forms part of the IR site 1 binding region along with residues of the L1 domain. The NMR structure of IGF-II shows that the corresponding residue Val43 in IGF-II is largely surface-exposed (5, 6) and so is already in a position to interact with the IR (and IGF-1R) and thus would not necessarily require an equivalent structural change. Consistent with the importance of Val43 to the IGF-1R and IR binding of IGF-II, IR binding is highly sensitive to mutation at the equivalent ValB12 residue of insulin with extensive mutagenesis pointing to the importance of both side-chain bulk and polarity (44, 45). The equivalent Val11 in IGF-I however appears less critical to its interaction with the IGF-1R. Val11 → Thr IGF-I binds the IGF-1R with 3.8-fold lower affinity than IGF-I (46) compared with the 10- to 30-fold decrease in affinity for Val14 → Thr IGF-II analyzed in the present study (Table 1).

Gln18 in IGF-II lies at the periphery of the hydrophobic patch described above and appears to play a role in modulating the
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specificity of IGF-II for the IGF-1R and IR. Mutation of this residue to alanine was without effect on IR binding but caused a 2-fold decrease in IGF-1R binding, indicating a difference in the way in which Gln15 interacts with these two receptors. Mutation of the corresponding Gln15 in IGF-I to alanine has a similar effect on IGF-1R binding as seen here for IGF-II (48).

That IGF-II can accommodate Gln and Ala equally in its interaction with the IR represents a small point of difference between the interactions of insulin and IGF-II with the IR. The corresponding insulin residue, TyrB16, has been extensively mutated, and there is a preference for an aromatic side chain and alanine over glutamine for IR binding (44, 45, 47). Mutation of TyrB16 to glutamine results in an 11-fold decrease in affinity for the IR-A, whereas mutation to alanine lead to only a 3-fold decrease (45). TyrB16 cross-links to the L1 domain beta helix that forms part of site 1 (44). Mutation of Gln15 in IGF-I to Tyr produces a 3.8-fold increase in IR-A binding and when mutated in combination with Thr4 → His, Phe49 → Thr, Ser51 → Ile this analogue has an equal affinity to insulin for the IR-A (34). Accordingly, we would predict that substitution of Gln18 in IGF-II with Tyr would result in an increase in IR-A binding.

Five of the six site 2 analogues (Glu12 → Ala, Asp15 → Ala, Phe19 → Ala, Leu23 → Ala, and Glu57 → Ala) had significant (>2.5-fold) decreases in affinity for the IGF-1R and IR isoforms. Only alanine substitution of Asp52 was without effect on receptor binding as was the case for the equivalent IGF-I analogue (Asp53 → Ala IGF-I) (22); the equivalent insulin analogue produced a ∼2.5-fold decrease in affinity for the IR-A on IM9 cells (35). Of these five, it was substitution of the charged residues Glu12, Asp15, and Glu57 that caused the most significant decreases in affinity for both IGF-1R and IR-A, as is the case for IGF-I binding the IGF-1R (22). In insulin it is Glu13 and the hydrophobic Leu17 and Leu13 of the six site 2 residues that contribute most to IR binding (equivalent to Asp15, Phe19, and Leu53 in IGF-II) (35). This suggests that within site 2 IGF-II shares more in common with IGF-I, both in terms of sequence identity and contribution of specific residues to binding, than with insulin and, in agreement with the work of Benyoulcef et al. (19), may explain why IGF-I and IGF-II can bind hybrid IGF-1R/IR receptors, whereas insulin does with only low affinity (49).

All of the analogues showed similar affinities, relative to IGF-II, for both the IR-A and IR-B. We can therefore conclude that these residues do not contribute to the differing affinity of IGF-II for these receptors.

The designation of insulin residues (and by inference IGF-I and IGF-II) into sites 1 and 2 is largely historical with site 1 identified first, and site 2 not until years later (9). Direct evidence for interaction of site 1 and site 2 residues with two distinct receptor binding regions is fundamentally lacking. In this study we decided to define our IGF-II analogues based upon their abilities to bind two different forms of the IGF-1R: the full-length IGF-1R and the mini-IGF-1R construct containing only the site 1 component of IGF-1R. The mini-IGF-1R comprises the first three domains (L1-CR-L2) fused to residues 692–706 of the α subunit (CTa) and thus lacks the loops of the FnIII-1 and FnIII-2 domains that are proposed to accommodate site 2 on the receptor (9, 17, 37). We hypothesized that residues that interact with site 2 of the receptor (or indeed any part of the receptor outside of the L1-CR-L2-CTa) would, when mutated, only show a decrease in affinity for the full-length and not the mini-IGF-1R. Conversely, residues that interact with site 1 of the receptor would show similar decreases in affinity for both the full-length and mini-IGF-1R. Of the nine analogues in this study that had a significant (>2-fold) decrease in affinity for the full-length IGF-1R (in either assay format), five showed a similar decrease in affinity for the mini-IGF-1R. These were Val14 → Thr, Gln18 → Ala, Phe28 → Ala, and Val43 → Met of the site 1 analogues and, perhaps unexpectedly, Asp15 → Ala from site 2. It is thus unlikely that Asp15 is part of the site 2 surface but is rather an extension of site 1 and more likely interacts with the L1 or CTa. Asp15 is part of the B chain α helix and is adjacent to Val14, Gln18, and the hydrophobic cluster of residues that form site 1.

In contrast, site 2 analogues Phe19 → Ala and Leu53 → Ala bound the mini-IGF-1R with essentially wild-type affinity (<2-fold change). Glu12 → Ala did not show a decrease in affinity relative to IGF-II for the mini-IGF-1R, whereas Glu57 → Ala had only a 1.9-fold decrease. However, all of these site 2 analogues had a significant (>2-fold) decrease in affinity for the full-length IGF-1R. These results support the notion that Glu12, Phe19, Leu53, and Glu57 form part of a second binding surface, which might interact with the FnIII domains of the IGF-1R.

The IGF-II site 1 and 2 residues are mapped onto a model of IGF-II in complex with the IGF-1R L1-CR-L2 domains in Fig. 5. The model is adapted from the Epa et al. model (50) of the IGF-I-IGF-1R (L1-CR-L2) complex, which assumes that Tyr24 of IGF-I contacts the L1 domain and Arg59 and Arg77 contact the CR domain, and is consistent with insulin:IR cross-linking studies, which suggest that the IR site 1 consists of the L1 and FnIII-2 insert domains (40, 44). This orientation of IGF-II within the L1-CR-L2 domains leaves the IGF-II site 2 residues identified in the present study (Glu12, Phe19, Leu53, and Glu57)
accessible to bind to the proposed FnIII site 2 on the receptor and is similar to that proposed by Gauguin et al. (22) for IGF-I. Unfortunately, the affinity of IGF-II for the equivalent mini-IR construct is not sufficiently high to enable the analogus set of experiments with the insulin receptor. Given, however, the similarity in effect on binding affinity of the majority of analogues (all but Gln18 → Ala-IGF-II) on both the IGF-1R and IR, similar results would be expected. At this stage this approach to define two binding regions using the two receptor forms has not been reported for insulin and IGF-I analogues. It will be interesting to see whether the equivalent analogues behave the same way or whether small differences in the ligand-receptor interactions exist.

With the exception of Glu12 → Ala, all the IGF-II analogues showed potency in stimulating IGF-1R and IR-A phosphorylation that correlated with their ability to bind these receptors. Glu12 → Ala in contrast showed reduced potency in IGF-1R and IR-A phosphorylation that was unexpected in light of its affinity. As well as reduced potency, Glu12 → Ala also showed a reduction in maximum response, stimulating IGF-1R phosphorylation to just 40% that of wild-type IGF-II. The mechanism for this loss of efficacy is the subject of ongoing investigation in our group; however, it is tempting to speculate that mutation at this position alters the ability or efficiency of that ligand to cross-link the two receptor halves and to thus activate the tyrosine kinase activity of the receptor. Glu12 → Ala was also interesting in that it showed a much greater decrease in affinity for the IGF-1R and IR in the whole cell binding assay than when binding to immunocaptured receptor. A similar observation was recently made with the equivalent IGF-I analogue Glu9 → Ala IGF-I, which interestingly also was unable to accelerate dissociation of IGF-I from the IGF-1R (22). The equivalent His810 residue in insulin (which plays a role in coordinating Zn2+ into the insulin hexamer) is important for insulin receptor binding and insulin activity. Substitution with Asp, which makes it more “IGF-like” at that position gives rise to a “super-active” insulin (51). Therefore this residue plays a vital role for all three ligands in receptor binding and subsequent activation.

With the elucidation of the IGF-1R and IR binding surfaces of IGF-II the present study allows a comparison of the receptor binding surfaces of insulin, IGF-I and IGF-II. We conclude that IGF-II uses almost identical residues for binding the IGF-1R and IR-A. Furthermore, IGF-II has broadly similar IGF-1R and IR binding surfaces to IGF-I and insulin with subtle differences in the relative contribution of particular amino acids (1, 9, 22, 35). Similar observations have been made on the receptor side of the interaction, with alanine scanning mutagenesis of the IGF-1R and IR demonstrating that, within broadly similar ligand binding regions of the receptor, there are subtle differences in the contribution of certain residues to the binding of the different ligands (20, 21, 25, 52–54). Evidence in the literature suggests that any of IGF-I, IGF-II, and insulin can with small modifications have the specificity of one of the other ligands suggesting that there is significant plasticity within the IR and IGF-1R to allow high affinity binding of ligand to be variously achieved (24, 34). It is an important distinction, however, that affinity does not necessarily correlate with biological outcome. For example, despite binding equipotency, insulin and the synthetic peptide mimetic S597 initiate divergent signaling outcomes through the IR (55, 56). The ultimate goal now is to understand how different ligands bind the same receptor to give different biological outcomes. An understanding of this process might then allow the design of analogues to produce a desired biological outcome.

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REFERENCES

1. Denley, A., Cosgrove, L. J., Booker, G. W., Wallace, J. C., and Forbes, B. E. (2005) Cytokine Growth Factor Rev. 16, 421–439
2. Rinderknecht, E., and Humbel, R. E. (1978) J. Biol. Chem. 253, 2769–2776
3. Cooke, R. M., Harvey, T. S., and Campbell, I. D. (1991) Biochemistry 30, 5484–5491
4. Sato, A., Nishimura, S., Ohkubo, T., Kyogoku, Y., Koyama, S., Kobayashi, M., Yasuda, T., and Kobayashi, Y. (1993) Int. J. Pept. Protein Res. 41, 433–440
5. Torres, A. M., Forbes, B. E., Aplin, S. E., Wallace, J. C., Francis, G. L., and Norton, R. S. (1995) J. Mol. Biol. 248, 385–401
6. Terasawa, H., Kohda, D., Hatanaka, H., Nagata, K., Higashihashi, N., Fujiiwara, H., Sakano, K., and Inagaki, F. (1994) EMBO J. 13, 5590–5597
7. Bentley, G., Dodson, E., Dodson, G., Hodgkin, D., and Mercola, D. (1976) Nature 261, 166–168
8. Baker, E. N., Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. M., Hubbard, R. E., Isaacs, N. W., Reynolds, C. D., Sakabe, K., Sakabe, N., and Vijayan, N. M. (1988) Philos. Trans. R Soc. Lond. B Biol. Sci. 319, 369–456
9. De Meyts, P. (2008) Trends Biochem. Sci. 33, 376–384
10. Adams, T. E., Epa, V. C., Garrett, T. P., and Ward, C. W. (2000) Cell Mol. Life Sci. 57, 1050–1093
11. De Meyts, P., and Whittaker, J. (2002) Nat. Rev. Drug Discov. 1, 769–783
12. Denley, A., Wallace, J. C., Cosgrove, L. J., and Forbes, B. E. (2003) Horm. Metab. Res. 35, 778–785
13. De Meyts, P., and Whittaker, J. (2004) Bioessays 26, 1351–1362
14. Schaffer, L. (1994) Eur. J. Biochem. 221, 1127–1132
15. McKern, N. M., Lawrence, M. C., Streltsov, V. A., Lou, M.-Z., Adams, T. E., Lovrecz, G. O., Elleman, T. C., Richards, K. M., Bentley, J. D., Pilling, P. A., Hoyne, P. A., Cartledge, K. A., Pham, T. M., Lewis, I. L., Sankovich, S. E., Stoichevska, V., Da Silva, E., Robinson, C. P., Frenkel, M. J., Sparrow, L. G., Fernley, R. T., Epa, V. C., and Ward, C. W. (2006) Nature 443, 218–221
16. Hao, C., Whittaker, L., and Whittaker, J. (2006) Biochem. Biophys. Res. Commun. 347, 334–339
17. Ward, C., Lawrence, M., Streltsov, V., Garrett, T., McKern, N., Lou, M. Z., Lovrecz, G., and Adams, T. (2008) Acta Physiol. (Oxf) 192, 3–9
18. Chan, S. I., Nakagawa, S., and Steiner, D. F. (2007) J. Biol. Chem. 282, 13754–13758
19. Benyoucef, S., Surinya, K. H., Hadaschik, D., and Siddle, K. (2007) Biochem. J. 403, 603–613
20. Whittaker, J., Groth, A. V., Mynarcik, D. C., Pluzek, L., Gadsboll, V. L., and Whittaker, L. J. (2001) J. Biol. Chem. 276, 43980–43986
21. Whittaker, J., Sorensen, H., Gadsboll, V. L., and Hinrichsen, J. (2002) J. Biol. Chem. 277, 47380–47384
22. Gauguin, L., Delaine, C., Alvino, C. L., McNeil, K. A., Wallace, J. C., Forbes, B. E., and De Meyts, P. (2008) J. Biol. Chem. 283, 20821–20829
23. Sakano, K., Enjoh, T., Numata, F., Fujiiwara, H., Marumoto, Y., Higashihashi, N., Sato, Y., Perdue, J. F., and Fujita-Yamaguchi, Y. (1991) J. Biol. Chem.
Defining Two IGF-II Receptor Binding Surfaces

Chem. 266, 20626–20635

24. Denley, A., Bonython, E. R., Booker, G. W., Cosgrove, L. J., Forbes, B. E., Ward, C. W., and Wallace, J. C. (2004) Mol. Endocrinol. 18, 2502–2512

25. Sorensen, H., Whittaker, L., Hinrichsen, J., Groth, A., and Whittaker, J. (2004) FEBS Lett. 565, 19–22

26. Schaffer, L., Larsen, U. D., Linde, S., Hejnaes, K. R., and Skriver, L. (1993) Biochim. Biophys. Acta 1203, 205–209

27. Sell, C., Dumenil, G., Deveaud, C., Miura, M., Coppola, D., DeAngelis, T., Rubin, R., Efstratiadis, A., and Baserga, R. (1994) Mol. Cell Biol. 14, 3604–3612

28. King, R., Wells, J. R., Krieg, P., Snoswell, M., Brazier, J., Bagley, C. J., Wallace, J. C., Ballard, F. J., Ross, M., and Francis, G. L. (1992) J. Mol. Endocrinol. 8, 29–41

29. Delaine, C., Alvino, C. L., McNeil, K. A., Mulhern, T. D., Gauguin, L., DeMeyts, P., Jones, E. Y., Brown, J., Wallace, J. C., and Forbes, B. E. (2007) J. Biol. Chem. 282, 18886–18894

30. Soos, M. A., O’Brien, R. M., Brindle, N. P., Stigter, J. M., Okamoto, A. K., Whittaker, J., and Siddle, K. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5217–5221

31. Soos, M. A., Field, C. E., Lammers, R., Ullrich, A., Zhang, B., Roth, R. A., Andersen, A. S., Kjeldsen, T., and Siddle, K. (1992) J. Biol. Chem. 267, 12955–12963

32. Kirsch, R. D., and Joly, E. (1998) Nucleic Acids Res. 26, 1848–1850

33. Wang, Z. X. (1995) FEBS Lett. 360, 111–114

34. Gauguin, L., Klaproth, B., Sajid, W., Andersen, A. S., McNeil, K. A., Forbes, B. E., and De Meyts, P. (2008) J. Biol. Chem. 283, 2604–2613

35. Jensen, M. (2000) Analysis of Structure-activity Relationships at the Insulin Molecule by Alanine-scanning Mutagenesis. Masters thesis, University of Copenhagen, Denmark

36. Frasca, F., Pandini, G., Scalia, P., Sciacca, L., Mineo, R., Costantino, A., Goldfine, I. D., Belfiore, A., and Vigneri, R. (1999) Mol. Cell Biol. 19, 3278–3288

37. Kristensen, C., Wiberg, F. C., and Andersen, A. S. (1999) J. Biol. Chem. 274, 37351–37356

38. De Meyts, P. (1994) Diabetologia 37, S135–S148

39. Bass, J., Kurose, T., Pashmforoush, M., and Steiner, D. F. (1996) J. Biol. Chem. 271, 19367–19375

40. Wan, Z. L., Huang, K., Xu, B., Hu, S. Q., Wang, S., Chu, Y. C., Katsoyannis, P. G., and Weiss, M. A. (2005) Biochemistry 44, 5000–5016

41. Denley, A., Wang, C. C., McNeil, K. A., Walenkamp, M. J., van Duyvenvoorde, H., Wit, J. M., Wallace, J. C., Norton, R. S., Karperien, M., and Forbes, B. E. (2005) Mol. Endocrinol. 19, 711–721

42. Walenkamp, M. J., Karperien, M., Pereira, A. M., Hilhorst-Hofstee, Y., van Doorn, J., Chen, J. W., Mohan, S., Denley, A., Forbes, B., van Duyvenvoorde, H. A., van Thiel, S. W., Slioums, C. A., Bax, J. J., Breuning, M. B., Romijn, J. A., and Wit, J. M. (2005) J. Clin. Endocrinol. Metab. 90, 2855–2864

43. Hua, Q. X., Shoelson, S. E., Kochoyan, M., and Weiss, M. A. (1991) Nature 354, 238–241

44. Huang, K., Xu, B., Hu, S. Q., Chu, Y. C., Hua, Q. X., Qu, Y., Li, B., Wang, S., Wang, R. Y., Nakagawa, S. H., Theede, A. M., Whittaker, J., De Meyts, P., Katsoyannis, P. G., and Weiss, M. A. (2004) J. Mol. Biol. 341, 529–550

45. Glendorf, T., Sorensen, A. R., Nishimura, E., Pettersson, I., and Kjeldsen, T. (2008) Biochemistry 47, 4743–4751

46. Hodgson, D. R., May, F. E., and Westley, B. R. (1995) Eur. J. Biochem. 233, 299–309

47. Hu, S. Q., Burke, G. T., and Katsoyannis, P. G. (1993) J. Protein Chem. 12, 741–747

48. Jansson, M., Uhlen, M., and Nilsson, B. (1997) Biochemistry 36, 4108–4117

49. Slabry, R., Schaffer, L., Lautrup-Larsen, I., Andersen, A. S., Shaw, A. C., Mathiesen, I. S., and Brandt, I. (2006) J. Biol. Chem. 281, 25869–25874

50. Cacioppo, V., and Ward, C. W. (2006) Protein Eng. Des. Sel. 19, 377–384

51. Schwartz, G. P., Burke, G. T., and Katsoyannis, P. G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6408–6411

52. Myhr, D. C., Williams, P. F., Schaffer, L., Yu, G. Q., and Whittaker, J. (1997) J. Biol. Chem. 272, 18650–18655

53. Myhr, D. C., Yu, G. Q., and Whittaker, J. (1996) J. Biol. Chem. 271, 2439–2442

54. Whittaker, J., and Whittaker, L. (2005) J. Biol. Chem. 280, 20932–20936

55. Jensen, M., Hansen, B., De Meyts, P., Schaffer, L., and Urso, M. B. (2007) J. Biol. Chem. 282, 35179–35186

56. Jensen, M., Palsgaard, I., Borup, R., de Meyts, P., and Schaffer, L. (2008) Biochem. J. 412, 435–445