

# Modularity in receptor evolution: insulin- and glucagon-like peptide modules as binding sites for insulin and glucose in the insulin receptor

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**Abstract:** Dwyer suggested that peptide receptors evolved from self-aggregating peptides. Root-Bernstein extended Dwyer's theory to include complementary molecules. Insulin is a self-aggregating peptide. Insulin is also complementary (and therefore binds) to glucagon. We have shown previously using similarities searches that the insulin receptor (IR) has several insulin-like and several glucagon-like sequences associated with insulin-binding regions. We demonstrate here that peptides derived from these insulin- and glucagon-like regions bind insulin with up to high-nanomolar affinity, providing experimental evidence for the evolution of the IR from insulin- and glucagon-like modules. Moreover, we demonstrate that insulin itself binds glucose (and cytochalasin B), and that many of the insulin-like regions of the IR (but not the glucagon-like regions of the insulin receptor) do likewise. These data suggest the function of insulin, and of the IR in glucose regulation has been directed by chemical selection for their mutual set of molecular interactions. This model may be generalizable to the evolution of other receptor and transporter systems. The relationship between molecular structure and function within living systems may be highly constrained by selection for molecular complementarity.

**Keywords:** co-evolution, modular evolution, molecular complementarity, chemical selection, molecular paleontology

## Introduction

### Basic theoretical questions and proposals

How did receptors evolve in early biotic systems? The problem often presents itself as a chicken-and-egg situation. Which came first: the ligand or the receptor? How can a ligand evolve and be selected for in the absence of its receptor, or a receptor evolve in the absence of its ligand? If ligand and receptor must co-evolve, what principles determine such co-evolution?

Surprisingly little research has been devoted to this question.<sup>1-5</sup> In the absence of any understanding of the selection pressures that shaped receptor evolution, it is consequently difficult to address the mechanistic and functional aspects of their structures, or to relate structure to function. We present here evidence that there is a very basic, conceptually simple set of principles relating to the evolution, structure, and function of the insulin receptor that may be more widely generalizable. Because there appears to be a "molecular paleontology" embedded within the modern insulin receptor that reflects its past evolution, the results also have very practical biochemical and clinical implications.

Unveiling the processes by which evolution produced the complex machinery associated with cellular function is an important and nontrivial task for life scientists.

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The origins of receptors present a particularly thorny problem since it is difficult to imagine how complex receptor-second-messenger systems could have evolved by gradual steps. Dwyer<sup>3,4</sup> has provided a key insight into such a step-wise process by proposing that some peptide receptors may have evolved from the self-aggregating peptides. During evolution, the exons encoding such self-aggregating peptides could have been duplicated and spliced to exons encoding conserved transmembrane spanning modules and enzymatic modules (or other transducers), resulting in primitive receptors that variation and selection could have modified and elaborated.<sup>1,2</sup> Many peptide receptors do appear to have ligand-like regions in their binding sites, including the LFA-3 protein and CD2 protein; immunoglobulins (which share homologies with the Fc receptor); bungarotoxin (an acetylcholine receptor antagonist that mimics the acetylcholine receptor binding site); alpha scorpion toxins (which mimic the sodium channel proteins they block); interleukin 1 and interleukin 2; transforming growth factor-beta; thionin and crambin (plant defense molecules); neurophysin; and interleukin 1.<sup>3-6</sup> Insulin, glucagon, and gastrin are also self-aggregating peptides that have multiple sequence homologies with regions of their receptors that are associated with ligand binding.<sup>1,2</sup>

Root-Bernstein<sup>1,2</sup> has suggested that, in addition to self-aggregation (or homo-complementarity), receptor evolution may also involve complementarity between different molecules (hetero-complementarity). For example, insulin and glucagon are molecularly complementary, binding to each other with mid-micromolar affinity.<sup>7,8</sup> Notably, the insulin receptor has multiple glucagon-like regions, while the glucagon receptor has multiple insulin-like regions.<sup>1,2</sup> These hetero-complementary regions are, like the homo-complementary regions described above, also associated with ligand binding.<sup>1,2</sup> Thus, both homo-complementarity and hetero-complementarity could have played roles in the evolution of receptors.

Molecular complementarity may also provide selection pressures that resulted not only in the selection of ligands for their particular structures, but also for their particular functions.<sup>9</sup> Why, for example, does insulin, rather than some other peptide sequence, regulate glucose uptake by cells? Molecular complementarity suggests the possibility that insulin was selected by evolution to modulate glucose activity because glucose binds directly to insulin. Insulin is known to have two high affinity (ca.  $1 \times 10^{-3}$  M) and four low affinity (ca.  $6 \times 10^{-2}$  M) binding sites for glucose.<sup>9-13</sup> We therefore hypothesized, and demonstrated, in a previous publication that the insulin-like sequences of the insulin receptor may

also bind glucose.<sup>14</sup> Because these potential glucose-binding regions are also associated with insulin binding, insulin binding to its receptor is glucose sensitive.<sup>14</sup> So it is possible that glucose, insulin, and the insulin receptor each play the roles they play in metabolism because they were selected for their mutual set of chemical interactions. These direct chemical interactions may have been the primitive biological control systems from which the complex second-messenger systems evolved that govern metabolism today.

As noted above, we have previously demonstrated that glucose binds to the IR modifying insulin binding to it.<sup>14</sup> We report here experiments further characterizing insulin receptor specificity for insulin and for glucose, in light of our evolution-based hypotheses. In particular, we more than double the number of tests of insulin and glucose binding to insulin-receptor peptides in comparison with our previous publication on this topic;<sup>14</sup> demonstrate that insulin and glucose binding does not occur with ascorbate-binding peptides<sup>15</sup> derived from the H1 histamine receptor (H1HR) or the beta 2 adrenergic receptor (B2AR); and place all of the data for the first time in the context of how the insulin and other small-molecule receptors may have co-evolved.

## Materials and methods

### Glucose binding to insulin and insulin receptor peptides

Initial experiments were designed to determine binding of glucose and cytochalasin B to insulin (bovine insulin, Sigma-Aldrich Chemical Co.) and individual peptides derived from the insulin receptor. Peptide regions previously identified as being either insulin-like (IL) or glucagon-like (GL) regions of the IR (see Table 1),<sup>1,2</sup> and peptides associated with ascorbate binding to the beta-2 adrenergic (B2AR) or H1 histamine (H1HR) receptors,<sup>15</sup> were synthesized by the Mass Spectrometry, Synthesis and Sequencing Facility of the Department of Biochemistry at Michigan State University, and purified to >99% purity as determined by HPLC. Insulin (2.5 mg/ml) and IR peptides (1.0 mg/mL) were dissolved as solutions of ca. 0.5 mM in pH 7.4 phosphate buffer (Fisher Scientific). A 1.0 M D-glucose (Sigma-Aldrich) was made as a stock solution in the same buffer and diluted by thirds. 100  $\mu$ L of each glucose concentration plus 100  $\mu$ L of buffer, and mixtures of 100  $\mu$ L of each peptide with 100  $\mu$ L of buffer or with 100  $\mu$ L of each glucose concentration were made in a crystal 96-well plate and a complete spectrum of each well recorded on a Spectromax Plus spectrophotometer. Data were collected using SoftMax Pro 4.0 software. Every combination was done in triplicate and the results averaged.

**Table 1** Summary of experiments carried out on horse radish peroxidase-linked insulin (Ins-HRP) using an ELISA-like methodology, human recombinant insulin (Humulin®, Lilly) measured free in solution using UV spectroscopy, glucose (UV spectroscopy) or cytochalasin B (cyto B) (UV spectroscopy) binding to insulin, glucagon, peptides derived from the insulin receptor (IR),<sup>1,2</sup> peptides derived from the H1 histamine receptor (H1HR),<sup>15</sup> and peptides derived from the beta 2 adrenergic receptor (B2AR)<sup>15</sup>

Kd for binding between receptor peptides, insulins, and glucose	Ins-HRP	Humulin	Glucose	Cyto B
Insulin			250 µM and 30 mM	13 µM
Glucagon	150 nM	89 µM	>100 mM	nd
<b>Insulin receptor peptides</b>				
91–103 (IL) FRVYGLESKDLF	70 nM	<b>5 µM</b>	37 mM	nd
95–113 (IL) GLESLKDLFPNLTVIRGSR	<b>21 nM</b>	<b>2 µM</b>	<b>11 mM</b>	<b>75 µM</b>
105–118 (IL) NLTVIRGSRLLFN	<b>1.8 nM</b>	<b>2 µM</b>	<b>35 mM</b>	nd
157–166 (GL) TIDWSRILDS	30 nM	150 µM	57 mM	112 µM
223–234 (ns) CKSHGCTAEGLC	>1 µM	>1 mM	>100 mM	nd
233–248 (IL) CCHSECLGNCSQPDD	<b>8.0 nM</b>	<b>44 µM</b>	<b>3mM</b>	nd
284–300 (GL) SFCQDLHHKCKNSRR QG	<b>8.5 nM</b>	<b>46 µM</b>	>100 mM	1 mM
390–405 (GL) EISGYLKIRRSYALVS	150 nM	330 µM	30 mM	166 µM
392–404 (GL) SGYLIKIRRSYALV	300 nM	82 µM	54 mM	180 µM
424–444 (GL) YSFYALDNQNLRLQWDWSKH	>1 µM	400 µM	>100 mM	>1 mM
453–464 (IL) TQGKLFHHYNPK	<b>7.6 nM</b>	<b>45 µM</b>	57 mM	nd
660–679 (IL) ERQAEDSELFELDYCLKGLK	370 nM	>1 mM	70 mM	117 µM
897–916 (IL) LCVSRKHFALEGRCLRLGL	<b>1.5 nM</b>	<b>6.2 µM</b>	<b>14 mM</b>	<b>25 µM</b>
<b>Histamine H1 receptor peptides</b>				
H1HR 77–87 GAVVNMNILE	nd	>1 mM	60 mM	nd
H1HR 105–115 SMDYVASTASI	>1 µM	>1 mM	>100 mM	nd
<b>Beta 2 adrenergic receptor peptides</b>				
B2AR 97–106 KMWTFGNFWC	>1 µM	>1 mM	60 mM	nd
B2AR 103–113 FWCEFWTSIDV	nd	>1 mM	75 mM	nd
B2AR 175–188 RATHQEAINCYANE	nd	>1 mM	65 mM	nd

**Notes:** Peptides from the IR are labeled as being insulin-like (IL) or glucagon-like (GL) or, have no significant similarity (ns) to either insulin or glucagon.<sup>1,2</sup> Combinations that were not investigated are indicated with “nd”, meaning “not done.” The lowest binding constants (Kd) are indicated by bolded figures and coincide almost completely with insulin-like (IL) peptides derived from the IR. Note that insulin binds best to insulin-like modules from the IR, and that there is also insulin binding to some glucagon-like modules as well. Insignificant binding was observed to peptides derived from receptors other than the IR. Binding of insulin-HRP to IR peptides (ELISA-like method) is about 200 to 1000 times stronger than Humulin binding (UV spectroscopy), as a result of decreasing the thermodynamic degrees of freedom when constraining the IR peptides to an ELISA plate (see text for interpretation of results). Glucose binding is limited almost exclusively to insulin-like modules derived from the IR. Glucose does not have significant affinity for glucagon or glucagon-like modules of the IR or peptides derived from other receptors. Cytochalasin B binding is stronger than glucose binding to the peptides, but follows exactly the same pattern, as would be expected for a glucose antagonist. About a third of these data appeared in a previous publication.<sup>14</sup> See Table 2 for a schematic diagram of where the various insulin-like and glucagon-like modules are located on the IR.

These curves were examined for the absorbance at which the greatest spectral shifts occurred (usually between 195 and 205 nm) and these data analyzed to produce binding constants (Kd) determined by the difference in absorbance between the absorbance obtained from the mixture of the peptide with glucose and the sums of the peptides (plus buffer) and glucose (plus buffer) individually.

## Cytochalasin B binding to insulin and insulin receptor peptides

Following a procedure described by us previously for measuring binding to receptors,<sup>15</sup> UV spectrophotometry was also used to measure cytochalasin B (Sigma-Aldrich) binding to insulin and to the IR peptides, but a different protocol was followed since the cytochalasin B is not soluble in the buffer,

and was supplied in pure DMSO. 10 mM cytochalasin B (DMSO) was serially diluted by thirds with phosphate buffer to produce eight dilutions. DMSO was similarly diluted to make solutions with matching DMSO concentrations. The same peptide solutions as above were utilized. 200 µL of a peptide in phosphate buffer was put in two wells of a crystal 96 well plate, and phosphate buffer alone in another two. 20 µL of the lowest concentration of cytochalasin B solution was added to one peptide well and one buffer well, and 20 µL of the lowest concentration of DMSO solution was added to the other peptide and buffer wells. The spectra of the wells was collected from 190 to 240 nm and saved. The process was repeated by adding the next higher concentration of cytochalasin B and DMSO, etc. until the highest concentration was added and the spectra recorded. In order to determine

whether cytochalasin B bound to the peptide, the buffer plus DMSO dilutions data were subtracted from all corresponding spectra; then the spectra for the peptide and cytochalasin B wells were added to provide expected values and this was compared with the actual values obtained from the mixing of peptide and cytochalasin B. The clearest binding curves were found in the range of 200–210 nm.

## Humulin® binding to peptides derived from the insulin receptor

Insulin receptor peptides, 1 mg per 1 mL, and serial dilutions by thirds were used with UV spectroscopy of each peptide against recombinant human insulin (Humulin®). Humulin R from Lilly was diluted to a constant concentration of 1 uL per 1 mL to make a stock solution. 100 ul of each peptide concentration was added to three wells of a crystal 96 well plate, and 100 uL of pH 7.4 phosphate buffer (Fisher Scientific) added to each. 100 ul of buffer were also added to three wells of 100 uL Humulin R stock solution. 100 ul of Humulin R stock solution were added to three wells of each dilution of the insulin receptor peptide. Finally, three wells were filled with 200 uL of buffer solution. Solutions were incubated at room temperature for 30 minutes to maximize binding before being read from 190 to 300 nm in a SPECTRAmax Plus spectrophotometer. Data were collected using software. Binding constants were calculated using an Excel program. The buffer reading at each absorbance was subtracted from the absorbances of all other wells. The remaining absorbances for the peptide concentrations was added to the remaining absorbance of the Humulin R to give an expected absorbance. The difference of the expected absorbance and the actual experimental absorbance of the peptide-Humulin combination was then determined and these differences plotted according to peptide concentration. The best binding curves were visible between 190 and 200 nm.

## Insulin-HRP binding to peptides derived from the insulin receptor

A modified version of enzyme-linked adsorption assay (ELSA) was used to measure insulin binding to the peptides derived from the insulin receptor.<sup>7,14</sup> 0.5 mg of insulin-HRP (insulin conjugated to horse radish peroxidase [Sigma-Aldrich]) was diluted in 2.0 mL of pH 7.4 phosphate buffer as a stock solution (5 µM). Serial dilutions of the insulin-HRP were then made from 1:100 to 1:333,333 by thirds, producing solutions that varied in insulin-HRP concentration from 50 nM to 15 pM. Each insulin receptor peptide was dissolved in buffer to produce a 50 µM stock solution and 100 µL were then added

to an ELISA plate (Costar), incubated for an hour, and then triply washed with a 1% solution of TWEEN in phosphate buffer. 200 µL of a 2% polyvinylalcohol (PVA) blocking agent in phosphate buffer was added to each well, incubated for an hour, and triply washed. 100 µL of each dilution of the insulin-HRP solution was then added to a well, incubated for an hour, and triply washed. Finally, 100 µL of ABTS single reagent (Chemicon, Temecula, CA, USA) was added to each well, incubated for 30 minutes, and the plate read at 405 nm. All combinations and controls were run in duplicate and the resulting values averaged.

## Data analysis

All data were analyzed and plotted using an Excel spreadsheet program.

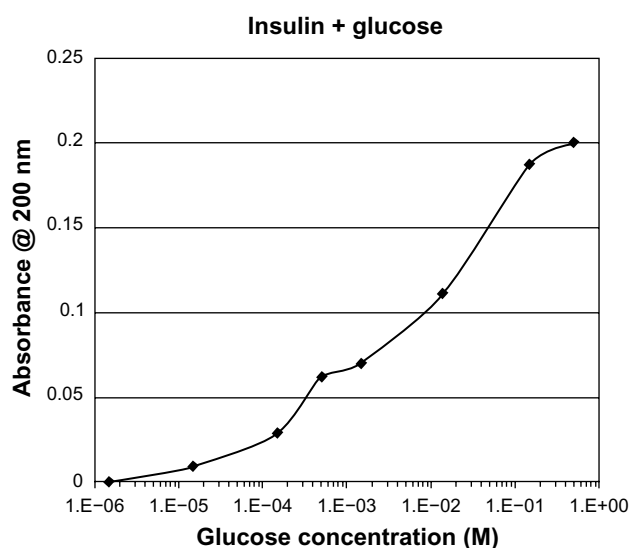
## Note on insulin receptor numbering

All IR sequences are provided in the SwissProt numbering system (<http://www.expasy.ch>) that begins with the pre-pro sequence. Many papers on the IR use a numbering system that begins with the edited transcript (eg, refs. 26 and 31) and thus have 27 amino acids subtracted from the number as compared with the SwissProt convention.

## Results

Table 1 summarizes the results of all of the experiments carried out involving insulin binding to IR, H1HR and B2AR peptides; glucose binding to IR, H1HR, and B2AR peptides; and cytochalasin B binding to IR peptides. We began by validating the use of UV spectrometry for this study by demonstrating that we could reproduce the phenomenon of glucose binding to insulin. Figure 1 shows that we were able to visualize the two-step binding curve that one would expect from previous reports which imply that insulin contains both high affinity (ca.  $1 \times 10^{-3}$  M), and low affinity (ca.  $6 \times 10^{-2}$  M), binding sites for glucose.<sup>10–13</sup> We found slightly lower binding constants of 250 uM and 30 mM (Table 1), which may simply reflect differences in the buffers employed by various investigators. We also confirmed that cytochalasin B, a potent glucose transport antagonist, binds to insulin with an even lower binding constant of 13 µM (Figure 2). No measurable binding was found between glucose and glucagon (data not shown).

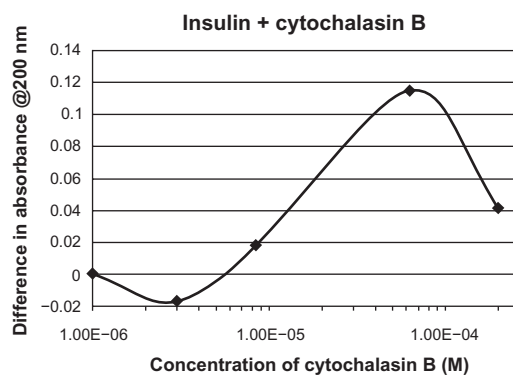
Figure 3 illustrates some of the data obtained from binding experiments between insulin-HRP and IR peptides. Binding constants were determined from the inflection points of the curves, and varied between 1.5 nM and greater than 1 mM (ie, effectively unmeasurable by this technique). The binding constants using both the insulin-HRP “ELSA” and



**Figure 1** UV spectroscopic study of glucose binding to insulin showing clearly that there are both high affinity (ca.  $1 \times 10^{-3}$  M) and low affinity (ca.  $6 \times 10^{-2}$  M) binding sites. The binding constants were determined from the inflection points of the curves. The same technique was used to determine glucose binding to IR peptides (Table 1).

Humulin-R-UV-spectroscopy methods are summarized in Table 1. Binding constants determined by UV spectroscopy were uniformly 200 to 1000 times higher than by insulin-HRP “ELSA”, but were extremely consistent with whether there was measurable binding or not. These data show that insulin binds to insulin-like modules of the IR, and to some glucagon-like modules, but does not bind significantly to a peptide that mimics neither insulin nor glucagon, nor to peptides derived from the beta-2 adrenergic receptor (B2AR) or the histamine receptor (H1HR).

Table 1 goes on to demonstrate that glucose and cytochalasin B bind to many, but not all, insulin-like regions of the IR, with affinities similar to those for their binding to insulin itself. Glucose and cytochalasin B do not, however, bind



**Figure 2** Cytochalasin B binds to insulin with a binding constant of ca.  $13 \mu\text{M}$ , confirming the existence of at least one high affinity glucose binding site. The binding constant was determined from the inflection point of the curve. Similar results were obtained for binding of cyto B to IR peptides that were found to bind glucose (Table 1).<sup>14</sup>

significantly to glucagon or to glucagon-like regions of the IR ( $K_d > 100 \text{ mM}$ ), nor to an IR peptide that mimics neither insulin or glucagon. No physiologically significant binding of glucose to H1HR or B2AR was observed.

Table 1 also demonstrates that insulin itself binds to both insulin-like and glucagon-like peptide regions of the IR. Binding of insulin-HRP is in the nanomolar range, but binding of unlabelled recombinant human insulin (Humulin) was generally between 100 and 1000 times less. No significant binding of insulin to H1HR or B2AR receptor peptides was found. Figure 3 illustrates some of the insulin-HRP binding data.

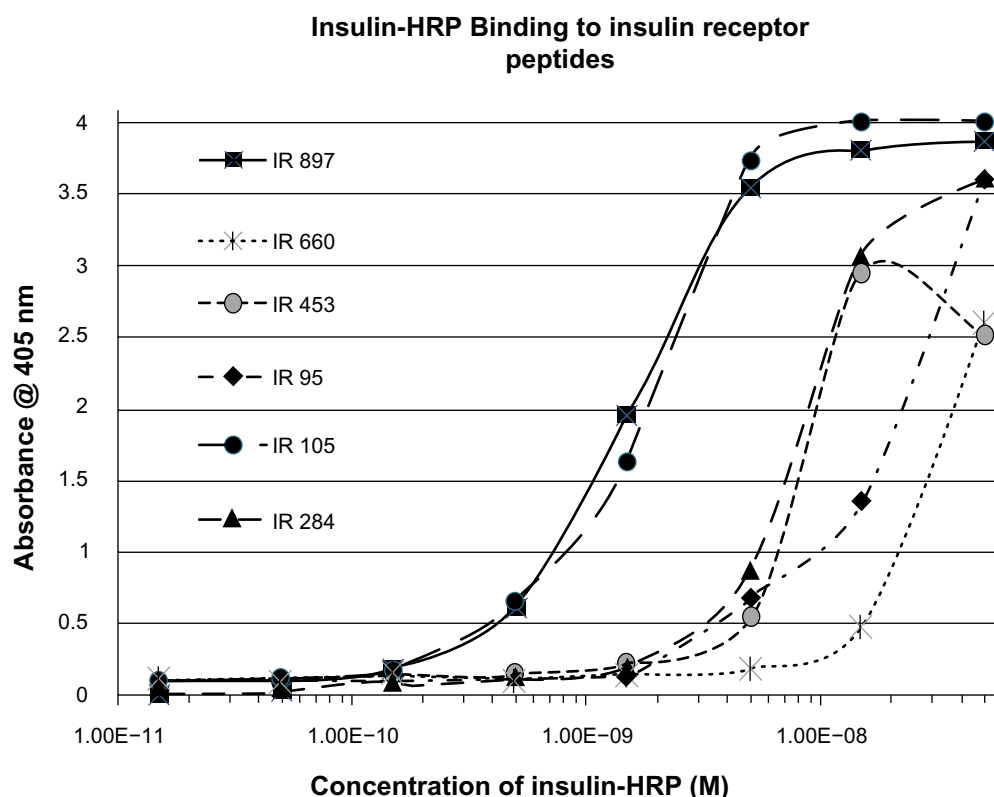
Table 2 plots the insulin-like and glucagon-like regions of the IR on the SwissProt (ID# P06213) sequence. Table 2 also shows where antibody interference studies, alanine-scanning mutagenesis studies, and mutations associated with impaired insulin binding (especially Leprechaunism) map onto this sequence. The table demonstrates that all of the extracellular insulin-like peptides map to regions that are known to be involved in insulin binding with one exception, which is the 897–917 peptide from the receptor B chain. The highest affinity glucose binding sites are localized to the same regions. On the other hand, Table 2 also illustrates the fact that insulin binding to the IR also involves a number of regions not associated with insulin- or glucagon-like modules.

## Discussion

The insulin receptor has multiple high and low affinity sites that interact in both positively and negatively cooperative manners, making insulin binding very complex.<sup>16</sup> The insulin-HRP ELSA-like experiments yielded binding constants (1.5 to 8.5 nM) that are about eight- to thirty-fold lower than those found for the high-affinity binding of native insulin to the native IR (ca. 250 pM).<sup>18,19</sup> The difference between insulin binding to native IR and the peptides in our study is hardly surprising, since ligand binding to receptors is usually mediated by the tertiary or quaternary structure of the receptor, which is lacking in the peptides. Our data may therefore suggest that the peptide modules we have characterized may cooperate through tertiary conformational effects to create higher affinity insulin binding sites in the native IR, or they may represent some of the lower affinity insulin binding sites (ca. 1 nM)<sup>16</sup> that are known to exist on the IR. In either case, it is likely that the insulin-like and glucagon-like modules characterized here play some role in insulin binding to the IR.

Our data also demonstrate that while insulin binds to insulin-like and glucagon-like modules on the IR, binding





**Figure 3** Some of the data from binding studies of insulin-HRP to peptides derived from the IR. These data, as well as additional experiments involving insulin-HRP binding to peptides derived from other receptors, were used to put together Table 1. Binding constants were derived from the inflection points of the curves.

constants differ considerably depending on the technique used to measure them. Using HRP-labeled insulin binding to IR peptides immobilized to an ELISA plate yields binding constants in the low nanomolar range. Using UV spectroscopy to measure insulin binding to IR peptides free in solution yields binding constants about 200 to 1000 times lower (low-micromolar range). The difference in binding constants is not surprising in light of basic thermodynamics. Immobilizing a protein or peptide to a surface (whether in a cell membrane or on an ELISA plate) eliminates several degrees of freedom, significantly improving the probability of binding. In a similar study, Huppa, et al<sup>17</sup> have shown that T cell receptor peptides binding to major histocompatibility (MHC) proteins also have about 100 to 1000 times lower affinity when both are in solution than when the MHC protein is immobilized on a surface. Both our data and the Huppa et al data suggest that it is therefore very important to consider the nature of the technique used to determine binding constants between molecules before interpreting their physiological significance. Solution-derived binding constants may indicate surface-bound binding constants that are significantly lower. The data must also be placed in the context of the physiological situation. In this instance, the IR is immobilized within the cell membrane and is better

mimicked by the insulin-HRP ELSA experiments than by binding between free peptides in solution. Indeed, this thermodynamic argument is also of importance in understanding the selection pressures that governed the evolution of peptide receptors: the evolution of specific, immobilized receptors on cell surfaces would have increased binding (and thus information transfer) through cell surfaces by the same order (100- to 1000-fold) – a huge selection advantage – over solution interactions or random tunneling through lipid membranes.

Significantly, our IR-derived peptide data are consistent with previous studies of insulin binding to the IR. Regions thought to be involved in insulin binding (Table 1) include its N-terminus (SwissProt numbers) 34–84;<sup>20</sup> 107–112;<sup>21,22</sup> 238–393;<sup>22,23</sup> and more specifically; 264–272,<sup>21</sup> and 344–352;<sup>21</sup> 496–619;<sup>24</sup> 677–785<sup>25,26</sup> and more specifically 728–737;<sup>20,21</sup> and 792–797.<sup>24</sup> Additionally, known mutations to the insulin receptor (summarized on the SwissProt website [<http://www.expasy.ch>] for human insulin receptor P06213) show that the 232–387, 496–619, and 677–797 as well as two additional regions, 113–119 and 925–937, are associated with loss of insulin binding resulting in leprechaunism, a genetic disease accompanied by severe insulin resistance. Longo et al<sup>27</sup> and Whittaker et al<sup>20</sup> for example, demonstrated that the Arg 86

**Table 2** Annotated sequence of the extracellular (amino acids 1–956) portion of the insulin receptor (IR)

10 20 30 40 50 60  
 MGTGRRGAA AAPLLVAVAA LLLGAAGHLY PGEVCPGMDI RNNLTRLHEL ENCSVIEGHL  
 70 80 90 100 110 120  
 QILLMFKTRP EDFRDLSPFK LIMITDYLLL **FRVYGLES**LK **DLFPNLT**VIR **GSRLFFNYAL**  
 130 140 150 160 170 180  
 VIFEMVHLKE LGLYNLMNIT RGSVRLEKNN ELCYLA **TIDW SRILDSVEDN YIV**NKDDNE  
 190 200 210 220 230 240  
 ECGDICPGTA KGKTNCPTV INGQFVERCW THSHCQKVCP **TICKSHGCTA EGLCC**HSECL  
 250 260 270 280 290 300  
**GNCSQPDDPT** KCVACRNFL DGRCVETCPP PYYHFQDWRC VNF **SFCQDLH HKCKNSRRQG**  
 310 320 330 340 350 360  
**CHQW**HNK CIPECPSGYT MNSSNLLCTP CLGPCPKVCH LLEGEKTIDS V TSAQELRGC  
 370 380 390 400 410 420  
 TVINGSLIIN IRGGNNLAAE LEANLGLIE **ISGYLKIRRS YALV**SLSFFR KLRLIRGETL  
 430 440 450 460 470 480  
 EIGNYSFYAL **DNQNLRLWD WSKH**NLTITQ **GKLFFHY**NPK **LCLSE**HKME **EVS**GTKGRQE  
 490 500 510 520 530 540  
 RNDIALKTNG DQASCENELL KFSYIRTSFD KILLRWEPYW PPDFRDLLGF **MLFYKEAPYQ**  
 550 560 570 580 590 600  
**NVTEFDGQDA CGSNS**WTVVD IDPLRSNDP KSNHPGWLM RGLKPWTQYA IFVKTLVTFS  
 610 620 630 640 650 660  
 DERRTYGAKS DIIYVQTDAT NPSVPLDPIS VSNSSSQIIL KWKPPSDPNG **NITHYLVFWE**  
 670 680 690 700 710 720  
**RQAEDSELFE LDY****CLKGLKL PSRTWSP**PFE **SEDSQ**KHNQS EYEDSAGECC SCPKTD SQIL  
 730 740 750 760 770 780  
 KELEESSFRK **TFEDYLHN**VV FVPRKTSSGT GAEDPRP//SRK RRLSGDVGNV TVAVPTVAAF  
 790 800 810 820 830 840  
 PNTSSTSVPT SPEEHRPF EK VVNKESLVIS GLRHFTGYRI **ELQAC**NQDTP EERCSVAAYV  
 850 860 870 880 890 900  
 SARTMPEAKA DDIVGPVTHE IFENNVLHLM WQEPKEPNGL IVLYEVS YRR YGDEELHL**Cv**  
 910 920 930 940 950  
**SRKH**FALERG **CRLRGL**SPGN YSVRI**R**ATSL AGNSW**T**EPT YFYVTDYLDV PSNIA

**Notes:** Bold letters represent insulin-like modules identified in Root-Bernstein.<sup>1,2</sup> Bold italicized letters represent glucagon-like modules identified in Root-Bernstein.<sup>1,2</sup> Underlined regions represent insulin binding regions identified using blocking antibodies.<sup>22–24</sup> Strike through regions represent insulin binding regions identified by photocrosslinking studies.<sup>16,20,25,26</sup> Amino acids highlighted in gray blocks have been identified by alanine-scanning mutagenesis to be involved in insulin binding.<sup>16,20</sup> Amino acids in large font are mutations associated with various forms of Leprechaunism, in which there is decreased or obliterated insulin binding to the IR (derived from annotations in SwissProt ID# P06213). The two Cys in black boxes covalently link the alpha chain of the IR (28–758) to the beta chain (759 ff.). This disulfide bond links to insulin-like modules, mimicking the structure of insulin itself. The double lines after amino acid 758 denote the place at which the IR is cleaved into alpha and beta chains. As can be seen by inspection of this Table, all of insulin-like modules correspond to regions of the IR identified by at least one, and in some cases as many as four different methods, as being important to insulin binding. We suggest that the insulin-like module spanning 897–916, which has been very little studied by previous investigators, may be far more important to insulin binding than has previously been thought. Glucagon-like modules are significantly less associated with identified regions or insulin binding. The Table also illustrates the fact that some regions of the IR that are essential to insulin binding do not correspond to insulin-like modules, so modular evolution does not explain all aspects of the IR structure.

(SwissProt, 113 – Table 1) is critical to activation of glucose transport. The same residue when mutated to Pro results in complete abolition of insulin binding (see SwissProt annotations for P06213). Ala scanning mutagenesis (reviewed in De Meyts and Whittaker)<sup>16</sup> has also identified the five amino acids following this Arg 86 (SwissProt numbering, 113) as being essential to insulin binding. This sequence of

amino acids sits right in the center of one of the insulin-like modules of the IR. Similarly, the insulin-like sequence at 222–250 has been shown to be involved in insulin binding through antibody studies,<sup>22,23</sup> affinity photolabeling,<sup>16</sup> and mutation (SwissProt P06213). In fact, all of the insulin-like modules (but none of the glucagon-like modules) listed in Table 1 are within one of the regions defined by

previous investigators as being associated with high affinity insulin binding, with one major exception, and that is the 897–916 region that we have identified as having both excellent insulin-binding and glucose-binding capacity. Given that this region is on the extracellular portion of the beta chain of the IR (Table 2), and is involved through a disulfide bond to the alpha chain of the IR (Table 2), it is in a perfect region to act as a highly sensitive “switch” modulating transmembrane information flow. As far as we can tell, no research has been performed on this region by those investigating insulin binding to the IR, since the focus has been almost exclusively on the alpha chain. We predict that the extracellular region of the beta chain will turn out to be critical for the binding of insulin and the transduction of its message intracellularly.

The data concerning glucose binding to insulin-like modules of the IR is also consistent with other research on the nature of glucose-binding sites. Our experiments reveal binding of glucose to insulin with a high affinity binding of 250  $\mu$ M and a low affinity of 30 mM, which are consistent with the binding constants reported previously by Anzenbacher and Kalous.<sup>10,11</sup> Similarly, insulin-like peptides in the IR that have binding constants in the range of 3 to 30 mM. These binding constants are physiologically relevant since normal blood glucose levels in human beings fluctuate around 5 mM, and under hyperglycemic conditions associated with diabetes can range up to 50 mM.<sup>28</sup> We have demonstrated elsewhere<sup>14</sup> that varying the concentration of glucose from 1 mM to 25 mM has very significant effects on insulin binding to its receptor, though to what extent the differences in binding are due to glucose binding to insulin, glucose binding to the IR, or both, could not be determined definitively. Our data were consistent with experiments by other investigators showing that glucose concentration affects insulin activity in a variety of assays (reviewed in<sup>16</sup>).

The IR sequences that we have observed to bind glucose are also consistent with what is known about glucose binding protein sequences more generally. Li et al<sup>29</sup> analyzed a number of different glucose-binding proteins and concluded that “a possible glucose binding site can be made by placing Asp and Asn around glucose for hydrogen bonding and Phe on both sides of glucose for hydrophobic interaction.” Notably, each of the four peptides listed in Table 1 that bind glucose most tightly have a variation of the Li et al motif, especially if one is willing to consider reasonable substitutions, such as Leu or Tyr for Phe. The glucose binding regions of the IR include 100–106: LeuLysAspLeuProAsn;

113–118: ArgLeuPhePheAsnTyr; 239–246: LeuGlyAsnCys-SerGlnProAsp; 902–907: ArgLysHisPheAlaLeu. It appears that Arg and Lys may also play a role in glucose binding in these peptides, perhaps forming hydrogen or ionic bonds. Notably, three of these sequences are cognates of a single previously identified glucose binding site on the B chain of insulin, in which Glu substitutes for Asp in the binding motif: LeuValGluAlaLeuTyrLeuValCysGlyGluArgGlyPhe (underlined in Table 1).<sup>12,13</sup> Our data are therefore consistent with other studies of glucose binding to proteins. We have argued previously that the IR may therefore represent a glucose sensor, and have demonstrated experimentally that insulin binding to the IR is certainly glucose concentration-sensitive.<sup>14</sup> The existence of glucose binding sites on the IR therefore links IR structure and function in a manner that is consistent with the hypothesis that evolution of receptor structure-function was governed by selection for molecularly complementary modules.

Notably, this evolutionary theory of IR structure and function has a number of potential clinical implications. It is also well-documented that people who develop type 1 (insulin-dependent) diabetes are characterized by having antibody and Tcells against insulin epitopes.<sup>30–38</sup> We predict from the data presented here that the insulin-like modules of the IR will also be targets of these anti-insulin antibodies, resulting in both insulin resistance and also malfunction of the glucose-sensor function of the IR. Since many people with diabetes also develop anti-glucagon antibodies,<sup>39,40</sup> it is possible that IR function can also be compromised by anti-glucagon antibodies in diabetes that cross-react with glucagon-like modules of the IR. As far as we know, no one has yet investigated any of the possibilities raised by this evolutionary approach to understanding IR structure and function.

In sum, the data presented here demonstrate that insulin-like modules almost certainly play functional roles in insulin binding in the modern human IR. This conclusion is supported by evidence that both the insulin-like and glucagon-like modules are very highly conserved in the IR throughout evolution from insects through human beings,<sup>2</sup> suggesting that these modules are critical components of IR function. Thus, the evidence supports the hypothesis that the IR evolved from an insulin-like molecule, possibly insulin itself. Whether glucagon-like modules are also essential components of the IR, perhaps either structurally, or as low-affinity binding sites, remains conjectural. Perhaps these glucagon-like modules are actually glucagon binding sites that allosterically modulate the IR. Further tests



will be required to investigate these possibilities. In any event, we conclude that homocomplementary (insulin-like modules of the IR binding insulin) and heterocomplementarity (insulin and insulin-like modules of the IR binding glucose) likely played critical roles in the evolution of the IR by linking structure and function.

The hypothesis that homocomplementarity and heterocomplementarity play critical selective roles in the evolution of receptors and transporters is generally testable. For example, we have demonstrated by similarity searching that the glucose transporter also contains insulin-like modules localized specifically to its transport core.<sup>41</sup> These insulin-like regions can be predicted to bind glucose at physiological concentrations, just as do the insulin-like modules of the IR characterized here. In addition, the glucagon receptor, like the IR, contains both glucagon-like and insulin-like sequences in regions associated with glucagon binding.<sup>1,2</sup> The same type of study performed here can be carried out as a test of the complementarity hypothesis for glucagon-glucagon receptor co-evolution. And yet another aspect of that hypothesis would suggest that just as insulin binds directly to glucose, glucagon might interact with some other component of the glucose regulation as well. Since insulin mediates glucose transport into cells, while glucagon mediates conversion of glycogen into glucose for release from the liver, one possible conjecture would be that glucagon will bind to glycogen and perhaps even act on it as a very weak enzyme to convert it to glucose. In this manner, molecular complementarity would integrate a wide range of structure-function relationships in the evolution of the glucose regulation system. These chemical interactions would then have become the basis for the evolution of the more complex regulatory systems that now govern cell function. If so, each of these elements should bear the stamp of the same kind of “molecular paleontology” revealed here.

## Disclosure

The authors report no conflicts of interest in this work

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