Real Time Kinetics of Insulin-like Growth Factor II (IGF-II) Interaction with the IGF-II/Mannose 6-Phosphate Receptor

THE EFFECTS OF DOMAIN 13 AND pH*

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The interaction of soluble forms of the human cation-independent insulin-like growth factor-II/mannose 6-phosphate receptor (IGF-IIR) with IGFs and mannansylated ligands was analyzed in real time. IGF-IIR proteins containing domains 1–15, 10–13, 11–13, or 11–12 were combined with rat CD4 domains 3 and 4. Following transient expression in 293T cells, secreted protein was immobilized onto biosensor chips. β-Glucuronidase and latent transforming growth factor-β1 bound only to domains 1–15. IGF-II bound to all constructs except a control, which contained a point mutation in domain 11. The affinity of domains 1–15, 10–13, 11–13, and 11–12 to IGF-II were 14, 120, 100, and 450 nM, respectively. Our data suggest that domain 13 acts as an enhancer of IGF-II affinity by slowing the rate of dissociation, but additional enhancement by domains other than 10–13 also occurs. As the receptor functions to transport ligands from either the trans-Golgi network or extracellular space to the endosomes, the interaction of IGF-IIR extracellular domains with IGF-II was analyzed over a pH range of 5.0–7.4. The constructs behaved differently in response to pH and in recovery after low pH exposure, suggesting that pH stability of the extracellular domains depends on domains other than 10–13.

The mammalian form of IGF-IIR1 processes growth promoters and inhibitors, recycles lysosomal enzymes, binds granzyme B to mediate cytotoxic T cell-induced apoptosis (1), has an imprinted gene (2), and has also been implicated in human intelligence (3). The glycosylated protein (270 kDa) has 15 extracytosolic repeat domains containing distinct binding sites for phosophomannosyl residues and IGF-II in mammals and marsupials. Mannosylated proteins bind to domains 1–3 and 7–9 (4, 5), and IGF-II binds to domain 11 (6). Approximately 90% of membrane-bound IGF-IIR is normally found within the cell. The remaining protein is present at the cell surface, where its extracellular domains can exist as monomers or dimers (7, 8). A 23-amino acid transmembrane domain and a cytoplasmic tail containing recognition sequences ensure rapid internalization at the plasma membrane (9). A soluble form of IGF-IIR has been reported in a variety of mammalian species, including humans (10–12), and may be involved in limiting the availability of IGF-II. At the cell surface, IGF-IIR is involved in binding a variety of ligands, including IGF-II and mannansylated proteins, such as leukemia inhibitory factor, which are internalized and degraded (13, 14). IGF-IIR also interacts with the mannose 6-phosphate groups of glycosylated latent transforming growth factor-β1 (LTGF-β1), leading to removal of the side chains and activation to TGF-β1, a growth inhibitor (15, 16). IGF-IIR also binds retinoic acid and urokinase-type plasminogen activator receptor, but the specific function and position of binding is not known (17, 18). Recently, it was shown that the uptake of granzyme B in T cell-mediated cell death was dependent upon IGF-IIR, and it is suggested that IGF-IIR is the receptor for cytotoxic T-cell-induced cell death (1). Evidence for an IGF-IIR signaling pathway is contradictory (19, 20). The mitogenic and cell survival signals of IGF-I and IGF-II primarily occur via the IGF-I and insulin receptors (21).

It is suggested that IGF-IIR is a tumor suppressor gene, since the loss of IGF-IIR leads to increased levels of the cell survival factor IGF-II and decreased levels of the growth suppressor TGF-β1 (22); resistance to T cell-mediated apoptosis and increased circulating proteolytic enzyme levels would also be predicted. IGF-II is often overexpressed in tumors, and its supply has been shown to be important in the growth of murine and human tumors (23, 24). IGF-IIR is mutated with frequent loss of heterozygosity within human hepatocellular, breast, lung, and colon tumors such that ligand binding is disrupted (25). In addition, the IGF2R gene contains a poly(G) tract in exon 38, which is thought to be a common mutational target in gastric, colon, and endometrial tumors with mismatch repair deficiencies (26, 27). IGF2R inactivation may be an early event in carcinogenesis, since IGF2R allelic loss occurs in surrounding phenotypically normal tissue within hepatitis virus-infected patients with dysplastic liver lesions and hepatocellular carcinomas (28). Mice with targeted disruption of the Igf2r gene are born 25–30% larger than control mice and have an ~3-fold increase in serum IGF-II (21). The phenotype can be rescued in crosses with IGF2 knockout mice, suggesting that there are no significant growth effects independent of IGF-II (29). However, expression of a soluble form of IGF-IIR delivered using a transgene under the control of the keratin 10 promoter suggested that there may be IGF-II-independent growth effects in certain tissues (30). The addition of purified and soluble IGF-IIR to primary rat hepatocytes decreased IGF-II-, epidermal growth factor-, and platelet-derived growth factor-dependent DNA synthesis (31). Moreover, IGF2R down-regulation increased both cell growth rate in vitro and tumor xenograft growth rate in vivo (32).

To analyze the high affinity interactions of IGF-IIR with its
ligands and to investigate the effect of pH on IGF-II/receptor interaction, soluble and stable recombinant forms of the extracellular ligand binding region of IGF-II were generated.

**EXPERIMENTAL PROCEDURES**

**Generation of Soluble IGF-IIR Constructs**—Domains 1–15 were constructed by cloning the 438-base pair XhoI/BamHI-digested PCR product from the human IGFII cDNA clone, J03528 (American Type Culture Collection) using j11for (5'-GCGTCTAGTGGCGGCTCGACAGCCGGGCTCGAG-3') at positions 252–273 and j13rev (5'-GCGGATCCGCTCTATGTACTGAAGCGAGAGCCGAC-3') at position 668–687 into pBluescript to produce pJL1. The 5.2-kb BglII/NruI fragment from IGFII cDNA was cloned into pJL1 digested with BglII/NruI, producing pJL2. The PCR fragment synthesized using primers j2for (5'-TCTGTTGCAAGCGCCTGGAAG-3') at positions 4982–4999 and j2rev (5'-GAAGAGTCGACCTTGAGGTTCTGACAGCCCCTTGTGCATC-3') at positions 4854–4873 and j12rev, the reverse complement of j11for, amplified domains 10–13. j15for and j15rev (5'-GAAGAGTCGACACCTTTCGATCTGAC-3') at positions 6100–6125 and j14rev amplified domains 11–13. jl5for and jl5rev (5'-GAAGAGTCGAACCTTTCGATCTGAC-3') at positions 668–687 and j16rev, the reverse complement of j15for, amplified a 0.15-kb fragment. A single base change of T to G is shown in boldface type and underlined. j2for (5'-GAAGAGTCGACCTTGAGGTTCTGACAGCCCCTTGTGCATC-3') at positions 6100–6125 and j14rev amplified domains 10–13, j15for and j15rev (5'-GAAGAGTCGACCTTGAGGTTCTGACAGCCCCTTGTGCATC-3') at positions 4854–4873 and j16rev, the reverse complement of j15for, amplified a 0.15-kb fragment. The 0.15- and 0.18-kb fragments were combined in a tube and amplified using j17for and j17rev. Then the resulting 0.3-kb fragment was digested with BstEI and StuI and cloned into pJL3 at positions 5308–5324 and jl3rev (5'-GAAGAGTCGACACCTTTCGATCTGAC-3') at positions 5325–5344, digesting StuI/XhoI digested pJL3, producing pJL4, which was digested with 2.5 units of SauI and EcoRV to remove IGF-IIR domains 10–13. Small domain 1–5 (IGF-IIR fragment, corresponding to nucleotides 253–6998, was cloned into the same expression vector.

Oligonucleotides j4for (5'-GAAGAGTCGACACCTTTCGATCTGAC-3') at positions 4233–4254 and j4rev (5'-GAAGAGTCGACCTTGAGGTTCTGACAGCCCCTTGTGCATC-3') at positions 6100–6125 were used to amplify IGF-II domains 10–13 cDNA (J03528). The BglII/EII fragment from pJL2, producing pJL3. j6for (5'-GACCGAGATATCGGTCTGACAGCCCCTTGTGCATC-3') at positions 4982–4999 and j6rev amplified domains 11–13. j5for and j5rev (5'-GAAGAGTCGACATCCTTTCGATCTGAC-3') at positions 5524–5549 amplified domains 11 and 12. Domain 10–13, 11–13, and 11–12 PCR fragments were digested with SalI and cloned into the expression vector, producing p10–13, p11–13, and p11–12, respectively. A unique version of 10–13 (10–13(I1572T)) was constructed by site-directed mutagenesis. j6for (5'-GACCGAGATATCGGTCTGACAGCCCCTTGTGCATC-3') at positions 4854–4873 and j6rev, the reverse complement of j6for, amplified a 0.15-kb fragment. A single base change of T to G is shown in boldface type and underlined. j7for (5'-GACTGCCAGGTCACCAAC-3') at positions 4690–4707 and jl3rev (5'-GACTGCCAGGTCACCAAC-3') at positions 4982–4999 were used to amplify IGF-IIR domains 10–13 cDNA. jl5for (5'-GAAGAGTCGACACCTTTCGATCTGAC-3') at positions 6100–6125 and jl4rev amplified domains 11–13. jl5for and jl5rev (5'-GAAGAGTCGAACCTTTCGATCTGAC-3') at positions 668–687 and jl4rev, the reverse complement of j15for, amplified a 0.15-kb fragment. The 0.15- and 0.18-kb fragments were combined in a tube and amplified using jl7for and jl7rev. Then the resulting 0.3-kb fragment was digested with BstEI and StuI and cloned into pJL3 at positions 5308–5324 and jl3rev (5'-GAAGAGTCGACACCTTTCGATCTGAC-3') at positions 5325–5344, digesting StuI/XhoI digested pJL3, producing pJL4, which was digested with 2.5 units of SauI and EcoRV to remove IGF-IIR domains 10–13. Small domain 1–5 (IGF-IIR fragment, corresponding to nucleotides 253–6998, was cloned into the same expression vector.

**Expression of Recombinant Chimeric IGF-IIR-CD4**—IGF-IIR constructs were transiently expressed in 293T cells by calcium phosphate nucleofection (36) using 20 μg of DNA. Following transfection, the cells were grown for 5 days in serum-free conditions (Dulbecco's modified Eagle's medium containing 2 mM glutamine and 1% (v/v) penicillin/ streptomycin). Supernatant was collected, and cells were cleared by centrifugation. Cell lysis was performed according to Devi et al. (41). Recombinant protein was detected using a CD4 sandwich enzyme-linked immunosorbent assay (34) and concentrated using Centriprep-10 (Amicon) under nonreducing conditions and Western blotting according to Sambrook et al., (38) using 1 μg/ml streptavidin-alkaline phosphatase conjugate (Sigma) and Lumi-Phos (Pierce).

**BiAcore Analysis**—All BiAcore experiments were performed on a BiAcore biosensor 2000 at 37 °C in HBS buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20) unless otherwise stated. Streptavidin was immobilized to a CM5 chip using EDC/NHS carbodiimide coupling reagents (39). All kinetic measurements were performed with the biotinylated receptor construct bound as the immobilized acceptor molecule except where stated. The amount of protein immobilized for the kinetic analysis was based on Equation 1,

\[
R_{\text{max}} = \frac{\text{Molecular weight of ligand} \times \text{Immobilization response}}{\text{Molecular weight of receptor} \times \text{Stoichiometry}}
\]

where \(R_{\text{max}}\) is the maximum level of response and was set at ~50–200 resonance units (Rs). 40 μl of sample was injected over immobilized protein at a flow rate of 40 μl/min. To determine stoichiometry, the flow cells were saturated with biotinylated protein. Ligand concentrations used were 17–270 nM human recombinant IGF-II (Gropep), 100 nM to 56 mM IGF-I, 35 mM insulin (Sigma), 10–1150 nM epidermal growth factor and fibroblast growth factor 2 (2) and a generous gift of Prof. John Heath, 50–100 nM latent TGF-b1 (R & D Systems), and 10–20 nM β-glucuronidase (generous gift of Prof. William Sly). Biotinylated IGF-II (Gropep) was immobilized onto a streptavidin-coated chip, and 10–13 and 10–13/I1572T) were passed over the chip. Affinities were calculated using BiAevaluation software.

**Kinetic Measurements at Reduced pH**—The pH of HBS was adjusted to 7.0, 6.5, 6.0, and 5.5 using a combination pH electrode, PHM3 (Radiometer Ltd.) at room temperature. These buffers were used to dilute IGF-II and wash flow cells through before analysis. After exposure to each pH, flow cells were rinsed with HBS buffer (pH 7.4) using the wash command, and IGF-II diluted in HBS (pH 7.4) was passed over the chip.

**Statistical Analysis**—The sample size was between three and six samples, and S.E. was used. Samples where compared using directional Mann-Whitney nonparametric analysis.

**RESULTS**

**Expression of IGF-IIR Fusion Proteins**—IGF-IIR chimeric proteins (Fig. 1) were constructed by amplifying IGFII cDNA and restriction enzyme digestion. Domain boundaries were designed around those proposed by Lobel et al. (40) and included two amino acids on either side of a domain. cDNA was cloned in frame with the rat CD4 leader sequence, CD4 domains 3 and 4, and a biotin-accepting peptide and expressed in 293T cells. The structure of rat CD4 domains 3 and 4 has been determined (41), and they have previously been found to be expressed at high levels when truncated (42) or expressed as a fusion protein (43). Levels of expression were quantified by CD4 enzyme-linked immunosorbent assay. In relation to the rat CD4 control construct, which was expressed at 1 μg/μl, domains 10–13, 10–13/I1572T), and 11–12 were expressed at

**Fig. 1.** The repeating domains (red boxes) of IGF-IIR. The regions containing the poly(G) tract and reported missense mutations are indicated (nucleotide position). The 43-base pair fibronectin type II domain is shown in green, the transmembrane domain is shown in blue, and the cytoplasmic region is shown in yellow. Predicted N-glycosylation sites are indicated by a blue circle. Constructs containing domains 1–15 (253–6998), 10–13, 10–13/I1572T), and 11–12 combined with CD4 (blue box) are shown. The point mutation in 10–13/I1572T) is indicated by a yellow band. Mannose 6-phosphate and IGF-II binding sites are indicated.

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10–15 ng/µl, but domains 1–15 (nucleotides 253–6998) were expressed at 0.8–2 ng/µl. Cell lysis techniques did not increase the yield of protein detected. The size of biotinylated domains 1–15, 10–13, 10–13(I1572T), and 11–12 conjugated to rat CD4 was confirmed by Western blot analysis (Fig. 2). The first chimeric protein containing domains 1–15 (nucleotides 253–7052) was not detectable in the spent supernatant by enzyme-linked immunosorbent assay or Western blot (data not shown) and was not used in further experiments.

**Surface Plasmon Resonance—Interaction of recombinant IGF-IIR with IGF-II was measured using surface plasmon resonance techniques.** To prevent mass transport limitations and ligand rebinding, the amount of immobilized ligand was kept to an Rmax of 50–200 Ru, and a high flow rate was used (44). The association rate (k_a) was measured as a function of the increase in Ru over time. Dissociation of IGF-II caused a decrease in Ru as IGF-II was removed by washing from the chip surface (k_d). A plot of dRu/dt over Ru gave a linear response (data not shown), which suggests that mass transport was not a limiting factor in the analysis of IGF-II binding using 67 nM IGF-II. Domains 3 and 4 of CD4 and 10–13(I1572T) were used as controls once the failure to bind IGF-II was confirmed (Fig. 3). All kinetic measurements of domains 1–15, 10–13, and 11–12 were subsequently performed after the subtraction of the Ru generated by 10–13(I1572T) to remove changes in signal arising from either the refractive index or nonspecific binding events. Measurement of the Rmax derived from steady state binding indicated that >90% of immobilized domains 10–13 and 11–13 were able to bind IGF-II. However, only 50–57% of immobilized domains 1–15 and 11–12 were active.

**Affinity to Mannosylated Proteins—**10–20 nM β-glucuronidase and 50–100 nM latent TGF-β1 were passed over immobilized biotinylated constructs, and binding was observed with domains 1–15 but not domains 10–13 and domains 11–12 (data not shown). The association rates of latent TGF-β1 and IGF-II were found to be similar, but not for β-glucuronidase, which was ~6-fold lower. Its dissociation from domains 1–15 was ~16-fold slower than the dissociation of IGF-II and latent TGF-β1 (Table I).

**Specificity of IGF-II for the IGF-IIR—**Insulin, epidermal growth factor, fibroblast growth factors 1 and 2, and IGF-I did not bind to any of the IGF-IIR constructs (data not shown), whereas IGF-II bound to domains 1–15, 10–13, 11–13, and 11–12 with high affinity at 37 °C, pH 7.4. Association and dissociation rates from domains 1–15, 10–13, 11–13, and 11–12 are shown in Table I. Since domain 10–13 and 11–13 bound IGF-II with similar affinity, domains 10–13 were used in later experiments.

**Immunoblotting of Biotinylated IGF-II—**Biotinylated IGF-II was immobilized on a BIACore chip. Nonbiotinylated 10–13 bound to the immobilized ligand with an affinity constant of 1.34 × 10^−9 M, which was ~96-fold lower than the interaction between immobilized 10–13 and IGF-II. It is possible that a conformational change occurs in the IGF-II protein upon biotinylation such that interactions with receptor molecules are retained but at reduced affinity. Nonbiotinylated 10–13(I1572T) did not bind to immobilized biotinylated IGF-II (data not shown).

**pH Dependence of IGF-II Ligand Binding—**The biosensor chip was washed with the appropriate pH buffer before 67 nM IGF-II diluted in the same buffer was passed over the immobilized IGF-IIR constructs. The amount of IGF-II binding was indicated by the Ru, since they are proportional to the analyte mass. Binding was measured at pH 7.4 after exposure to each low pH buffer.

**Domains 1–15—**Decreasing pH did not significantly affect IGF-II association with domains 1–15 until pH 5.5, when the association rate was decreased ~28-fold (Table II). At pH 6.5, the dissociation rate was significantly increased by 2-fold (p = 0.015), and the amount of IGF-II binding was significantly reduced (p = 0.04). No binding was detectable at pH 5.0 (Fig. 4A). Optimum affinity occurred at pH 7.4. The association rate and amount of IGF-II bound to immobilized domains 1–15 at pH 7.4 was not affected by previous exposure to low pH, since
The effect of pH on the interaction between IGF-II and IGF-IIR-CD4 chimeras containing domains 1–15, 10–13, and 11–12

67 nM IGF-II was passed over immobilized biotinylated domains 1–15, 10–13, and 11–12 at 37 °C. The pH of the buffer used to wash through the flow cells and dilute IGF-II was adjusted accordingly. Association rate (k_a), dissociation rate (k_d), and equilibrium (K_d) constants were determined using BiaEvaluation software. Values represent the mean ± S.E.

### Table II

<table>
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<tr>
<th>Domains</th>
<th>Parameters</th>
<th>pH</th>
<th>7.4</th>
<th>7.0</th>
<th>6.5</th>
<th>6.0</th>
<th>5.5</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–15</td>
<td>k_a × 10^6 (M^{-1} s^{-1})</td>
<td>1.4 ± 0.2</td>
<td>1.4 ± 0.08</td>
<td>1.3 ± 0.04</td>
<td>1.0 ± 0.2</td>
<td>0.05 ± 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>k_d × 10^{-2} (s^{-1})</td>
<td>2.0 ± 0.3</td>
<td>3.0 ± 0.3</td>
<td>3.0 ± 0.03</td>
<td>6.0 ± 0.3</td>
<td>7.0 ± 0.5</td>
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<tr>
<td></td>
<td>K_d (nm)</td>
<td>14</td>
<td>21</td>
<td>23</td>
<td>60</td>
<td>1400</td>
<td></td>
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<tr>
<td>10–13</td>
<td>k_a × 10^6 (M^{-1} s^{-1})</td>
<td>0.2 ± 0.09</td>
<td>0.2 ± 0.02</td>
<td>0.2 ± 0.02</td>
<td>0.2 ± 0.01</td>
<td>0.1 ± 0.03</td>
<td>0.8 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>k_d × 10^{-2} (s^{-1})</td>
<td>2.4 ± 0.3</td>
<td>2.9 ± 0.03</td>
<td>2.0 ± 0.02</td>
<td>7.0 ± 0.05</td>
<td>10.5 ± 2.0</td>
<td>12.3 ± 1.6</td>
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<td></td>
<td>K_d (nm)</td>
<td>120</td>
<td>150</td>
<td>100</td>
<td>350</td>
<td>1050</td>
<td>1540</td>
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<tr>
<td>11–12</td>
<td>k_a × 10^6 (M^{-1} s^{-1})</td>
<td>0.2 ± 0.02</td>
<td>0.4 ± 0.09</td>
<td>0.4 ± 0.03</td>
<td>0.3 ± 0.07</td>
<td>0.3 ± 0.04</td>
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<tr>
<td></td>
<td>k_d × 10^{-2} (s^{-1})</td>
<td>9.1 ± 1.2</td>
<td>8.0 ± 0.5</td>
<td>7.7 ± 0.3</td>
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<td>8.3 ± 0.4</td>
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<td></td>
<td>K_d (nm)</td>
<td>460</td>
<td>200</td>
<td>190</td>
<td>260</td>
<td>210</td>
<td>420</td>
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</table>

**Fig. 4.** Sensorgram data of 67 nM IGF-II binding to domains 1–15 (A), 10–13 (B), and 11–12 (C) at 37 °C at pH 7.4 (dark blue), 7.0 (pink), 6.5 (yellow), 6.0 (light blue), 5.5 (burgundy), and 5.0 (brown).

levels returned to those measured during the initial pH 7.4 measurements. The dissociation rate remained significantly higher (p = 0.03) when measured at pH 7.4 immediately after exposure to pH 6.5 and below (Fig. 5A).

**Domains 10–13**—Unlike domains 1–15, binding of IGF-II to immobilized 10–13 was detectable at pH 5.0. The association rate of IGF-II was significantly decreased by 2-fold at pH 5.5 (p = 0.03), although at pH 5.0, the rate was increased by ~4-fold (Table II). The dissociation rate and total amount of IGF-II bound was significantly decreased from pH 6.0 (p = 0.02) (Fig. 4B). By pH 5.0, the dissociation rate and Ru had decreased 5-fold. Optimum affinity occurred between pH 7.4 and 6.5. The association and dissociation rate and amount of IGF-II binding did not return to previous values when measured at pH 7.4 following each stage of low pH exposure (Fig. 5B).

**Domains 11–12**—The association rate was significantly increased by 2-fold between pH 7.0 and 6.5 (Table II). In addition, the amount of bound IGF-II was also increased by 2-fold at pH 6.0 (p = 0.02). By pH 5.5, the dissociation rate was decreased, although only by 1.4-fold, and this was found not to be significant (Fig. 4C). Optimum affinity occurred between pH 7.0 and 5.5. The association rate and amount of IGF-II binding did not return to previous values when measured at pH 7.4 following each low pH exposure (Fig. 5C).
DISCUSSION

Affinities of IGF-IIR-CD4 chimeras were measured using surface plasmon resonance, a method that enables measurement of protein interaction in real time, providing information on the amount of ligand bound and, unlike immunoblot analysis, association and dissociation rates. A biotin-accepting peptide sequence was included in the chimeras so that high affinity, stable immobilization of constructs could be achieved on a streptavidin-coated biosensor chip, since the affinity of streptavidin for biotin is on the order of $10^{-15}$ M and is stable over a wide temperature and pH range (45). We confirmed that a point mutation at nucleotide position 4862, converting a T to C, altering the amino acid content from isoleucine to threonine in domain 11, abolished IGF-II binding (46) and was utilized as a control. Similar amounts of chimeric proteins containing domains 10–13 and 10–13 (I572T) were secreted, which suggests that the point mutation did not aberrantly affect protein structure such that secretion was affected.

Human recombinant IGF-II bound to immobilized constructs with differing affinity. The affinity of domains 1–15 for IGF-II was 14 nM, compared with 120, 100, and 450 nM for domains 10–13, 11–13, and 11–12, respectively. The differences observed may be due to differing stability of the truncated proteins, steric hindrance at the chip surface, or missing IGF-II-binding enhancing domains. Domain 13, which is lacking in the domains 11–12 construct, is thought to enhance IGF-II binding (37). Our data support this and suggest that domain 13 enhances IGF-II affinity by slowing its rate of release from the receptor.Domains 1–15 had the highest affinity for IGF-II, suggesting that other unknown IGF-II binding enhancing domains may also exist. Previously reported affinity values of IGF-II for full-length receptor have ranged from 0.2 to 5 nM for bovine IGF-II (47, 48) and 15 nM for placental purified human IGF-II (6). Differences may be due to experimental approach, since measurements have been determined both by affinity blots and surface plasmon resonance, with variations due to either the quality of purified proteins or stability of recombinant proteins. There may also be species and tissue differences for IGF-II receptor affinity (49). β-Glucuronidase and ITGF-β1 bound to immobilized domains 1–15 only. The affinity of this construct for β-glucuronidase was 2.4 nM, similar to previous values for the full-length purified, nonrecombinant receptor (50). Domains 1–3 and 7–9 bound β-glucuronidase with affinities of 0.3 and 0.9 nM, respectively (51). ITGF-β1 had a faster association and dissociation rate than β-glucuronidase, presumably because of the number of mannosylated residues on each protein molecule. ITGF-β1 may bind to the IGF-II via mannose 6-phosphate groups located on the latency-associated protein (16). Proteases involved in the activation of ITGF-β1 remove the latency-associated protein, which may then be internalized for degradation (52). It is not understood how ITGF-β1 avoids trafficking to the lysosomes. The ~17-fold faster dissociation rate of ITGF-β1 compared with β-glucuronidase may be one mechanism preventing ITGF-β1 from internalization and degradation before activation. Other factors may bind to the IGF-II-ITGF-β1 complex and prevent internalization. If these factors are not available, ITGF-β1 may dissociate before it is internalized.

IGF-IIR is exposed to a range of pH environments in vivo, since it is involved in ligand internalization at the cell surface, protein sorting within the trans-Golgi network and transport to the endosomes (53). The membrane-bound receptor binds mannosylated proteins with maximal binding within a pH range of 6.5–7.5, and optimum binding of β-glucuronidase to domains 1–3 and 7–9 occurs at pH 6.9 and 6.4–6.5, respectively (50, 51). In addition, the 46-kDa cation-dependent mannose 6-phosphate receptor, which displays sequence similarity to each exocrtytoplasmic domain of IGF-IIR, displays weak binding at neutral pH and has a pH optimum at 6.0–6.3 (54). Domains 10 and 13 may be involved in the pH dependence of IGF-II binding, since the optimum affinity of domains 11–12 had shifted downwad compared with domains 1–15 and 10–13. However, domains other than 10, 11, 12, and 13 may also be involved, since domains 1–15 responded differently to changes in pH compared with domains 10–13 and domains 11–12. The exact structural features contributing to pH dependence of ligand binding and release is not known for either type of protein and awaits characterization of receptor structure bound to ligands.

The reduction in association rate and amount of IGF-II bound to domains 1–15 at low pH was recovered by increasing the pH to 7.4, although dissociation was not. In contrast, the measured parameters affected by pH in domains 10–13 and domains 11–12 did not recover by reexposure to pH 7.4. Our data emphasize the stability of the extracellular domains 1–15 to repeated low pH exposure and perhaps suggest a role for the non-ligand binding domains of the receptor. Although the stability of IGF-IIR to pH exposure may be important during recycling, IGF-IIR may be continuously replaced from a pool once irreversible denaturation due to continuous low pH exposure has occurred. Evidence for a pool of IGF-IIR has been identified in Chinese hamster ovary cells, where labeled IGF-IIR was degraded with a half-life of 2.5–16 h (55).

In summary, the expression of functional recombinant soluble IGF-IIR in human embryonic kidney cells and their immobilization onto biosensor chips provided a method for characterizing the extracytosolic repeat domains. We have demonstrated that domain 13 acts to slow the rate of IGF-II release but domains other than 10, 11, 12, and 13 are also necessary for optimum IGF-II binding at neutral pH and suggest a functional role for the nonbinding domains of the cation-independent IGF-IIR.

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Real Time Ligand Kinetics of IGF-IIR