Supporting Materials and Methods

Expression vectors for recombinant human growth hormone (hGH) and hGH receptor (hGHR) extracellular domain (ECD) production were from Genentech. For the hGHR ECD, a truncated version was used (residues 29-238), which has unaltered activity and superior expression level compared to the 1-245 version (1). The proteins were expressed into the periplasm of *Escherichia coli* essentially as described (2), except that BL21 cells from Novagen were used as host, and the mutant hGHR ECD variants were expressed at 20°C. Kunkel mutagenesis (3) was used for all variants, and the entire gene was sequenced to confirm that only the desired mutations were incorporated. The periplasmic protein fraction was prepared as described (2). All the hGH variants were purified by using a Resource Q ion exchange column (Amersham Pharmacia) with some variants further purified on a Superdex 75 gel filtration column (Amersham Pharmacia). hGHR ECDs were purified over a hGH affinity column as described (2), except that 4.5 M MgCl₂ was used in the elution step. The W104A and W169A ECD mutants were purified by ion exchange and gel filtration chromatography. All protein variants were analyzed for purity by analytical HPLC, and the presence of the mutation was confirmed by electrospray mass spectrometry. Purity of the protein samples exceeded 95%. Typical yields for the hGHR ECDs were between 1 and 2 mg purified protein from 1 liter of culture. Protein concentration was determined by using molar extinction coefficients at 280 nm of 16.2 mM⁻¹ cm⁻¹ and 55 mM⁻¹ cm⁻¹ for hGH and hGHR ECD, respectively, and these values were adjusted appropriately for mutations (4).
**Surface Plasmon Resonance.** All experiments were carried out on a Biacore 2000 instrument at 25°C. hGHR ECDs were coupled to a Biacore Pioneer C1 sensor chip by disulfide bond formation through an engineered cysteine on the S237C mutant. This unpaired cysteine was typically modified by glutathione during expression. The glutathione modification was removed by treatment with 1 mM DTT for 30 min on ice. The DTT and glutathione were then removed from the sample by a desalting step on a PD-10 column (Amersham Pharmacia) equilibrated with 10 mM sodium phosphate buffer, pH 7.4. The ECD sample was then concentrated to 0.5 – 1.0 mg/ml for the subsequent immobilization step. The immobilizations and kinetic measurements were carried out by using filtered and degassed HBS buffer (10 mM HEPES/150 mM NaCl/3 mM EDTA/0.005% Tween 20, pH 7.4). The S237C hGHR ECD mutant was immobilized on the C1 sensor chip following Biacore protocol. Briefly, the flow rate was set to 5 µl min⁻¹ and a 25 µl mixture of 100 mM N-hydroxysuccinimide (NHS) and 390 mM N-ethyl-N'-(3-dimethyl-aminopropyl-carbodiimide hydrochloride (EDC) was injected. It was followed by a 40 µl injection of 80 mM 2-2(-pyridinyldithio)ethaneamine hydrochloride (PDEA) in 50 mM sodium carbonate, pH 9.5 buffer. A 50-100 µg ml⁻¹ solution of the S237C-ECD in 10 mM sodium acetate, pH 4.5, was then injected to achieve a level of 20-100 functional response units (RUs). The unreacted functional groups were blocked with a 30 µl injection of 50 mM reduced glutathione in 20 mM sodium acetate/1 M NaCl, pH 4.5 buffer.

**Data Analysis.** Association \((k_{on})\) and dissociation \((k_{off})\) rate constants were determined by using a modified tri-molecular decaying surface model of the program Clamp (5, 6). The original decaying surface model was introduced to describe a system in which molecule
A is covalently immobilized to the surface, and first B and then C are injected. Molecule A captures molecule B in an oriented fashion allowing for characterization of the B-C binding interaction:

\[
A + B \leftrightarrow AB \quad [1]
\]

\[
A + B \leftrightarrow AB \quad [2]
\]

Association \((k_{\text{on1}})\) and dissociation \((k_{\text{off1}})\) rate constant values corresponding to the first binding step are determined by fitting data from the first injection by using the simple 1:1 Langmuir binding model. These values are then used when determining the \(k_{\text{on2}}\) and \(k_{\text{off2}}\) rate constant values for the AB-C interaction. The surface decay model accounts for the continuous decay of AB during the second injection step due to B dissociating from A. The model assumes that molecule C binds to the AB complex by interacting only with molecule B (no direct A-C contact) and the B to A and C to B binding interactions are independent. Therefore, it also considers an \(A + BC \leftrightarrow ABC\) equilibrium with corresponding \(k_{\text{on3}}\) and \(k_{\text{off3}}\) values and assumes that the BC complex and molecule B dissociate from the immobilized A with the same dissociation constant values \((k_{\text{off3}}\) equals \(k_{\text{off1}})\).

The binding model that describes our system differs from the above model in two important aspects: i) there is a direct A-C (ECD1-ECD2) contact, and ii) a separate BC
complex, which would be equivalent to hGH:ECD2, does not exist, because the
dissociation of the ternary complex follows a stepwise mechanism where ECD2
dissociates in the first step \((k_{\text{off}2})\), followed by dissociation of hGH in the second step
\((k_{\text{off}1})\). Therefore, both the \(k_{\text{on}3}\) and the \(k_{\text{off}3}\) values were set to zero.

\[
k_{\text{on}1} \\
\text{hGH} + \text{ECD1} \leftrightarrow \text{hGH:ECD1} \quad [1] \\
k_{\text{off}1} \\
k_{\text{on}2} \\
\text{hGH:ECD1} + \text{ECD2} \leftrightarrow (\text{hGH:ECD1}):\text{ECD2} \quad [2] \\
k_{\text{off}2}
\]

For each data set, the first hGH injection step was fit by using a simple 1:1 Langmuir
model (Scheme 1), and three parameters were determined for the hGH + ECD1-binding
interaction, \(k_{\text{on}1}\), \(k_{\text{off}1}\), and the maximal response unit value. These values were then included
in the mathematical description of the subsequent steps; formation and breakdown of the 1:2
complex upon binding and dissociation of hGHR ECD2. Correction for the dissociation of
the 1:1 hGH:ECD1 complex to hGH + ECD1 was necessary, because it occurs in the entire
course of the experiment whenever the 1:1 complex is present due to non-saturating levels
of ECD2 binding. Three parameters were calculated by fitting the ECD2-binding curves by
using the modified decaying surface model; the association rate constant, \(k_{\text{on}2}\); the
dissociation rate constant \(k_{\text{off}2}\), and a response factor that reflects to the molecular weight
relations of the three components. Equilibrium dissociation constant values ($K_d$) for the second ECD binding were determined by the mass action relation $K_d = k_{off2} / k_{on2}$. 


