The mammalian insulin-like growth factor (IGF)-II/cation-independent mannose 6-phosphate receptor (IGF2R) binds IGF-II with high affinity. By targeting IGF-II to lysosomal degradation, it plays a role in the maintenance of correct IGF-II levels in the circulation and in target tissues. Loss of IGF2R function is associated with tumor progression; therefore, the IGF2R is often referred to as a tumor suppressor. The interaction between IGF2R and IGF-II involves domains 11 and 13 of the 15 extracellular domains of the receptor. Recently, a hydrophobic binding region was identified on domain 11 of the IGF2R. In contrast, relatively little is known about the residues of IGF-II that are involved in IGF2R binding and the determinants of IGF2R specificity for IGF-II over the structurally related IGF-I. Using a series of novel IGF-II analogues and surface plasmon resonance assays, this study revealed a novel binding surface on IGF-II critical for IGF2R binding. The hydrophobic residues Phe\textsuperscript{19} and Leu\textsuperscript{53} are critical for IGF2R binding, as are residues Thr\textsuperscript{16} and Asp\textsuperscript{52}. Furthermore, Thr\textsuperscript{16} was identified as playing a major role in determining why IGF-II, but not IGF-I, binds with high affinity to the IGF2R.

The mammalian insulin-like growth factor-II (IGF-II)/cation-independent mannose 6-phosphate receptor (IGF2R) has multiple functions due to its ability to interact with a wide variety of ligands. This transmembrane receptor has 15 homologous extracellular repeat domains and a short intracellular domain, which lacks kinase activity (1). The IGF2R plays a major role in targeting mannose 6-phosphate-labeled proteins via the trans-Golgi network and from the cellular membrane to lysosomes for degradation (2). It also binds and internalizes IGF-II, thereby maintaining correct levels of IGF-II locally and in the circulation. These functions are central in the processes of embryonic development and normal growth. Mice lacking the IGF2R have increased levels of circulating IGF-II, are born larger, and die soon after birth due to cardiac hyperplasia. This phenotype is overcome by concomitant deletion of the IGF-II gene (3). Likewise, loss of IGF2R activity in many cancers has been associated with increased tumor cell growth and tumor progression. This can arise from the loss of heterozygosity or mutation of the receptor (4, 5), leading to an increase in bio-available IGF-II, which then acts via the type 1 IGF receptor (IGF-1R) to promote cancer growth and survival.

The extracellular domains of the IGF2R share significant similarity with the single extracellular domain of the cation-dependent mannose 6-phosphate receptor (2). Domains 3 and 9 and more recently domain 5 of the IGF2R were shown to contain mannose 6-phosphate binding sites (6–8) and act as binding sites for ligands, including the urokinase plasminogen activator receptor, plasminogen, transforming growth factor-\(\beta\), and retinoic acid. A distinct IGF-II binding site is located on domain 11, and the additional presence of domain 13 is required for high affinity IGF-II binding equivalent to intact IGF2R (9–11).

The crystal structure of the IGF2R domain 11 has been solved (12), and specific mapping of the IGF-II binding site on this domain has recently been reported (13). Residues corresponding to the mannose 6-phosphate binding site of the cation-dependent mannose 6-phosphate receptor were mutated to Ala, and Tyr\textsuperscript{1542}, Thr\textsuperscript{1570}, Phe\textsuperscript{1567}, and Ile\textsuperscript{1572} within this hydrophobic patch were shown to be critical for IGF-II binding. Ile\textsuperscript{1572} had previously been identified as essential for IGF-II binding (10). Interestingly, mutation of Glu\textsuperscript{1544} (which lies at the edge of this binding site) to Ala or Lys leads to an increase in binding affinity (13). Although the binding site on domain 11 has been mapped, it is not yet clear whether IGF-II contacts domain 13.

IGF-I and IGF-II share >60% sequence identity, yet the IGF2R only binds IGF-II with high affinity. Little is known about this interaction, since relatively few IGF-II mutants have been made, and a structure of the IGF2R-IGF-II complex has not been solved. Residues Phe\textsuperscript{48}, Arg\textsuperscript{49}, and Ser\textsuperscript{50} have been
strongly implicated in IGF2R binding (14). However, these residues are conserved between IGF-I and IGF-II and therefore would not drive the specificity of binding. Ala^54 and Leu^55 of IGF-II differ from the corresponding residues in IGF-I (Arg^54, Arg^56) and are important for IGF2R binding (15). In addition, we recently showed that IGF-II residues in the nonconserved C domain are important for IGF2R binding and play a role in conferring the specificity of binding (16). However, the effects of mutation on residue Ala^54 or Leu^55 or the C domain are insufficient to account for the complete lack of binding by IGF-I, suggesting that another determinant provides the IGF-II specificity.

In this study, we have significantly added to our understanding of the IGF2R binding site on IGF-II using a mutagenesis approach and surface plasmon resonance with isolated IGF2R domains. We have revealed a novel hydrophobic patch critical for IGF2R binding, which encompasses residues Phe^19 and Leu^23 and includes Thr^16 and Asp^52. We have also identified the major determinant on IGF-II responsible for the specificity of IGF2R binding.

**EXPERIMENTAL PROCEDURES**

*Materials—Oligonucleotides (Table 1) were purchased from Geneworks Pty. Ltd. (Adelaide, South Australia). Restriction enzymes were from New England Biolabs (Hitchin, UK) or Geneworks Pty. Ltd. Long^™RIGF-I, human IGF-I, and IGF-II were purchased from GroPep 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. Europium-labeled IGF-II was performed by comparing analytical C4 HPLC profiles with proteomics Facility) and were shown to have the correct masses and to be greater than 95% pure. Quantitation of analogues was performed by comparing analytical C4 HPLC profiles with profiles of standard Long^™RIGF-I preparations (23).

*Circular Dichroism—CD spectra were recorded on a JASCO J-815 CD spectrometer. Spectra were recorded from 250 to 185 nm with a 1.0-nm step size using a 1.0-s response time and 1.0-nm bandwidth. Three accumulations were recorded for each spectrum. Samples were analyzed in a quartz cuvette with a 0.1-cm path length. Spectra were background-corrected by subtraction of the spectrum of buffer alone. In order to allow direct comparison of spectra without the interference from small differences in quantification of protein concentration, all spectra were normalized to the ellipticity measured at 207 nm (24).

*IGF-1R Binding Assay—Receptor binding affinities were measured using an assay similar to that described for analyzing epidermal growth factor binding to the epidermal growth factor receptor (25) and outlined in Ref. 17. Briefly, P6 cells were serum-starved prior to lysis with lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 10% (v/v) glycerol, 1 mM 2-hydroxyethyl disulfide, pH 9.1, and dilution to less than 0.1 mg/ml. Cleavage of the fusion partner and a final reverse phase HPLC cleanup were achieved as previously described (21, 22). Purified proteins were analyzed by mass spectroscopy and N-terminal sequencing (Dr. Chris Bagley and Chris Cúrsaro (Adelaide Proteomics Facility)) and were shown to have the correct masses and to be greater than 95% pure. Quantitation of analogues was performed by comparing analytical C4 HPLC profiles with profiles of standard Long^™RIGF-I preparations (23).

**TABLE 1**

| Oligonucleotides used to generate mutant IGF-II cDNA constructs | Mutation | Sequence |
|---|---|---|
| E12K | 5'-CTG TCG GGT GGT AAA CTG GGT GAT ACC-3' |
| V41T | 5'-GAT GAA CTA GCT GAG ACC GAT ACC CGA GAG-3' |
| T16A | 5'-GAA CTA GCT GAG ACC GAT ACC CGA GAG-3' |
| E18A | 5'-GAT GAT ACC CGT GCG TTT GGT GCG GAT C-3' |
| F19A | 5'-GAT ACC CGT GCG TTT GGT GCG GAT C-3' |
| F19S | 5'-GAT ACC CGT GCG TTT GGT GCG GAT C-3' |
| F19Y | 5'-GAT ACC CGT GCG TTT GGT GCG GAT C-3' |
| F19L | 5'-GAT ACC CGT GCG TTT GGT GCG GAT C-3' |
| F19A | 5'-GAT ACC CGT GCG TTT GGT GCG GAT C-3' |
| F19S | 5'-GAT ACC CGT GCG TTT GGT GCG GAT C-3' |
| F19Y | 5'-GAT ACC CGT GCG TTT GGT GCG GAT C-3' |
| F19L | 5'-GAT ACC CGT GCG TTT GGT GCG GAT C-3' |

**Refolding and Purification of IGF-II Analogues—Inclusion bodies containing the IGF-II fusion peptides were processed essentially as described (20) with some modifications. Briefly, washed inclusion bodies from a 0.5-liter fermentation were solubilized in 8 M urea containing 40 mM glycine, 0.1 M Tris, and 16 mM dithiothreitol at pH 2.0. Inclusion bodies were immediately desalted on a Superdex 75 column (1 × 30 cm (GE Healthcare) in two 2-ml batches) using the same buffer but with 1.6 mM dithiothreitol. Fractions containing the IGF-II fusion protein were identified by SDS-PAGE and reverse phase chromatography and pooled prior to folding in 2.5 M urea, 12.5 mM glycerol, 0.7 M Tris, 5 mM EDTA, 0.5 mM dithiothreitol, 1 mM 2-hydroxyethyl disulfide, pH 9.1, and dilution to less than 0.1 mg/ml. Cleavage of the fusion partner and a final reverse phase HPLC cleanup were achieved as previously described (21, 22). Purified proteins were analyzed by mass spectroscopy and N-terminal sequencing (Dr. Chris Bagley and Chris Cúrsaro (Adelaide Proteomics Facility)) and were shown to have the correct masses and to be greater than 95% pure. Quantitation of analogues was performed by comparing analytical C4 HPLC profiles with profiles of standard Long^™RIGF-I preparations (23).**

**Construction of Expression Plasmids Encoding Human IGF-II Analogues—The IGF expression vector was developed by King et al. (19), and the cDNA encoding IGF-II was introduced into the vector as previously described (20). The QuikChange site-directed mutagenesis kit (Stratagene) was used to incorporate the 14 different mutations into IGF-II (Table 1). 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^1]pGH-(1–11)) after isopropyl β-D-thiogalactoside induction. Inclusion bodies were isolated as previously described (19).

**J-815 CD spectrometer. Spectra were recorded from 250 to 185 nm with a 1.0-nm step size using a 1.0-s response time and 1.0-nm bandwidth. Three accumulations were recorded for each spectrum. Samples were analyzed in a quartz cuvette with a 0.1-cm path length. Spectra were background-corrected by subtraction of the spectrum of buffer alone. In order to allow direct comparison of spectra without the interference from small differences in quantification of protein concentration, all spectra were normalized to the ellipticity measured at 207 nm (24).**

**IGF-1R Binding Assay—Receptor binding affinities were measured using an assay similar to that described for analyzing epidermal growth factor binding to the epidermal growth factor receptor (25) and outlined in Ref. 17. Briefly, P6 cells were serum-starved prior to lysis with lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 10% (v/v) glycerol, 1 mM 2-hydroxyethyl disulfide, pH 7.5) for 1 h at 4 °C. Lysates were centrifuged for 10 min at 3500 rpm at 4 °C, and then 100-μl aliquots were added to wells of a white Greiner Lumitrac 600 plate previously coated with anti-IGF-1R antibody 24-31 (2.5 μg/ml) (26). Approximately 500,000 fluorescence counts of europium-labeled IGF-II were added to each well along with increasing concentrations of unlabeled competitor 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) and 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 Labortories Technologies Polarstar fluorimeter (Morninigton, Australia).**
IGF2R Fragments—IGF2R domain 11 (IGF2R-d11) and IGF2R domains 11–13 (IGF2R-d11–13) were prepared as described previously (12, 16). Briefly, IGF2R-d11 was expressed as inclusion bodies in E. coli and refolded, whereas IGF2R-d11–13 was produced as soluble protein in a mammalian expression system. The latter approach was also used for preparation of IGF2R domains 11 and 12 (IGF2R-d11-12), IGF2R domains 10–13 (IGF2R-d10–13), and IGF2R domains 11–14 (IGF2R-d11–14). The required regions of IGF2R were amplified by PCR using appropriate primers, which also introduced a C-terminal carboxypeptidase A-cleavable histidine tag (Lys-His6STOP). After cloning into the pEEl4 vector (27), the construct was verified by sequencing and transfected into Lec32.8.1 Chinese hamster ovary cells (28) using Pfx-8 lipids (Invitrogen). Following expression under methionine sulfoxamine selection, secreted proteins were purified by immobi- lized metal ion affinity chromatography and gel filtration (Che- lating Sepharose Fast Flow and HiLoad 16/60 Superdex 200; GE Healthcare).

Kinetic Studies of IGF2R Binding Using Surface Plasmon Resonance—Each IGF2R fragment was coupled to the CM5 biosensor chip using a similar method to that previously described (16). Briefly, the IGF2R fragment to be bound was prepared in 10 mM sodium acetate, pH 4.6, at 18 μg/ml and injected over the NHS/EDC activated chip surface in HBS running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4). The surfaces were then deacti- vated by 1 M ethanolamine. A blank flow cell was used as a reference on all chips. Binding analyses were performed at 25 °C using a BIAcore 2000 biosensor (BIAcore, Uppsala, Sweden) within the Flinders Advanced Analytical Laboratories (Flinders University, South Australia).

The IGF2R-d10–13, IGF2R-d11–13, and IGF2R-d11–14 fragments were coupled to the biosensor surface to give a final resonance value of 2000 response units, whereas IGF2R-d11 and IGF2R-d11-12 were coupled to final values of 500 and 1000 response units, respectively. The proportion of active surface was >90% for all surfaces except for the IGF2R-d11–14 surface, which was 70% active. IGF-II, IGF-I, and IGF analogues were injected over the chip surfaces at the following concentrations: IGF-II, E12K IGF-II, V14T IGF-II, Q18A IGF-II, F19A IGF-II, F28L IGF-II, and V43M IGF-II at concentrations of 100, 50, 25, 12.5, and 6.25 nM; T16A IGF-II, F19A IGF-II, F19G IGF-II, F19Y IGF-II, D52K IGF-II, D52E and D52N IGF-II, L53A IGF-II, IGF-I, and A13T IGF-I at 500, 400, 200, 100, and 50 nM in HBS running buffer for 2.5 min at a flow rate of 40 μl/min to mini- mize mass transfer effects. Dissociation of bound analyte in HBS buffer alone was measured at the same flow rate for 15 min. All flow cells were regenerated by injection of 60 μl of 10 mM HCl. Reference flow cell data were subtracted from all runs to account for bulk refractive index due to the buffer.

All kinetic data were analyzed using the BIAevaluation 3.2 software (Biacore AB, Uppsala, Sweden). IGF2R-d11 and IGF2R-d11-12 curves were fitted globally across all concentra- tions to a steady state affinity model. The steady state model describes the affinity of the interaction at equilibrium. The IGF2R-d10–13, IGF2R-d11–13, and IGF2R-d11–14 curves were fitted using a two-state conformational change model and were derived from fitting of the 50 nM curves. This model describes a 1:1 binding interaction with a conformational change upon binding, resulting in two association and dissociation rates. This model resulted in the best fitting of the data and has been previously used for analysis of binding to IGF2R fragments (13). Each mutant was analyzed in duplicate in a minimum of two separate experiments. The affinity constant (K_a) derived from eight separate experiments on two separate chips for IGF-II on IGF2R-d10–13 was 1.5 ± 0.4 × 10^8 M^{-1}, and a similar variation between experiments was seen on all surfaces with all mutants analyzed.

RESULTS

Production and Characterization of IGF-II Analogues—Fourteen novel IGF-II analogues were made to define the IGF2R binding site. Only Val^43 had previously been mutated, and substitutions were designed to probe the properties of each residue. Analogues with Ala substitutions included T16A IGF- II, Q18A IGF-II, F19A IGF-II, and L53A IGF-II. The potential role of hydrophobicity at position 19 was investigated by fur- ther substitutions with Ser, Tyr, and Leu. The effect of charge reversal at position 12 (Glu) was probed by substitution with Lys. Val^14 was changed to the equivalent residue in insulin (Thr), and the smaller hydrophobic residue of Leu was used in place of Phe at position 28. Val^43 was substituted with Met. The equivalent mutation in IGF-I (V44M), identified as the first missense mutation in the IGF-I gene, leads to an IGF-I with severely impaired IGF-1R binding (29). The contribution of the Asp^52 side chain was probed by substitution with Asn, Gln, and Glu.

Far UV circular dichroism was used to test the structural integrity of the analogues. Misfolding of IGFs can be identified by a significant change in the far UV CD spectrum (30). The following analogues exhibited spectra indistinguishable from IGF-II: E12K IGF-II, V14T IGF-II, Q18A IGF-II, F19A IGF-II, F19Y IGF-II, F19S IGF-II, F19G IGF-II, D52K IGF-II, D52E and D52N IGF-II, L53A IGF-II, IGF-I, and A13T IGF-I. T16A IGF-II, Q18A IGF-II, F19A IGF-II, and D52E IGF-II had CD spectra that differed from IGF-II (T16A, D52K, and D52E) retained the ability to bind the IGF-1R with high affinity (only 2–3-fold lower affinity; Table 2). D52E IGF-II had the greatest change in far UV CD spectrum, yet its IGF-1R binding affinity was only 3-fold lower than IGF-II (Table 2). We therefore believe this mutant is correctly folded, but the introduced changes appear to have little overall effect on secondary structure, and we believe that correct folding was achieved.

Further evidence of structural integrity was provided by ana- lyzing IGF-1R binding. Misfolded IGF-I has a 30-fold lower affinity for the IGF-1R (30), and a similar reduction in IGF-1R affinity is seen for misfolded IGF-II (31).^{6} All of the mutants that had CD spectra that differed from IGF-II (T16A, D52K, and D52E) retained the ability to bind the IGF-1R with high affinity (only 2–3-fold lower affinity; Table 2). D52E IGF-II had the greatest change in far UV CD spectrum, yet its IGF-1R binding affinity was only 3-fold lower than IGF-II (Table 2). We therefore believe this mutant is correctly folded, but the difference in CD spectrum represents local structural perturbations.

Although the CD spectra indicated no structural perturba- tions for V14T IGF-II, F28L IGF-II, and V43M IGF-II, their

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^{6} C. Delaine, C. L. Alvino, K. A. McNeil, J. C. Wallace, and B. E. Forbes, unpub- lished observation.
IGF-1R binding was significantly perturbed. This result was not unexpected, since all three residues correspond to IGF-I residues found in the "classic" binding surface that is essential for IGF-1R binding (32). Mutation of Val11 in IGF-I (equivalent to Val14 in IGF-II) to Thr also results in a reduction in IGF-1R binding affinity (33). Mutation of Val44 IGF-I or Val43 insulin (Val43 in IGF-II) to Met or Leu, respectively, disrupts receptor binding without causing gross structural perturbation (34, 35).

**IGF2R Binding**—Having established that all of the analogues were correctly folded, we then used surface plasmon resonance to test their ability to bind five different IGF2R fragments. The five IGF2R fragments included two low affinity fragments, IGF2R-d11 and IGF2R-d11–12, and three high affinity fragments, IGF2R-d10–13, IGF2R-d11–13, and IGF2R-d11–14. Association constants (Kₐ) of 5.4 × 10⁶ and 10.75 × 10⁶ m⁻¹ were observed for IGF-II binding to IGF2R-d11 and IGF2R-d11–12 were similar to previous reports (13, 16) (Table 3). The Kₐ val-

| **Ligand** | **IC⁻⁵₀** | **Affinity relative to IGF-II** |
|-----------|-----------|-------------------------------|
| IGF-I     | 1.4 ± 0.3 | 2.4                           |
| Insulin   | >100      | <0.01                         |
| IGF-II    | 3.4 ± 0.2 | 1                             |
| E12K      | 8.5 ± 2.7 | 0.4                           |
| V14T      | 28.6 ± 4.5| 0.1                           |
| T16A      | 12.1 ± 3  | 0.3                           |
| E18A      | 4.8 ± 1.2 | 0.7                           |
| F19A      | 7.7 ± 1.3 | 0.4                           |
| F19S      | 5.4 ± 1.7 | 0.6                           |
| F19Y      | 6.5 ± 1.1 | 0.5                           |
| F19L      | 4.6 ± 1.5 | 0.7                           |
| F28L      | 21.6 ± 3.3| 0.2                           |
| V43M      | >100      | <0.01                         |
| D52N      | 2.0 ± 2.7 | 1.7                           |
| D52K      | 5.9 ± 0.9 | 0.6                           |
| D52E      | 10.7 ± 4.5| 0.3                           |
| L53A      | 7.3 ± 0.4 | 0.5                           |

**TABLE 3**

| **IGF2R-d11** | **IGF2R-d11–12** |
|---------------|-----------------|
| IGF-II        | 10.75           |
| E12K          | 7.46            |
| V14T          | 2.99            |
| T16A          | 9.52            |
| Q18A          | 34.25           |
| F18A          | 7.66            |
| F19A          | 41.70           |
| F19S          | 8.76            |
| F19Y          | 1.90            |
| F28L          | 0.26            |
| V43M          | 7.66            |
| D52N          | 0.26            |
| D52K          | 0.51            |
| D52E          | 4.85            |
| L53A          | 39.20           |
|              | 21.45           |

**Table 2**

| **IC⁻⁵₀** | **Affinity relative to IGF-II** |
|-----------|-------------------------------|
| IGF-II    | 2.4                           |
| Insulin   | <0.01                         |
| IGF-II    | 1                             |
| E12K      | 0.4                           |
| V14T      | 0.1                           |
| T16A      | 0.3                           |
| E18A      | 0.7                           |
| F19A      | 0.4                           |
| F19S      | 0.6                           |
| F19Y      | 0.5                           |
| F19L      | 0.7                           |
| F28L      | 0.2                           |
| V43M      | <0.01                         |
| D52N      | 1.7                           |
| D52K      | 0.6                           |
| D52E      | 0.3                           |
| L53A      | 0.5                           |

**Figure 1**. Far UV circular dichroism spectra of IGF-II analogues. For comparison, the CD spectra of the respective IGF-II analogues are superimposed on the CD spectra of both IGF-I and IGF-II. The wild type IGFs have similar CD profiles, with a deep minimum at 207 nm and a shoulder at 220 nm. However, IGF-II shows an increase of ~25% in the depth of the band at ~220 nm. Most of the IGF-II analogues have CD spectra indistinguishable from that of IGF-II. For the IGF-II analogues that do show significantly different CD profiles (T16A IGF-II, D52K IGF-II, and D52E IGF-II) the change is less than the difference between IGF-I and IGF-II.
ues derived for IGF-II binding to the IGF2R-d10–13, IGF2R-d11–13, and IGF2R-d11–14 were 1.5 \times 10^8, 1.27 \times 10^8, and 1.65 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}, respectively (Table 4). These values were also similar to previous reports (16, 36).

All 14 mutants were tested for their ability to bind the IGF2R fragments. Each mutant bound to both low affinity fragments, IGF2R-d11 and IGF2R-d11-12, with a similar affinity (Figs. 2 and 3 and Table 3). There was very little effect on IGF2R binding with the E12K, Q18A, and F28L IGF-II mutants. V43M IGF-II and V14T IGF-II had 2- and 3-fold lower affinities for the IGF2R-d11-12 but had little effect on IGF2R-d11 binding.

In contrast, no binding was detectable with F19A IGF-II, F19S IGF-II, or F19Y IGF-II even at a concentration of 500 nM. However, F19L IGF-II had 6.4- and 3.9-fold higher affinities than IGF-II for IGF2R-d11 and IGF2R-d11-12. This would suggest that Phe19 is involved in a hydrophobic interaction with the IGF2R, and replacing the aromatic ring with a bulky aliphatic side chain can even enhance binding.

Both Leu\textsuperscript{53} and Thr\textsuperscript{16} are also critical for binding to the low affinity fragments, with no binding detectable even at a concentration of 500 nM. In addition, mutation of Asp\textsuperscript{52} to Lys, Glu, or Asn has a significant effect on binding to IGF2R-d11 and IGF2R-d11-12, with the greatest effect seen by substitution with lysine (20-fold lower affinity than IGF-II).

As seen with IGF2R-d11 and IGF2R-d11-12, each mutant bound to all high affinity fragments, IGF2R-d10–13, IGF2R-d11–13, and IGF2R-d11–14, to a similar extent (Figs. 2 and 4 and Table 4). There was little effect on IGF2R binding by the E12K, Q18A, and V43M substitutions, with E12K showing no more than 2.3-fold lower affinities for IGF2R-d10–13, IGF2R-d11–13, and IGF2R-d11–14. Interestingly, although F28L IGF-II bound to IGF2R-d11 and IGF2R-d11-12, with only 1.1–

### Table 4

| IGF2R Binding Site on IGF-II |
|-----------------------------|

| IGF2R-d10–13 | $k_{a1}$ | $k_{d1}$ | $k_{a2}$ | $k_{d2}$ | $K_a$ | $K_a^*$ |
|--------------|---------|---------|---------|---------|------|------|
| IGF-II       | 6.87    | 1.21    | 3.01    | 1.50    | 1.50 | 1.00 |
| E12K         | 6.19    | 1.92    | 2.38    | 2.10    | 0.68 | 0.45 |
| V14T         | 6.72    | 1.13    | 3.76    | 1.71    | 1.97 | 1.31 |
| T16A         | —       | —       | —       | —       | —    | —    |
| Q18A         | 8.16    | 1.64    | 2.86    | 2.51    | 1.46 | 0.97 |
| F19A         | —       | —       | —       | —       | —    | —    |
| F19S         | —       | —       | —       | —       | —    | —    |
| F19Y         | —       | —       | —       | —       | —    | —    |
| F19L         | 4.51    | 0.73    | 4.05    | 1.53    | 2.56 | 1.70 |
| F28L         | 16.75   | 4.40    | 1.35    | 1.78    | 0.75 | 0.50 |
| V43M         | 9.52    | 1.68    | 2.48    | 1.69    | 1.59 | 1.06 |
| D52N         | 4.27    | 3.14    | 2.47    | 3.03    | 0.26 | 0.17 |
| D52K         | 1.31    | 6.28    | 2.63    | 7.60    | 0.03 | 0.02 |
| D52E         | 3.43    | 2.31    | 2.70    | 3.27    | 0.27 | 0.18 |
| L53A         | —       | —       | —       | —       | —    | —    |

| IGF2R-d11–13 | $k_{a1}$ | $k_{d1}$ | $k_{a2}$ | $k_{d2}$ | $K_a$ | $K_a^*$ |
|--------------|---------|---------|---------|---------|------|------|
| IGF-II       | 6.25    | 1.21    | 3.42    | 1.77    | 1.27 | 1.00 |
| E12K         | 5.92    | 1.58    | 2.04    | 2.18    | 0.76 | 0.60 |
| V14T         | 5.10    | 0.99    | 3.64    | 1.79    | 1.50 | 1.19 |
| T16A         | —       | —       | —       | —       | —    | —    |
| Q18A         | 6.26    | 1.48    | 2.78    | 2.66    | 0.86 | 0.68 |
| F19A         | —       | —       | —       | —       | —    | —    |
| F19S         | —       | —       | —       | —       | —    | —    |
| F19Y         | —       | —       | —       | —       | —    | —    |
| F19L         | 3.84    | 0.63    | 4.28    | 1.31    | 2.61 | 2.06 |
| F28L         | 9.96    | 3.98    | 1.43    | 2.08    | 0.46 | 0.36 |
| V43M         | 6.74    | 1.52    | 2.46    | 2.01    | 1.00 | 0.79 |
| D52N         | 2.07    | 2.02    | 2.51    | 3.35    | 0.18 | 0.14 |
| D52K         | 1.77    | 4.01    | 1.72    | 5.82    | 0.06 | 0.04 |
| D52E         | 2.11    | 1.81    | 2.60    | 3.11    | 0.22 | 0.17 |
| L53A         | —       | —       | —       | —       | —    | —    |

| IGF2R-d11–14 | $k_{a1}$ | $k_{d1}$ | $k_{a2}$ | $k_{d2}$ | $K_a$ | $K_a^*$ |
|--------------|---------|---------|---------|---------|------|------|
| IGF-II       | 7.58    | 1.25    | 3.41    | 1.71    | 1.65 | 1.00 |
| E12K         | 5.78    | 1.74    | 2.25    | 2.39    | 0.71 | 0.43 |
| V14T         | 7.25    | 1.05    | 3.48    | 1.51    | 2.25 | 1.36 |
| T16A         | —       | —       | —       | —       | —    | —    |
| Q18A         | 7.42    | 1.51    | 2.66    | 2.54    | 1.04 | 0.63 |
| F19A         | —       | —       | —       | —       | —    | —    |
| F19S         | —       | —       | —       | —       | —    | —    |
| F19Y         | —       | —       | —       | —       | —    | —    |
| F19L         | 3.90    | 0.67    | 4.40    | 1.47    | 2.41 | 1.46 |
| F28L         | 11.35   | 3.67    | 1.57    | 1.92    | 0.66 | 0.27 |
| V43M         | 8.38    | 1.45    | 2.43    | 1.99    | 1.40 | 1.03 |
| D52N         | 2.98    | 2.26    | 2.71    | 3.44    | 0.24 | 0.14 |
| D52K         | 0.27    | 4.76    | 2.28    | 9.80    | 0.01 | 0.00 |
| D52E         | 2.33    | 2.07    | 2.90    | 3.21    | 0.22 | 0.13 |
| L53A         | —       | —       | —       | —       | —    | —    |
1.4-fold lower affinity than IGF-II, there was a greater effect of the F28L IGF-II mutation on binding to the high affinity fragments (a 2.7–3.7-fold decrease). Again, no binding was detectable with F19A IGF-II, F19S IGF-II, F19Y IGF-II, L53A IGF-II, and T16A IGF-II to IGF2R-d10–13, IGF2R-d11–13, and IGF2R-d11–14. F19L IGF-II also showed improved binding over IGF-II for the high affinity fragments, predominantly due to slower dissociation rates. However, this effect was only 2-fold on the high affinity fragments versus up to 6.4-fold higher affinity with the low affinity fragments. The D52N, D52K, and D52E IGF-II substitutions had a similar decrease in binding to the high affinity IGF2R fragments, as was seen with IGF2R-d11 and IGF2R-d11–12, with the D52K substitution having the greatest effect.

An additional IGF-I mutant (A13T IGF-I) was made using a similar approach as previously described (22). No significant structural changes were detectable by CD analysis following introduction of Thr at this position, and IGF-1R binding affinity was identical to IGF-I (data not shown), suggesting that A13T IGF-I was correctly folded. Interestingly, there is no detectable binding of IGF-I to all IGF2R fragments using surface plasmon resonance. However, the A13T IGF-I did bind IGF2R-d10–13, albeit with a 25-fold lower affinity than IGF-II (Fig. 5). A similar result was seen for the other high affinity fragments (IGF2R-d11–13 and IGF2R-d11–14) and the low affinity fragments (IGF2R-d11 and IGF2R-d11-12) (data not shown).

**DISCUSSION**

With the lack of a structure of the IGF-II/IGF2R complex and only limited IGF-II mutagenesis data, we undertook a comprehensive study aimed at significantly improving our understanding of the IGF-II/IGF2R interaction and the molecular mechanism underlying the specificity of the interaction. A series of 14 novel IGF-II analogues were produced recombinantly and tested for their binding to five different IGF2R fragments. The IGF2R fragments encompassed either domain 11, which is essential for IGF-II binding, or both domains 11 and 13, which together are required to achieve high affinity binding (9–11). The binding data reveal a completely new IGF-II surface important for interaction with the IGF2R. This surface encompasses residues Thr16, Phe19, Asp52, and Leu53 of IGF-II, which form a continuous patch (see Fig. 6). This binding surface lies adjacent to residues Ala54 and Leu55, previously identified as important for IGF2R binding (15), but is further away from residues Phe48, Arg52, and Ser50, also reported to be involved in IGF2R binding (14).

The involvement in IGF2R binding of the hydrophobic residues Phe19 and Leu53, along with the adjacent Leu55, is consistent with the suggestion that IGF-II binds to the IGF2R using hydrophobic interactions (10, 13). Substitution of Phe19 with
polar residues, including the similarly sized Tyr, is detrimental to IGF2R binding. However, an increase in binding affinity to both low and high affinity IGF2R fragments is achieved by substitution with a bulky aliphatic residue (Leu). Consistent with this, a hydrophobic patch on the IGF2R is critical for IGF-II binding (10, 13).

Probing the contribution of Asp52 by substitution with Lys, Glu, or Asn suggests that although charge is important, with the greatest effect seen with the Lys substitution, size also is important at this position for IGF2R binding. A significant reduction in binding was observed for D52E IGF-II.

Mutation of those residues that proved important for IGF-1R binding (Val14, Phe28, and Val43) had little or no effect on IGF2R binding, and it is noteworthy that they are located on the opposite side of the IGF-II molecule (Fig. 6). This observation is consistent with studies showing that mutation of Phe26 and Tyr27 also had little or no effect on IGF2R binding, despite a significant decrease in IGF-1R binding (14, 37).

Interestingly, all of the mutations that had an effect on IGF2R binding disrupted binding to both the low affinity and high affinity IGF2R fragments. We can therefore deduce that these residues are making contact with domain 11 only, and our observation supports the suggestion that the role of domain 13 is to stabilize the complex, resulting in high affinity binding (9–11).

Thr16 of IGF-II is the only residue analyzed in this study that differs from its equivalent residue in IGF-I (Ala13) and therefore can be used to probe the IGF-II binding preference of the IGF2R. Previous studies have identified that Ala54, Leu55, and the C domain of IGF-II contribute to the specificity of the IGF-II/IGF2R interaction. However, mutation of either Ala54 or Leu55 to the equivalent residues in IGF-I (Arg and Arg, respectively) only resulted in a 3–4-fold lower binding affinity (15). Similarly, substituting the IGF-II C domain with the IGF-I C domain only reduced binding by 6-fold (16). Therefore, although Ala54, Leu55, and the IGF-II C domain contain determinants important for IGF2R binding and do play a role in specificity, they are not the major determinants. Since mutation of Thr16 to Ala resulted in a dramatic loss in binding to all IGF2R fragments tested (with only a 3.5-fold reduction in IGF-1R binding), we believe this residue represents the major determinant for the specificity of the IGF-II/IGF2R interaction. In support of this, we showed that A13T IGF-I does bind to the IGF2R fragments, albeit with lower affinity than IGF-II (Fig. 5),
whereas there is no detectable binding of IGF-I on either low or high affinity IGF2R fragments.

The ability of the IGF2R to bind IGF-II evolved in mammals after the divergence of monotremes and correlates with the emergence of placental development (38). The cation-independent mannose 6-phosphate receptors of nonmammalian species, including chicken and Xenopus, are unable to bind IGF-II due to differences in the sequence of the IGF-II binding site (39). Importantly, Thr16 is conserved in IGF-II of all mammalian species in which the IGF2R has an IGF-II binding motif.

Thr16 could play a direct role in IGF2R binding. Thr16 of IGF-II is found in a deep pocket and is partially surface-exposed, whereas this pocket does not exist in IGF-I, since the corresponding residue (Ala13) is more exposed and forms part of a flatter surface (Fig. 7A). The presence of the bulkier and somewhat polar Thr at position 16 could also be influencing the local structure in this region. There are significant differences between IGF-I and IGF-II in the local structures around the residues Phe19, Asp52, and Leu53, residues identified in this study to be important for
IGF2R binding (Fig. 7B). These structural perturbations may result from the accommodation of the critical Thr residue in IGF-II.

In summary, we have defined a novel binding surface on IGF-II that is important for binding to domain 11 of the IGF2R and confirmed that high affinity binding is achieved predominantly through hydrophobic interactions. We have also identified Thr\(^16\) as the major determinant of IGF-II binding specifically through hydrophobic interactions. We have also identified that high affinity binding is achieved predominantly through hydrophobic interactions. We have also identified Thr\(^16\) as the major determinant of IGF-II binding specifically through hydrophobic interactions. We have also identified Thr\(^16\) as the major determinant of IGF-II binding specifically through hydrophobic interactions.

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