Peptides Identify the Critical Hotspots Involved in the Biological Activation of the Insulin Receptor

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We used phage display to generate surrogate peptides that define the hotspots involved in protein-protein interaction between insulin and the insulin receptor. All of the peptides competed for insulin binding and had affinity constants in the high nanomolar to low micromolar range. Based on competition studies, peptides were grouped into non-overlapping Sites 1, 2, or 3. Some Site 1 peptides were able to activate the tyrosine kinase activity of the insulin receptor and act as agonists in the insulin-dependent fat cell assay, suggesting that Site 1 marks the hotspot involved in insulin-induced activation of the insulin receptor. On the other hand, Site 2 and 3 peptides were found to act as antagonists in the phosphorylation and fat cell assays. These data show that a peptide display can be used to define the molecular architecture of a receptor and to identify the critical regions required for biological activity in a site-directed manner.

The insulin receptor (IR) is a member of the tyrosine kinase receptor superfamily of growth factor receptors (1). This receptor is composed of two α- and two β-subunits derived by proteolytic processing from a single gene product that forms a disulfide-linked heterotetramer (β-α-α-β). The α-subunits are entirely extracellular and contain the ligand binding domain, whereas the β-subunits contain a transmembrane domain and a cytoplasmic domain displaying the tyrosine kinase activity. The sequence of IR is highly homologous to the insulin-like growth factor receptor (IGF-1R) with homology varying from about 40–70%, depending on the position within the α-subunit (2). The IR is primarily involved in metabolic functions, whereas the IGF-1R mediates growth and differentiation. However, both insulin and IGF-1 can induce mitogenic and metabolic effects in normal cells. It is unclear whether each ligand elicits these activities via its own receptor, or whether insulin exerts its mitogenic effects through its weak affinity binding to the IGF-1 receptor, and IGF-1 exerts its metabolic effects through the insulin receptor (3, 4).

Although much is known about the IR in terms of its mode of action and signaling pathways, there is a paucity of information regarding the molecular architecture of the insulin binding site, the contact sites for insulin binding, and the mechanisms by which insulin induces its biological effects. Several models have been proposed for insulin signaling including some in which cross-linking of the two receptor subunits is required for activity (5–9). For example, it is known that insulin binding to detergent-solubilized IR induces a conformational change that has been correlated with autophosphorylation of the receptor’s kinase domain. The IR can return to its original conformation following dissociation of the insulin molecule (10, 11).

To understand more fully the interaction of insulin with its receptor, we used random peptide phage display libraries to identify binding domains on the surface of the insulin receptor. The process creates and uses diverse peptide surrogates to identify surface hotspots (i.e. critical protein-protein interaction sites) on protein targets. Hotspot identification aids in the discovery of the single or multiple sites required for agonist or antagonist action.

We used several highly diverse (1010–1011 independent clones per library) randomized peptide phage libraries ranging in size from 20 to 40 amino acids to obtain the surrogate peptides. After analysis of the primary binders to determine sequence motifs, consensus regions, and multiple independent hotspots on the surface of the receptor, secondary and tertiary libraries were prepared by defined mutational strategies called “doping.” These libraries allowed us to identify the optimal amino acids at each position that are required for binding the surrogate to the IR as well as isolating peptides with biological activity.

EXPERIMENTAL PROCEDURES

Preparation of the Random Peptide Phage Display Libraries—DNA fragments coding for peptides containing 20–40 random amino acids were generated by the following protocol. An oligonucleotide was synthesized containing the sequence (NNK)40 where N is A, C, T, or G and K is G or T. This oligonucleotide was used as the template in PCR reactions along with two shorter oligonucleotide primers, both of which are biotinylated at their 5′-ends. The resulting 190-bp fragment was purified, and the phagemid pCANTAB5E (Amersham Biosciences) was digested with SfiI and NotI. The resulting 150-bp fragment was purified, and the phaged library was ligated overnight at 15 °C. The ligation product was purified, and elec-
troporations were performed at 1500 V in an electroporation cuvette (0.1-mm gap; 0.5-ml volume) containing 12.5 μg of DNA and 500 μg of Escherichia coli strain TG1 electrocompetent cells. Immediately after the pulse, 12.5 ml of prewarmed (40 °C) 2 mM YT medium containing 2% polyethylene glycol (YT-AG) was added, and the transformants were grown at 37 °C for 1 h. Cadmium acetate solutions were prepared in 0.1 M NaHCO3 and an aliquot was plated onto 2× YT-G containing 100 μg/ml ampicillin (YT-AG) to determine the number of transformants. Depending upon the amount of DNA electroplated, the diversity of the random 40-mer peptide cell library was found to be between 1.5x1010 and 1.5x1011 independent clones. The phage library was produced by rescue of the cell library according to standard phage preparation protocols. Phage titers were usually 4x1010 colony-forming units/ml. In previous studies sequencing of randomly selected clones from the cell library indicated that about 54% of all clones were in-frame (12). The short FLAG sequence, DYKD, was included at the N terminus as an immunoaffinity tag. In addition, the E-tag epitope (GAPVPYDPLFER) was engineered into the carboxyl terminus of the peptide. Additional phage libraries of random 20-mer peptides were constructed in a similar manner, except for the size of the initial oligonucleotide. The diversity of this cell library was found to be >1x1011 clones, and sequencing revealed that 77% of the clones were in-frame (13).

**Panning of the Libraries on Insulin Receptor**—Six wells of a 96-well microtiter plate were coated with antigen (in 50 μl of 50 mM NaHCO3, pH 8.0) at concentrations of 50-500 μg/ml overnight at 4 °C. The next day, unbound antigen was removed, and the coated wells were blocked with 300 μl of 2% nonfat milk in PBS (NFP-BS) for 1 h at room temperature. 100 μl of a phage library was added to the antigen-coated wells, and the plates were incubated for 3 h at room temperature. Each well was washed 13 times with PBS-2% NFM, and the phage was eluted with 100 μl of 50 mM glycine/HCl containing 0.1% bovine serum albumin (pH 2.2) following a 5-min incubation. The eluted phage from each well were pooled, neutralized with 100 μl of 1:5 Tris/HCl (pH 8.0), added to 10 ml of log-phase E. coli TG1 (OD600nm = 1.0), and amplified in 2× YT-glucose medium (1.0), and amplified in 2× YT-AM medium containing ampicillin and kanamycin), and incubated overnight at 37 °C. The next day the infected bacterial cells were centrifuged at 3500 rpm at 4 °C for 15 min, and the pellet was discarded. The supernatant contained the phage and was precipitated with one-fourth volume of 30% polyethylene glycol-8000 in 1.5 mM NaCl by incubation on ice for 1 h. The precipitate was centrifuged at 19,000 rpm at 4 °C for 30 min, and the phage pellet was resuspended in approximately 1 ml of NFM-PBS. The phage was then used for the next round of panning. Normally, 3-4 rounds of panning are done, and 96 random clones are picked from rounds 3 and 4 and grown in 96-well cluster plates as a master stock.

**Phage ELISA Analysis**—40 μl of master stock was transferred from each master to another set of cluster tubes containing 400 μl of 2× YT-AG and helper phage (final concentration of 5x1010/ml). The tubes were incubated at 37 °C with constant shaking for 2 h. The cultures were centrifuged at 2500 x g at 4 °C for 20 min, the supernatant was discarded, and the bacterial pellet was resuspended in 400 μl of 2× YT-AK medium and incubated overnight at 37 °C. The cells were removed by centrifugation at 2500 x g, and the supernatants were used in ELISA assays. Each well of a Maxisorp plate (Nunc, Denmark) was coated with 100 μl of target (1 μg/ml) overnight at 4 °C. The wells were blocked with NFM-PBS for 1.5 h at room temperature. Phage was added at 100 μl/well, and the plates were incubated for 3 h at room temperature. After washing three times with PBS-Tween 20, plates were probed with an anti-M13 antibody and conjugated to horseradish peroxidase (HRP) (1:3000 in PBS-NFM) for 1 h at room temperature followed by an addition of 100 μl of ABTS for 15 min at room temperature. The OD was measured using a SpectraMax microplate spectrophotometer (Molecular Devices) at 405 nm after a 20-30-min incubation at room temperature. Positive binders were PCR-amplified using a CLONTECH Advantage PCR kit with the following primers, which are specific for the pCANTAB vector: R1, 5’-CCG GAT CCC TCT CCA GGA GAC CAG TTA CGG CCG ATT GCG C-3’; R2, 5’-GGT CCT TCT CCA GGA GAC CAG TTA CGG CCG ATT GCG C-3’; and T7-3, 5’-GAC TCT TCT CCA GGA GAC CAG TTA CGG CCG ATT GCG-3’. Sequencing was performed using standard protocols on an Applied Biosystems 373 DNA sequencer, and the translated sequences were aligned to determine the consensus regions.

### Second Generation of Phage Peptide Libraries

**Secondary and tertiary libraries were constructed essentially as previously described, except that the templates used in the PCR reaction had the appropriate peptide sequences. Amino acid mutations (5-20%) were introduced at the level of oligonucleotide synthesis, and libraries with >109 independent clones were consistently obtained.**

**Generation of the CP42/J101 Phage**—A phage display library, Ph.D-CTC, in which constrained nonapeptides are expressed at the amino terminus of protein III of the filamentous phage, was purchased from New England Biolabs (MA). Prior to biopanning, the IR was immobilized on 96-well Maxisorp microtiter plates (Nunc) coated with either protein A, insulin receptor antibody 18-44 (Neomarkers, CA), or hFc (Jackson ImmunoResearch, PA) (100 μg/ml at 1:100 dilution in 0.1 M NaHCO3, pH 9). The antibody was then left either overnight at 4 °C or for 1 h at room temperature. Subsequently, wells were blocked for 30 min at room temperature with 200 μl of binding buffer (100 mM Hepes, pH 8.0, 100 mM NaCl, 10 mM MgCl2, 0.25% bovine serum albumin (w/v), 0.025% (w/v) Triton X-100) containing 2% (w/v) defatted skim milk (blocking buffer). Wells were washed with binding buffer prior to biotinylated insulin receptor (100 μg/ml at 1 μg/ml in binding buffer) was added. Plates were then incubated for 2 h at room temperature. Two receptors were used: the soluble ectodomain of IR (sIR), which was truncated just before the transmembrane domain (14) that was immobilized by the 18-44 antibody, and IR-Fc, consisting of the ectodomain fused to the Fc domain of the human IgG heavy chain (15). Plates were probed with protein A fragment antibody. Biopanning of the IR was carried out following the manufacturer’s instructions. Briefly, 2x1010 plaque-forming units of the phage library (complexity, 2x109) was added to plates immobilized with IR for 2-4 h at room temperature. Unbound phage was washed away, and bound phage was eluted by lowering the pH. The eluted phage was amplified, and the process was repeated for four rounds.

Sequences that were represented by more than one clone were tested for IR binding using phage ELISAs in microtiter plates with immobilized sIR. Phages (5x1010 plaque forming units in 100 μl) were blocked in binding buffer and incubated for 2 h at room temperature. Negative controls included wells containing phages without inserts (i.e. the M13KE phage from Bio-Lab) as well as wells without immobilized sIR. After incubation, the wells were washed five times in 200 μl of binding buffer, incubated with 100 μl 1:5000 HRP-M13 conjugate (Amersham Biosciences) in blocking buffer for 30 min, washed with binding buffer, and then developed using an o-phenylenediamine dihydrochloride/sodium perborate solution (Sigma). The reaction was then stopped with H2SO4 and read at 492 nm.

**Solid Phase Peptide Synthesis**—Peptides were synthesized manually on a 490S peptide synthesizer. Solid phase synthesis was performed on TentaGel S Ram resin (Rapp Polymere, Tübingen, Germany). Fmoc (N-[(9-fluorenylethoxycarbonyl) amino] acids purchased from Novabiochem were coupled using a DIC/HOAt coupling strategy. Peptides were cleaved as amides from the resin using 90% trifluoroacetic acid, 5% trisopropylsilane, 3% thioanisol, and 2% phenol and precipitated by addition of diethylther followed by lyophilization. Disulfide bonds were formed in 1 M 2-mercaptoethanol for 2 days, and the product was lyophilized and purified by reverse phase high pressure liquid chromatography (HPLC). The identity of each peptide was confirmed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry.

**Radioreceptor Assays**—Receptors were incubated with 125I-labeled insulin or IGF-1 at various concentrations of peptide. Briefly, human IR or human IGF-1 was purified from transfected cells after solubilization with Triton X-100. The assay buffer contained 100 mM Hepes (pH 7.8), 100 mM NaCl, 10 mM MgCl2, 0.5% human serum albumin, 0.2% gamma globulin, and 0.025% Triton X-100. The receptor concentration was chosen to give 30-60% binding of 2000 cpm (3 pmol) of 125I-labeled ligand (125I) to Triton X-100, and a dilution series of the substance to be tested was added. After equilibration for 2 days at 4 °C, each sample (200 μl) was precipitated by the addition of 400 μl of 25% polyethylene glycol 6000, centrifuged, washed with 1 ml of 15% polyethylene glycol 6000, and counted in a gamma counter.

**Phosphorylation Assay**—Wheat germ agglutinin-purified recombinant human insulin receptor was mixed with either insulin or peptide tritium-labeled peptide at 100 μM and Triton X-100, 0.025% bovine serum albumin, and counted in a gamma counter. Following a 1-h incubation at room temperature, the reactions were stopped by the addition of 50 μl EDTA. The reactions were transferred streptavidin-coated 96-well microtiter plates (NUNC, 2 R. E. Brissette and N. I. Goldstein, unpublished data.)
cat. no. 236001) and incubated for 1 h at room temperature. The plates were washed three times with Tris-buffered saline (10 mM Tris, pH 8.0, 150 mM NaCl), and a 2000-fold dilution of HRP-conjugated phosphotyrosine antibody (Transduction Laboratories, cat. no. E120H) in Tris-buffered saline was added. The plates were incubated for 30 min and washed three times with Tris-buffered saline. TMB. One substrate from Kem-En-Tec (Denmark) was added, and the reaction was stopped with 1% H2SO4 after 15 min. The absorbance, representing the extent of substrate phosphorylation, was measured in a spectrophotometer at 450 nM.

Marine Fat Cell Assay—Epididymal fat pads were dissected from BDII male mice (Charles River, Germany) and minced. Then, adipocytes were prepared by collagenase degradation during shaking for 1.5 h, at 36 °C in 110 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, 25 mM Hepes, pH 7.9, 4% human serum albumin, 1.1 mM glucose, and 0.4 mg/ml collagenase type 1 (Worthington Biochemical Corp.). Cells were then filtered through two layers of gauze and centrifuged briefly at 500 g in 110 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, 25 mM Hepes, pH 7.9, 1% human serum albumin), and then washed twice (110 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, 25 mM Hepes, pH 7.9, 1% human serum albumin), and the absorbance, representing the extent of substrate phosphorylation, was measured in a spectrophotometer at 450 nM.

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Representative surrogate peptides binding to hotspots on the IR

| Peptide Library source<sup>a</sup> | Site<sup>b</sup> | Sequence | Affinity<sup>c</sup> |
|----------------------------------|---------------|----------|------------------|
| Motif 1: FY X WF                  | Secondary DGI | 1        | FHNENFYDWFVRQVS  | 700   |
| H2C-D117                        | Secondary DGI | 1        | GRVDWLRQANFYDWFVAELG | 230   |
| S175                            | Secondary DGI | 1        | KRGGGTFYEWFESALRKHAG | 840   |
| G3-D110                         | Primary DGI   | 1        | GSLEDJFYDFWERQLG  | 40    |
| RP9-S377                        | By design     | 1        | DLYKDFYDAIDQLVGRSARAGTRD | 250   |
| Motif 2: FXXX[L]XX[L]            | Primary DGI   | 1        | PLAELWAYFEHSEQRSSH | 16,000 |
| 20E2-D118                       | Secondary DGI | 1        | LDLALDRMLRFEERPSL | 1,200 |
| Motif 3: LXXLXXYF                | Primary DGI   | 1        | KHLCVLEELFWGASLFGYCSG | 1,000 |
| S174                            | Primary DGI   | 1        | KWLDEQEWAVQCEVGYGRGCP | 550   |
| S173                            | Secondary DGI | 1        | S175 Secondary DGI 1 LDALDRLMRYFEERPSL | 230   |
| Motif 4: long C-C loop           | Primary DGI   | 2        | S175 Secondary DGI 1 GRVDWLQRNANFYDWFVAELG | 250   |
| F8-D124                         | Primary DGI   | 2        | S175 Secondary DGI 1 FHENFYDFVRQVS | 700   |
| Motif 5: short C-C loop          | Primary DGI   | 2        | S175 Secondary DGI 1 FHENFYDFVRQVS | 700   |
| D8-D123                         | Primary DGI   | 2        | S175 Secondary DGI 1 FHENFYDFVRQVS | 700   |
| Motif 6: small cyclic J101       | Primary PhD-C7C| 3        | ACVWPTYWNCG       | 5,000 |

<sup>a</sup> The primary DGI libraries were constructed in-house and consisted of 20- or 40-mer random peptides. The secondary libraries were prepared as described (17). The Ph.D.-C7C library was purchased from New England Biolabs.

<sup>b</sup> Assignment of a surrogate to a specific site was determined using a competition ELISA assay. A specific phage is allowed to bind to the IR-Fc in the presence of a competing synthetic peptide representing the same or different sites. Binding of the phage is determined with anti-M13 antibody conjugated to HRP.

<sup>c</sup> IR was incubated with [125]labeled insulin at various concentrations of peptide, and the K<sub>i</sub> calculated.

Because of the relatively low affinity of most of the primary Site 1 peptides, secondary libraries were prepared using several doping strategies. Following panning, several secondary peptides were identified with IR/IGF-1R ratios of >3. One of the peptides, S175, was synthesized and shown to have an affinity of ~230 nM (Table I). Other secondary libraries were generated based on the H2C (motif 1) and 20E2 (motif 2) prototypes. Design of the libraries was such that it enabled us to detect preferences within these motifs as well as flanking amino- and carboxyl-terminal residues. Libraries were characterized by sequencing random clones (round 0) before panning. After three to four rounds of panning, clones were tested for binding to the IR-Fc. The positive clones were sequenced, and the predicted peptide sequences were analyzed for amino acid preferences. Based on codon usage and library design (doping scheme), the expected frequency of occurrence of each of the 20 amino acids was calculated at every single position within the peptide. This was compared with the actual occurrence within the library by calculating the frequency for each amino acid in round 0 clones. By comparing the frequency in round 3 and 4 clones with the calculated and actual occurrences within the library, we were able to determine the expected amino acid preferences. These preferences can range from strong (>2–5-fold over the expected) to weak (1.5–2-fold change). In certain instances, preference for a certain type of amino acid (e.g. charged, hydrophobic, etc.) at a given position, rather than one specific amino acid, was seen. Based on this analysis, one...
peptide (designated RP9-S371) was synthesized incorporating the amino acid preferences for individual positions as well as other considerations such as overall charge. RP9-S371 was found to bind to the IR with the highest affinity (40 nM) of any of our Site 1 peptides (Table I) and activates the receptor in the absence of insulin (Fig. 1A).

Several of the surrogates were tested for insulin-like activity in a standard murine fat cell assay (18). The effect of the peptides on the incorporation of [3H]glucose was determined, and the results were expressed as an increase relative to the full insulin response. Given the fact that all of our surrogates were considerably smaller than insulin and unable to interact at both Site 1 and Site 2, we were surprised to find that certain Site 1 peptides had full agonist activity. This is best shown by S175 (Fig. 2A) that showed a full agonist response in the fat cell assay with an ED$_{50}$ of 5 nM. Other Site 1 peptides acted as either full (e.g. H2C-D117) or partial (e.g. 20E2-D118) agonists with ED$_{50}$ values of approximately 20 nM. Surprisingly, RP9-S371 had weak activity in the fat cell assay despite the fact that its sequence was based on amino acid preferences at each position, and the peptide had a relatively high affinity. Site 2 peptides did not induce agonist activity in the fat cell assay, but D8-D123 was found to be an antagonist at a sub-maximal insulin dose, whereas F8-D124 had no biological activity in the assay (Fig. 2B). The Site 3 peptide, J101, was found to be an antagonist with an ED$_{50}$ of 4 nM. Interestingly, the Site 1 peptide G3-D110 was also found to be an antagonist. This suggests that the Site 1 core motif FXYWF is critical, but not sufficient, for agonism and that the flanking amino acids play a role in the biological activity of the peptides.

Several hundred peptides were tested in the fat cell assay, and several dozen were tested in the kinase assay. Figs. 1 and 2 show only a few representative peptides in the various groups. Many peptides had no effect at all or showed effects only at very high concentrations, thus providing the controls confirming that the results observed were not caused by non-specific effects of adding hydrophobic peptides to the assays.

**DISCUSSION**

This is the first report that utilizes phage display to simplify the complex interaction domains between insulin and the IR into the basic units (or hotspots) that are crucial for these protein-protein interactions. In addition, several peptides were identified that had either agonist or antagonist activity in an insulin-dependent cellular model. Several laboratories have isolated peptides that mimic the biological functions of protein hormones or growth factors. Wrighton et al. (19) and Livnah et al. (20) described the isolation of peptides binding to the erythropoietin (EPO) receptor with full agonist activity in vivo without significant sequence homology to the natural ligand. Importantly, x-ray crystallography revealed that one of these
peptides spontaneously formed non-covalent homodimers that allowed the dimerization of two EPO receptors. In addition, another group (21) isolated two families of small peptides binding to the human thrombopoietin receptor (TPOR) that competed with the binding of the natural ligand TPO. The one with the highest affinity was subsequently dimerized and shown to function as a TPO agonist in vivo. Although these reports indicate the ability to develop peptide mimetics, it is not clear if these molecules activate their receptors by binding to a hotspot or by simply inducing dimerization of two receptor subunits. Because the IR already exists in nature as a heterotetramer, activation must occur through a conformational change in the receptor rather than multimerization (10, 11).

Therefore, identification of the IR hotspots is critical for a complete understanding of the molecular events involved in insulin activation of its receptor.

We were able to identify at least two independent hotspots (Sites 1 and 2) on the IR. A third site was also found (marked by J101) that appears to lie close to, but not overlapping, Site 2. Analysis of the binding of J101 to various truncated receptors suggests that this peptide recognizes a region of the IR near, but outside of, Site 2 and may actually enhance the binding of Site 2 peptides. Phage/peptide competition studies showed that Sites 1 and 2 did not overlap and had different biological properties. A model for the hotspots within the IR binding site is shown as a cartoon in Fig. 3. Thus, while all surrogates competed for insulin binding to its receptor, agonist activity was only observed for certain Site 1 peptides. These results suggest that binding to Site 1 of the IR is required and sufficient for receptor activation, although it is not clear from the present study whether binding of one or two peptide molecules is required. The role of Site 2 (and Site 3), other than to provide additional contacts and increase binding affinity, is less clear. One possibility is that Site 2 may mark the hotspot on the IR that is responsible for insulin potency and selectivity based on the fact that Site 2 peptides are IR specific and do not bind to the closely related IGF-IR.

None of the peptides identified in this study had any sequence homology with insulin, but there was a significant number of important hydrophobic residues found in the various motifs. This is consistent with the notion that most of the residues that constitute the binding sites on the surface of insulin are also hydrophobic. However, there is no evidence in the present study that the contact points between the peptides and the receptor must be identical to the contacts used by insulin. There is evidence only that they appear to be close enough in space to give rise to binding competition. Similar results were observed with peptide mimetics binding to the EPO receptor (19, 20).

In the present model of IR activation (5), the receptor exists as a heterotetramer where insulin is not required to induce dimerization but induces its agonist effect by cross-linking separate binding sites in the two adjacent subunits of the heterotetramer. Unless peptides such as S175 induce unexpected conformational effects, agonism by Site 1 surrogates would not appear to require such cross-linking and most likely occurs via a mechanism different from that of insulin. One possibility could be that receptor activation requires binding of two peptide molecules. It is intriguing to ask whether or not such differences might allow insulin-desensitized receptors or even non-functional mutated receptors to be activated by the surrogate peptides. Studies to elucidate the molecular mechanism of activation of the IR by the surrogates are currently ongoing. Also, a natural extension of the work described here would be to use the peptides identified to create homodimers and heterodimers in order to investigate whether a more insulin-like activation mechanism can be achieved.

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