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# Insulin–Insulin-like Growth Factors Hybrids as Molecular Probes of Hormone:Receptor Binding Specificity

Květoslava Křížková,<sup>†,‡</sup> Martina Chrudinová,<sup>†,‡</sup> Anna Povalová,<sup>†,‡</sup> Irena Selicharová,<sup>†</sup> Michaela Collinsová,<sup>†</sup> Václav Vaněk,<sup>†</sup> Andrzej M. Brzozowski,<sup>§</sup> Jiří Jiráček,<sup>†</sup> and Lenka Žáková<sup>\*,†</sup>

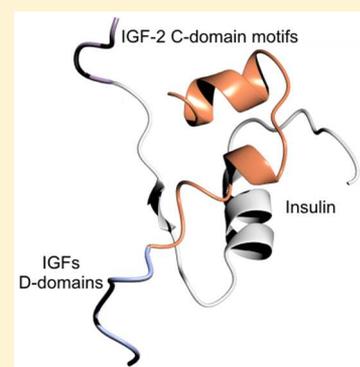
<sup>†</sup>Institute of Organic Chemistry and Biochemistry, Academy of Science of the Czech Republic v.v.i., Flemingovo nám. 2, 166 10 Praha 6, Czech Republic

<sup>‡</sup>Charles University in Prague, Faculty of Science, Department of Biochemistry, Hlavova 8, 128 43 Praha 2, Czech Republic

<sup>§</sup>York Structural Biology Laboratory, Department of Chemistry, University of York, Heslington, York YO10 5DD, United Kingdom

## Supporting Information

**ABSTRACT:** Insulin, insulin-like growth factors 1 and 2 (IGF-1 and -2, respectively), and their receptors (IR and IGF-1R) are the key elements of a complex hormonal system that is essential for the development and functioning of humans. The C and D domains of IGFs (absent in insulin) likely play important roles in the differential binding of IGF-1 and -2 to IGF-1R and to the isoforms of IR (IR-A and IR-B) and specific activation of these receptors. Here, we attempted to probe the impact of IGF-1 and IGF-2 D domains (D<sub>I</sub> and D<sub>II</sub>, respectively) and the IGF-2 C domain (C<sub>II</sub>) on the receptor specificity of these hormones. For this, we made two types of insulin hybrid analogues: (i) with the C-terminus of the insulin A chain extended by the amino acids from the D<sub>I</sub> and D<sub>II</sub> domains and (ii) with the C-terminus of the insulin B chain extended by some amino acids derived from the C<sub>II</sub> domain. The receptor binding affinities of these analogues and their receptor autophosphorylation potentials were characterized. Our results indicate that the D<sub>I</sub> domain has a more negative impact than the D<sub>II</sub> domain does on binding to IR, and that the D<sub>I</sub> domain Pro-Leu-Lys residues are important factors for a different IR-A versus IR-B binding affinity of IGF-1. We also showed that the additions of amino acids that partially “mimic” the C<sub>II</sub> domain, to the C-terminus of the insulin B chain, change the binding and autophosphorylation specificity of insulin in favor of the “metabolic” IR-B isoform. This opens new venues for rational enhancement of insulin IR-B specificity by modifications beyond the C-terminus of its B chain.



Insulin, insulin-like growth factors 1 and 2 (IGF-1 and -2, respectively), and their cognate cell surface receptors, together with IGF-binding proteins (IGFBP-1–6), form a complex hormonal/signaling system that is essential for the development and functioning of humans. Its deregulation leads to increased cancer risk, diabetes mellitus type 1 and 2, and other disorders, such as obesity and metabolic syndrome.<sup>1,2</sup>

Insulin is a small (51 amino acids) protein hormone that is the main regulator of glucose homeostasis. The mature insulin is the post-translational product of a single-chain (pre)-proinsulin, where the C peptide connecting A and B chains is proteolytically, and specifically, cleaved off. This results in a two-chain (A1–A21 and B1–B30) hormone, with two interchain disulfide bridges (A7–B7 and A20–B19) and one intrachain disulfide bridge (A6–A11) (Figure 1). The overall tertiary structure, chain organization, and arrangement of the disulfide bridges are conserved in members of the insulin-like family such as IGFs, relaxins, bombyxins, insulin-like peptides, etc.<sup>3</sup>

In contrast to insulin, IGF-1 and IGF-2 are single-chain hormones (70 and 67 amino acids, respectively) involved mainly in cell growth and protection against apoptosis.<sup>4,5</sup> Both IGFs share a high degree of sequence homology, which is also

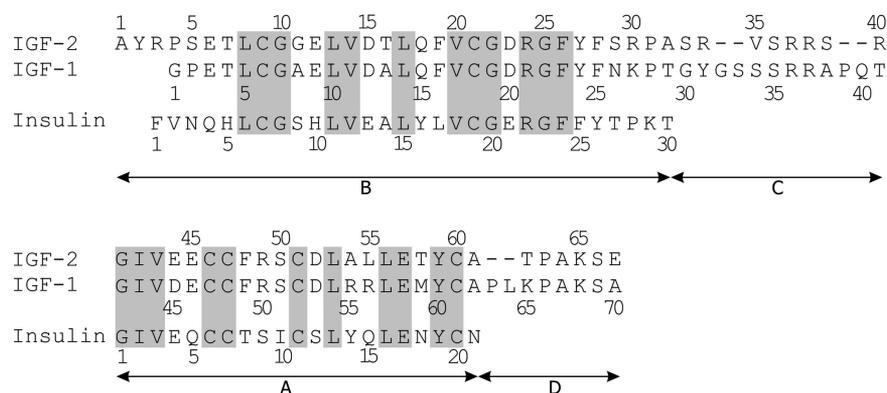
extended to insulin (see Figure 1). They consist of four domains, B, C, A, and D, with their B and A domains corresponding to the B and A chains of insulin. C segments of IGFs that span the B and A domains are structural analogues of insulin C peptide, but without sequence similarity. The unique D domains of IGFs [without equivalents in (pro)insulin] extend the C-terminus of the A domains (Figure 1).

Insulin and IGFs elicit their biological effects by binding to the insulin receptor isoforms (IR-A and IR-B), and the insulin-like growth factor receptor 1 (IGF-1R), while circulating IGF-binding proteins modulate bioavailability of both IGFs.<sup>6</sup> Moreover, IGF-2 binds specifically to the distinct insulin-like growth factor receptor 2 (IGF-2R), which is a cation-independent mannose 6-phosphate receptor that is likely responsible for the clearance of IGF-2 from the cell surface and for the preclusion of IGF-2:IGF-1R/IR interactions.<sup>4</sup> The IR and IGF-1R receptors are transmembrane glycoproteins consisting of two  $\alpha$  and two  $\beta$  subunits, which are connected by

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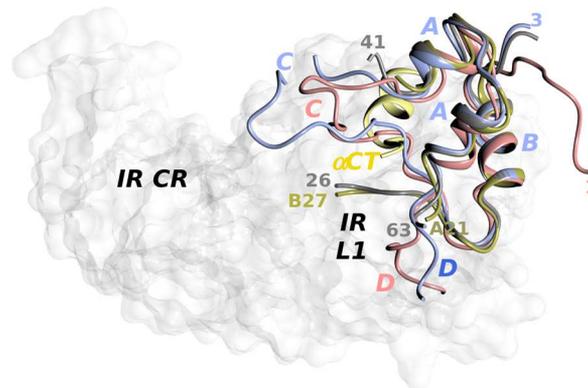


**Figure 1.** Alignment of primary sequences of human IGF-1, IGF-2, and insulin. The gray background highlights evolutionarily conserved residues. Arrows indicate the residues associated with the A–D domains of IGFs and the A and B chains of insulin.

disulfide bridges to create ( $\alpha\beta$ )<sub>2</sub> dimers. The extracellular  $\alpha$  parts of the IR contain hormone-binding regions, and the membrane-anchored parts  $\beta$  contain intracellular tyrosine kinase domains.<sup>7–9</sup> The functional heterogeneity of IR arises from an alternative splicing of exon 11 located at the C-terminus of the  $\alpha$  subunit, which results in two isoforms, IR-B and IR-A, with different C-terminal  $\alpha$ CT peptides (IR-B and IR-A,  $\pm 12$  amino acids). The IR isoforms are expressed in a tissue-specific manner in humans. IR-B is the main IR form for insulin glycemic response-sensitive tissues (liver, muscles, and adipose tissue), while IR-A is a dominant IR isoform in the brain.<sup>10–12</sup> Insulin and IGF-1 bind preferentially to their cognate receptors (IR-A/IR-B and IGF-1R, respectively) at subnanomolar concentrations. However, both hormones can also cross-bind to their receptors (but with significantly lower affinities), with the exception of IGF-2 that binds with relatively high affinity to both IR-A and IGF-1R.<sup>13–15</sup>

Simultaneous engagement of two, distinct hormone-binding sites, so-called site 1 and site 2, on insulin and IGF receptors is required for the high-affinity binding complex.<sup>16</sup> The recent crystallographic studies showed that site 1 on both IR and IGF-1R receptors is similar, involving some L1 domain surface and the  $\alpha$ CT peptide, which interact with the respective binding sites 1 on insulin and IGFs (in A and B chains/domains)<sup>17,18</sup> or IGF-1.<sup>19</sup> However, the nature of IR/IGF-1R-binding site 2 is still understood only on the basis of mutagenesis studies.<sup>20–23</sup>

Although there is now relatively good insight into the roles of A and B chains/domains of insulin and IGF-1 in the hormone:IR/IGF-1R site 1 interactions, the functional impact of the C and D domains of IGFs is much less understood. It is envisaged, however, that they play some role in a differential binding of IGF-1 and -2 to IR-A, IR-B, and IGF-1R, and activation.<sup>24,25</sup> The studies of the functional importance of the IGF-1 C domain (C<sub>I</sub> domain), investigated in the context of different constructs of insulin,<sup>26,27</sup> IGF-1,<sup>25,28</sup> and IGF-2,<sup>24</sup> revealed that it may play an important role in the IGF-1:IGF-1R interaction, and in eliciting biological activity of this hormone. However, it is detrimental to IGF-1:IR binding. It was also proposed that the C<sub>I</sub> domain may interact with the CR domain of the IGF-1R,<sup>29,30</sup> but the recent crystal structure of the complex of IGF-1 with IR L1-CR domains, mediated by the IGF-1R  $\alpha$ CT segment<sup>19</sup> (Figure 2), did not yet clarify contacts of the C and D domains with the receptor. In contrast to IGF-1, the interaction of the shorter C domain of IGF-2 (C<sub>II</sub>) with the CR domain is not anticipated.<sup>31,32</sup> Although the role of the C<sub>II</sub> domain has been much less studied, it has been shown that

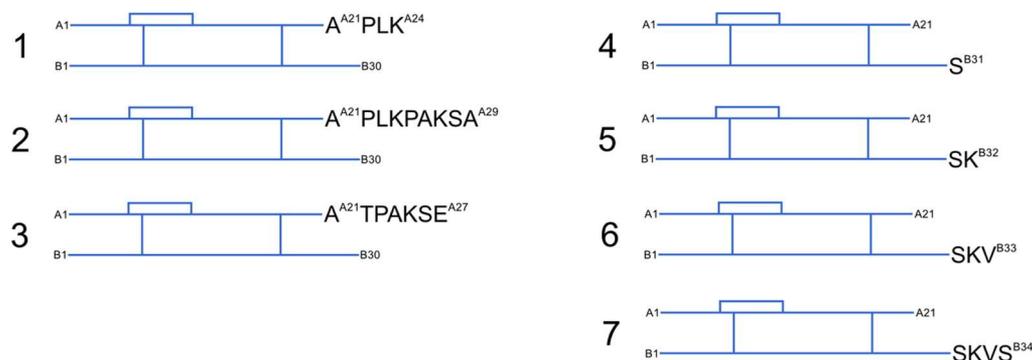


**Figure 2.** Structures of insulin, IGF-1, and IGF-2 on the insulin receptor L1-CR domains. All individual hormone structures have been superimposed on the crystal structure of the IR L1-CR/IGF-1/IGF-1R  $\alpha$ CT-peptide complex (PDB entry 4xss), with the IR as the white surface, IGF-1 in the 4xss complex colored dark gray, and the  $\alpha$ CT peptide colored bright yellow. The insulin molecule (gold/dark yellow) was derived from the homologous IR L1-CR/insulin/1R  $\alpha$ CT-peptide structure (PDB entry 4oga). Free, noncomplexed IGF-1 [blue, PDB entry 1gzz (to show C and D domains, not defined in the 4xss structure)], IGF-2 [pink, PDB entry 1igl (NMR model 1)], and insulin were superimposed on the 8–18 C $\alpha$  atoms of the B domain  $\alpha$  helix in the IGF-1 4xss complex. There is a 36–38 gap observed in the free IGF-1 (1gzz) structure. A–D denote domains in these hormones (color coding as in the individual molecules). Numbers assist terminal residues seen in the individual structures. The conformations of the C and D domains on the IR are putative, i.e., unchanged from their noncomplexed structure, hence their clash with the IGF-1R  $\alpha$ CT peptide.

the C<sub>I</sub>  $\rightarrow$  C<sub>II</sub> swap in IGF-1 doubled the binding affinities of this IGF-1 analogue for IR-A and IR-B but diminished its binding affinity for IGF-1R to  $\sim 25\%$ .<sup>24</sup>

The role of D<sub>I</sub> and D<sub>II</sub> domains has been studied by several groups,<sup>24,33,34</sup> which showed that both D<sub>I</sub> and D<sub>II</sub> domains play some roles in the activation of IR and IGF-1R, but they are possibly less important in this process than the C domains.

Although the receptor-binding surfaces of insulin and both IGFs have a similar side chain pattern,<sup>7,21,23,35</sup> it is assumed that each molecule uses a slightly different IR-A, IR-B, or IGF-1R binding mechanism,<sup>32</sup> triggering subsequently specific signaling cascades.<sup>14,36,37</sup> Moreover, the differential binding of insulin and IGFs to so-called hybrid receptors (receptor heterodimers formed by IR-A/B and IR-A/IGF-1R  $\alpha\beta$  subunits)<sup>38,39</sup> brings even more complexity to the IGF/insulin system.



**Figure 3.** Schematic representation of structures of insulin analogues 1–7 prepared in this study. Insulin A and B chains and disulfide bridges are shown as blue lines. The additional residues derived from the D<sub>I</sub>, D<sub>II</sub>, or C<sub>II</sub> domains are drawn in single-letter codes, with insulin numbering.

Here, we aimed to elucidate further the roles of the D<sub>I</sub>, D<sub>II</sub>, and C<sub>II</sub> domains in the specific functionality of these hormones: their binding to IR-A, IR-B, and IGF-1R and their impact on the autophosphorylation of these receptors. The human insulin molecule was selected here as a template, as it has a high affinity for both IR isoforms; hence, it is much more sensitive probe against these receptors. To address these issues, we made (i) insulin analogues with the C-terminus of the A chain extended by amino acids from the D domains of IGF-1 and IGF-2 and (ii) insulin analogues with the C-terminus of the B chain extended by amino acids mimicking the C<sub>II</sub> domain (Figure 3). Despite a single-chain organization of IGF-1, the structure of the IGF-1/IGF-1  $\alpha$ -CT/IR L1-CR complex<sup>19</sup> showed that the conformation of IGF-1 B domain residues 21–26 is almost identical to the structure of equivalent insulin B22–B27 residues on the same receptor (Figure 2). This suggests that at least the first residues of the C<sub>I</sub> domain (invisible in this complex) can follow the direction of the end part of the B domain. As the IGF-2 and IGF-1 IR binding modes should be similar, the insulin molecule can be considered as a useful structural scaffold for the study of the role of C<sub>II</sub> residues in binding of hormones to receptors. Receptor binding affinities of all analogues and their abilities to stimulate autophosphorylation of the receptors were characterized, to correlate the impact of the hormones' modifications on their receptor specificity.

## EXPERIMENTAL PROCEDURES

**Synthesis of Analogues 1–3. Synthesis of Insulin Chains.** The individual modified A chains and wild-type B chain were prepared by total chemical solid-phase synthesis, and SH groups of cysteines were converted to S-sulfonates as previously described.<sup>40,41</sup> Briefly, Wang resins preloaded with Fmoc amino acids (Novabiochem-Merck) were used to synthesize the human insulin B chain and modified insulin A chains (A<sup>A21</sup>-T-P-A-K-S-E<sup>A27</sup>-, A<sup>A21</sup>-P-L-K-P-A-K-S-A<sup>A29</sup>-, and A<sup>A21</sup>-P-L-K<sup>A24</sup>-A chains) on an automatic solid-phase synthesizer (ABI 433A, Applied Biosystems, Foster City, CA). The used coupling reagents were HBTU/HOBt in DMF. The Cys, His, Gln, and Asn side chains were protected with Trt. The side chains of Tyr, Asp, Glu, Ser, and Thr were protected with tBu, and the lysine side amino group was protected with Boc. Peptides were cleaved from the resin with a TFA/H<sub>2</sub>O/thioanisole/EDT/phenol/TIS mixture (92/2.2/2.2/1/2.2/0.4) and precipitated from cold Et<sub>2</sub>O. Crude chains (100  $\mu$ mol) were dissolved and stirred in 25 mL of sulfitolysis buffer [100 mM Tris, 250 mM Na<sub>2</sub>SO<sub>3</sub>, 80 mM Na<sub>2</sub>S<sub>4</sub>O<sub>6</sub>, and 7

M guanidine hydrochloride (pH 8.6)] for 3 h at room temperature (RT). The chains were desalted on a Sephadex G-10 column (4 cm  $\times$  85 cm) in 50 mM NH<sub>4</sub>HCO<sub>3</sub> and purified using reverse-phase high-performance liquid chromatography (RP-HPLC) (Nucleosil C18 column, 250 mm  $\times$  21 mm, 5  $\mu$ m).

**Recombination of Insulin Chains.** The method for the formation of disulfide bonds in insulin analogues has been described previously in detail.<sup>40,41</sup> Briefly, S-sulfonate derivatives of the insulin A chain (30 mg) and B chain (15 mg) were dissolved in 2 and 1 mL of degassed 0.1 M Gly/NaOH buffer (pH 10.5), respectively. The exact molar concentration of each chain was determined by UV spectroscopy at 280 nm using molar extinction coefficients of 3480 and 3230 M<sup>-1</sup> cm<sup>-1</sup> for the A and B chains, respectively. Dithiothreitol (aliquots from Pierce, catalog no. 20291) was added rapidly to the mixture of both chains to give an SH/SSO<sub>3</sub> molar ratio of 1.1/1. The mixture was stirred in a capped vessel for 30–45 min at RT. After the reduction, aerated 0.1 M Gly/NaOH (pH 10.5) buffer was added to a final 2/3 ratio of degassed and aerated buffers. The resulting solution was stirred for an additional 48 h at 4 °C in an open vessel to permit air oxidation.<sup>42</sup> Glacial acetic acid (4 mL) was added to the mixture to terminate the reaction. The resulting mixture was applied to a low-pressure column (Sephadex G-50 in 1 M acetic acid, 2 cm  $\times$  75 cm). The fractions containing analogues were purified using RP-HPLC (Nucleosil C18 column, 250 mm  $\times$  8 mm, 5  $\mu$ m). The molecular weight of products was confirmed by a HR mass spectroscopy instrument (LTQ, Orbitrap XL, Thermo Fisher Scientific, Waltham, MA). The purity of the analogues was analyzed by RP-HPLC (Nucleosil C18 column, 250 mm  $\times$  4.6 mm, 5  $\mu$ m) and was >95%.

**Synthesis of Analogues 4–7. Synthesis of Peptide Precursors.** The G-F-F-Y-T-P-K(Pac)-T-S and G-F-F-Y-T-P-K(Pac)-T-S-K-V-S peptides were synthesized manually by a stepwise coupling of the corresponding Fmoc-protected amino acid on a 2-chlorotrityl resin using HBTU/DIPEA in 1-methyl-2-pyrrolidinone (NMP). The completeness of the reaction was controlled by a Kaiser test and quantified by measuring the absorbance of the piperidine–dibenzofulvene complex after Fmoc group deprotection. Synthesized peptides were cleaved from the resin with a DCM/AcOH/trifluoroethanol mixture (6/2/2) for 2 h at RT. The residues were evaporated to dryness and treated with a DCM/TFA/TIS/H<sub>2</sub>O mixture (44/50/3/3) for 2 h at RT. The mixture was evaporated *in vacuo* and treated with diethyl ether. The solid residue after the diethyl ether

extraction was dissolved in 40% acetonitrile in water with 0.1% TFA and purified using RP-HPLC.

G-F-F-Y-T-P-K(Pac)T-S-K(Pac)V and G-F-F-Y-T-P-K(Pac)-T-S-K(Pac) peptides were synthesized using an automatic solid-phase synthesizer (ABI 433A, Applied Biosystems) by a similar method described above but using 2-chlorotrityl resin.

**Enzymatic Semisynthesis.** Analogues 4–7 were prepared by the enzymatic semisynthesis starting from *des*(B23–B30)-octapeptide-insulin (DOI) and respective peptides. Analogue 4 was prepared according to the previously described protocol.<sup>43,44</sup> Semisyntheses of analogues 5–7 were performed according to the slightly different protocol published by Nakagawa and Tager<sup>45</sup> because of the lower solubility of their precursor peptides. Briefly, a peptide (30 mM) and DOI (7.7 mM) were dissolved in an *N,N*-dimethylacetamide (DMA)/1,4-butanediol/0.2 M Tris-HCl mixture (pH 8.0) (7/7/6), supplemented with 10 mM Ca(Ac)<sub>2</sub> and 1 mM EDTA in a total volume of 400  $\mu$ L. The reaction was initiated by the addition of 2 mg of TPCK-treated trypsin. The pH of the solution was adjusted to 6.9–7.0 by *N*-methylmorpholine. The reaction mixture was incubated at 37 °C and monitored by RP-HPLC (Nucleosil 120-5 C-18 column, 250 mm  $\times$  4.6 mm). After 24–48 h, the reaction was stopped by the addition of acetone and the product was isolated by RP-HPLC (Nucleosil C18 column, 250 mm  $\times$  8 mm). The molecular weight was confirmed by HR mass spectroscopy (LTQ, Orbitrap XL, Thermo Fisher Scientific).

**Enzymatic Deprotection of Precursors of Insulin Analogues.** The enzymatic deprotection of Pac-protected analogues was performed according to the previously described protocol.<sup>43,46</sup> The respective precursor of an insulin analogue (4, 5, 6, or 7,  $\sim$ 1 mg of each) with a phenylacetyl protecting group (Pac) on N<sup>ε</sup> groups of lysine(s) was dissolved in 1 mL of 50 mM potassium phosphate buffer (pH 7.5). Protease inhibitor cocktail (5  $\mu$ L, Sigma-Aldrich, catalog no. P8465) was added. The reaction was initiated by the addition of soluble penicillin amidohydrolase (PA) and monitored via RP-HPLC (Nucleosil C18 column, 250 mm  $\times$  4 mm). The reaction proceeded at 37 °C. After 16 h, an extra amount of PA was added. After completion of the deprotection ( $\sim$ 28 h), an analogue was purified by RP-HPLC as described above. The molecular weight was confirmed by HR mass spectroscopy (LTQ, Orbitrap XL, Thermo Fisher Scientific). The purity (>96%) of analogues 4–7 was verified by RP-HPLC.

**Cell Cultures.** IM-9 cells (ATCC) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin in humidified air with 5% CO<sub>2</sub> at 37 °C.

Mouse embryonic fibroblasts used for binding and signaling were derived from animals with targeted disruption of the IGF-1 receptor gene<sup>47</sup> and stably transfected with expression vectors containing either A (R<sup>-</sup>/IR-A) or B (R<sup>-</sup>/IR-B) isoforms of human insulin receptor or human IGF-1 receptor (R<sup>+</sup>).<sup>14,48</sup> The cell lines were kindly provided by A. Belfiore (University of Magna Graecia, Catanzaro, Italy) and R. Baserga (Thomas Jefferson University, Philadelphia, PA). Cells were grown in DMEM medium with 5 mM glucose (Biosera) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.3  $\mu$ g/mL puromycin, 100 units/mL penicillin, and 100  $\mu$ g/mL streptomycin in humidified air with 5% CO<sub>2</sub> at 37 °C.

**Receptor Binding Studies.** *Human IM-9 Lymphocytes (human IR-A isoform).* Receptor binding studies with the insulin receptor in membranes of human IM-9 lymphocytes

(containing only the human IR-A isoform) were performed and K<sub>d</sub> values determined according to the procedure described recently in detail by Morcavallo et al.<sup>13</sup> Binding data were analyzed using the Excel algorithms specifically developed for the IM-9 cell system in the laboratory of P. De Meyts (A. V. Groth and R. M. Shymko, Hagedorn Research Institute, Gentofte, Denmark, a kind gift of P. De Meyts) using a method of nonlinear regression and a one-site fitting program and taking into account potential depletion of free ligand. Each binding curve was determined in duplicate, and the final dissociation constant (K<sub>d</sub>) of an analogue was calculated from at least three ( $n \geq 3$ ) binding curves (K<sub>d</sub> values) determined independently. The dissociation constant of human <sup>125</sup>I-labeled insulin (PerkinElmer) was set to 0.3 nM.

*Mouse Embryonic Fibroblasts (human IR-B isoform).* Receptor binding studies with the insulin receptor in membranes of mouse embryonic fibroblasts derived from IGF-1 receptor knockout mice that solely expressed the human IR-B isoform were performed as described in detail previously.<sup>40,49</sup> Binding data were analyzed, and the dissociation constant (K<sub>d</sub>) was determined with GraphPad Prism 5 using a method of nonlinear regression and a one-site fitting program and taking into account potential depletion of free ligand. Each binding curve was determined in duplicate, and the final dissociation constant (K<sub>d</sub>) of each analogue was calculated from at least three ( $n \geq 3$ ) binding curves (K<sub>d</sub> values) determined independently. The dissociation constant of human <sup>125</sup>I-labeled insulin (PerkinElmer) was set to 0.3 nM.

*Mouse Embryonic Fibroblasts (human IGF-1R).* Receptor binding studies with the IGF-1 receptor in membranes of mouse embryonic fibroblasts derived from IGF-1R knockout mice and transfected with human IGF-1R were performed as described previously.<sup>40</sup> Binding data were analyzed and the dissociation constants determined by the same method that was used for IR-B. The dissociation constant of human [<sup>125</sup>I]IGF-1 (PerkinElmer) was set to 0.2 nM. Here we should note that the use of bovine serum albumin in the binding buffer (e.g., Sigma-Aldrich A6003) void of “IGF-binding-like” proteins, which interfere with the binding assay, is essential.<sup>50</sup>

The significance of the changes in binding affinities of the analogues, related to the insulin binding for all types of receptors, was calculated using a two-tailed *t* test.

**Stimulation of Cells.** Cells (cell lines R<sup>+</sup>, R<sup>-</sup>/IR-A, and R<sup>-</sup>/IR-B) were seeded in 24-well plates (4  $\times$  10<sup>4</sup> cells per well) in 300  $\mu$ L of DMEM and incubated for 24 h. Cells were afterward starved for 4 h in serum-free medium. A ligand (insulin, IGF-1, IGF-2, or an analogue) at final concentrations of 10<sup>-8</sup> M was added to the medium in each well for 10 min. The reaction was terminated by removal of the medium, and the mixture was washed with ice-cold 0.9% NaCl and snap frozen until the next manipulation. The series of ligands were tested four times using different batches of cells.

**Immunoblotting.** Cells were lysed in 50  $\mu$ L of lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS (w/v), 10% glycerol (v/v), 0.01% Bromphenol Blue (w/v), 0.1 M DTT (w/v), 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 0.5% protease inhibitor cocktail (Sigma-Aldrich) by sonication. Proteins were routinely analyzed using immunoblotting and horseradish peroxidase-labeled secondary antibodies (Sigma-Aldrich). Cell extracts (10  $\mu$ L containing 10  $\pm$  0.8  $\mu$ g of proteins) were separated on 10% SDS–polyacrylamide gels and electroblotted to a PVDF membrane. The membranes were probed with the following antibodies: anti-phospho-IGF-1R  $\beta$  subunit (Tyr1131)/IR $\beta$

Table 1. Receptor Binding Affinities of Human Insulin, IGF-1, IGF-2, and the Insulin Analogues Reported in This Work

analogue	$K_d \pm SE$ (nM) ( <i>n</i> ) for human IR-A in IM-9 lymphocytes	relative binding affinity <sup>a</sup> for human IR-A (%)	$K_d \pm SE$ (nM) ( <i>n</i> ) for human IR-B in mouse fibroblasts	relative binding affinity for human IR-B (%)	$K_d \pm SE$ (nM) ( <i>n</i> ) for human IGF-1R in mouse fibroblasts	relative binding affinity for human IGF-1R (%)	
human insulin <sup>b</sup>	0.55 ± 0.04 (7) <sup>1</sup> 0.36 ± 0.06 (5) <sup>2</sup> 0.43 ± 0.00 (5) <sup>3</sup> 0.25 ± 0.02 (5) <sup>4</sup>	100	0.67 ± 0.17 (4)	100 ± 25	292 ± 31 (3) <sup>c</sup>	100 ± 11	0.08 ± 0.01
A <sup>A21</sup> PLK <sup>A24</sup> -insulin (1)	1.19 ± 0.08*** (3) <sup>2</sup>	30.3 ± 2.0	2.51 ± 0.63*** (4)	26.7 ± 6.7	877 ± 378* (3)	33.3 ± 14.4	0.03 ± 0.01
A <sup>A21</sup> PLKPAKSA <sup>A29</sup> -insulin (2)	1.44 ± 0.06*** (3) <sup>2</sup>	25.0 ± 1.0	2.51 ± 0.36*** (4)	26.7 ± 3.8	274 ± 29 (3)	107 ± 11	0.09 ± 0.01
A <sup>A21</sup> TPAKSE <sup>A27</sup> -insulin (3)	0.51 ± 0.04* (3) <sup>3</sup>	84.3 ± 6.6	1.26 ± 0.23*** (4)	53.2 ± 9.7	124 ± 22* (3)	235 ± 42	0.19 ± 0.03
S <sup>B31</sup> -insulin (4)	0.48 ± 0.01*** (3) <sup>4</sup>	52.1 ± 1.1	0.56 ± 0.11 (3)	120 ± 24	280 ± 41 (3)	104 ± 15	0.09 ± 0.01
S <sup>B31</sup> K <sup>B32</sup> -insulin (5)	1.28 ± 0.18*** (3) <sup>1</sup>	43.0 ± 6.0	2.03 ± 0.36*** (3)	33.0 ± 5.9	257 ± 16 (2) <sup>d</sup>	114 ± 7	0.09 ± 0.01
S <sup>B31</sup> KV <sup>B33</sup> -insulin (6)	0.41 ± 0.02 (3) <sup>2</sup>	87.8 ± 4.3	0.61 ± 0.07 (3)	110 ± 13	195 ± 33 (3)	150 ± 25	0.12 ± 0.02
S <sup>B31</sup> KV <sup>S</sup> B <sup>B34</sup> -insulin (7)	0.57 ± 0.19 (3) <sup>3</sup>	75.4 ± 25.1	0.45 ± 0.13* (4)	149 ± 43	234 ± 99 (2) <sup>d</sup>	125 ± 53	0.10 ± 0.03
human IGF-1	23.8 ± 6.6*** (3) <sup>4</sup>	1.1 ± 0.3	224 ± 16*** (4)	0.30 ± 0.02	0.24 ± 0.05*** (5) <sup>c</sup>	1217 ± 254	100 ± 21
human IGF-2	2.92 ± 0.14*** (3) <sup>4</sup>	8.6 ± 0.4	35.5 ± 5.6*** (4)	1.9 ± 0.3	2.32 ± 0.72*** (3)	126 ± 39	10.3 ± 3.2

<sup>a</sup>The relative receptor binding affinity (potency) is defined as ( $K_d$  of human insulin or IGF/ $K_d$  of analogue) × 100. <sup>b</sup>The  $K_d$  of human insulin for IR-A was determined in four independent measurements (1–4). The individual values of  $K_d$  of insulin analogues are relative to one of these  $K_d$  values of human insulin (e.g., 1 to 1, etc.). <sup>c</sup>From ref 61. <sup>d</sup>The  $K_d$  value represents the mean of two independent measurements ± range. Asterisks indicate that binding of the ligand to a particular receptor differs significantly from that of insulin (\* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001).

(Tyr1146), (Cell Signaling Technology), anti-human IR  $\beta$  subunit (Invitrogen), and anti-IGF-1R  $\beta$  subunit (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were developed using the SuperSignal West Femto maximum sensitivity substrate (Pierce) and analyzed using the ChemiDoc MP Imaging System (Bio-Rad). The signal density generated by each ligand in a particular experiment was expressed as the contribution of phosphorylation relative to the respective human insulin (R<sup>-</sup>/IR-A and R<sup>-</sup>/IR-B) IGF-1 (R<sup>+39</sup>) signal in the same experiment. Mean ± standard error of the mean (SEM) values were calculated from four independent experiments. The significance of the changes in stimulation of autophosphorylation related to the stimulation by insulin was calculated using one-way analysis of variance.

## RESULTS

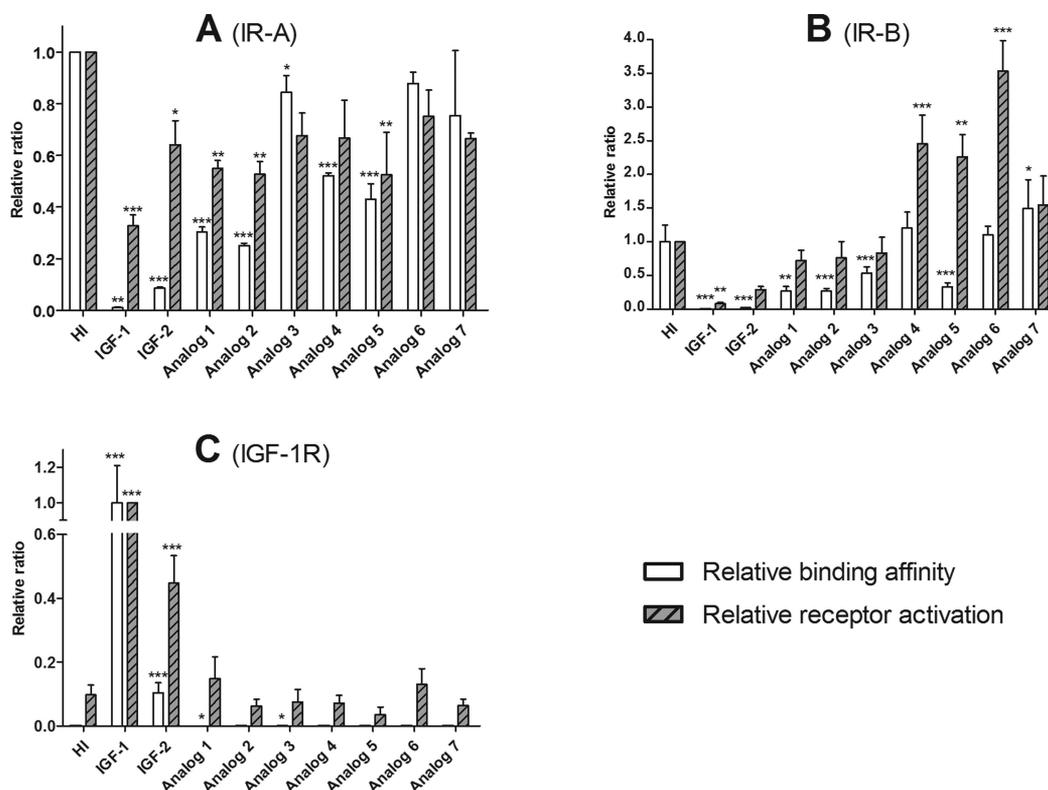
**Design of the Analogues.** The first three insulin analogues [1–3 (Figure 3)] have the C-terminus of the A chain extended by amino acids from the N-terminal parts of the IGF's D domains. In analogue 1, the D<sub>I</sub> domain is represented only by its first three P-L-K amino acids, which are an "insert" (or addition) to the D<sub>II</sub> domain (Figure 1); hence, they can be considered as the D<sub>I</sub> domain "unique" feature. Here, we were interested in their "isolated" (i.e., purely D<sub>I</sub>-like "signature") effect on binding of these hormones. Analogues 2 and 3 contain the entire D domains of IGF-1 and IGF-2, respectively. Additionally, insulin's A chain C-terminal Asn<sup>A21</sup> has been substituted in analogues 1–3 with alanine that is present in both IGFs at that site; hence, the A21 site could be considered here as part of the D domains.

The studies of the effect of the C<sub>II</sub> domain on insulin functionality were originally planned on hormone analogues containing the full (or large fragments of) C<sub>II</sub> domain, which would be enzymatically introduced into *des*(B23–B30)-octapeptide-insulin (DOI), as the extensions of insulin C-terminal octapeptide B22–B30.<sup>43</sup> An alternative recombination

of the already extended B chain with the A chain was also tried as described previously.<sup>40,41</sup> However, all these attempts were unsuccessful (see below); hence, for synthetic reasons, only the first four amino acids of the C<sub>II</sub> domain (with arginines also replaced with lysines) were added after B30. This work resulted in analogues 4–7 (Figure 3).

**Synthesis of the Analogues.** Analogues 1–3 were prepared by the total chemical synthesis and chain recombination (folding) of insulin A and B chain S-sulfonates.<sup>40,41</sup> Addition of amino acids from the D domains of IGFs to the C-terminus of insulin A chain resulted in reduced yields of chain recombination (2–5%), in comparison with the average recombination efficiencies of native insulin (~8–12%).<sup>40</sup> Analogue 3 (with the D<sub>II</sub> domain) was obtained with a yield (5.4%) better than those of analogues 1 and 2 containing D<sub>I</sub> domain motifs (~2%). Furthermore, all trials for synthesizing the A<sup>A21</sup>PL<sup>A23</sup>-insulin analogue, which was designed to probe the effect of P-L substitution alone (like P-L-K), have never yielded a sufficient amount of the material for its biological characterization.

In the first attempt toward the total chemical synthesis of an insulin analogue with the whole C<sub>II</sub> domain, the insulin B chain extended by the C<sub>II</sub> S-R-V-S-R-R-S-R amino acids of IGF-2 was successfully made. However, its recombination with the insulin A chain S-sulfonate form<sup>40,41</sup> failed because of the insolubility of this B chain derivative in the recombination buffer. Therefore, a fully protected (*t*-Bu, Boc) G-F-F-Y-T-P-K-T-S-K-V-S-K-K-S-K peptide containing C-terminal insulin octapeptide and amino acids of the C<sub>II</sub> domain was prepared. Here, arginine residues in the original C<sub>II</sub> were substituted with lysines to allow both easier side chain protection and enzymatic coupling with DOI. The N<sup>ε</sup>-Boc-Lys protection was necessary to shield this sequence against trypsin proteolysis during analogue semisynthesis. Although a similar approach was already used in the preparation of insulin with the C<sub>I</sub> domain,<sup>27</sup> the semisynthetic attachment of this peptide to DOI failed



**Figure 4.** Comparison of relative binding affinities (white bars) for IR-A (A), IR-B (B), and IGF-1R (C) and relative abilities to activate these receptors (gray bars) of human insulin (HI), IGF-1, IGF-2, and insulin analogues containing sequences derived from the D domain of IGF-1 (1 and 2) or IGF-2 (3) or from the C domain of IGF-2 (4–7). The experimental values are related to binding potency and biological activity of HI (for IR-A and IR-B) or IGF-1 (for IGF-1R). Asterisks indicate that binding of the ligand or autophosphorylation of a particular receptor induced by the ligand differs significantly from that of insulin (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). See also [Table 1](#) and [Experimental Procedures](#).

again because of its insolubility. Hence, we employed a more limited and pragmatic approach, in which the C-terminus of insulin B chain octapeptide was systematically expanded by the subsequent residues from the C<sub>II</sub> domain. This strategy resulted in four C<sub>II</sub> derivatives of insulin B23–30 octapeptide, with addition of S, S-K, S-K-V, or S-K-V-S amino acids, and all lysine free N<sup>ε</sup>-groups were phenylacetyl-protected. In contrast to the whole C<sub>II</sub> domain-modified B23–30 octapeptide, these peptides were successfully attached enzymatically to the DOI. However, the yields of the individual semisyntheses of analogues 4–7 were only within the range of ~1.5–5%. Analogue 7 was obtained with the lowest yield, and the efficiencies of semisyntheses for 4–7 were directly proportional to the length of the respective peptide. Furthermore, the final removal of the phenylacetyl protection required use of the cocktail protease inhibitors to protect these analogues against proteolytic contaminants present in the penicillin amidohydrolyse solution.

**Receptor Binding Studies.** The binding affinities of all analogues, human insulin, and human IGF-1 and IGF-2 for human IR-A, IR-B, and IGF-1R are listed in [Table 1](#).

**IR-A Binding Affinities.** All insulin analogues 1–7 have either similar, or lower, affinity for IR-A in comparison with that of human insulin. Hybrid molecules 1–3 containing motifs from the D domains of IGF-1 and IGF-2 have different impacts on IR-A binding. Whereas analogue 3 (whole D<sub>II</sub> domain) has a binding affinity comparable to that of HI (84%), the binding potencies of both analogues 1 (with the APLK fragment of the D<sub>I</sub> domain) and 2 (with the whole D<sub>I</sub> domain) decreased to less (or approximately) than one-third of HI binding potency.

The addition of amino acids mimicking the C<sub>II</sub> domain to human insulin (analogues 4–7) has lowered their level of IR-A binding to 43–88%. Interestingly, analogues with only one (S<sup>B31</sup>, 4) or two (S<sup>B31</sup>K<sup>B32</sup>, 5) additional amino acids are less active than analogues with three (6) or four (7) extra residues from the C<sub>II</sub> domain ([Table 1](#), [Figure 4A](#), and [Figure S1](#)).

**IR-B Binding Affinities.** The additions of the D domain amino acids of IGF-1 and -2 to HI have a similar negative effect on the binding of analogues to both IR isoforms, but the negative impact of the addition of the whole D<sub>II</sub> domain (analogue 3) is more significant on IR-B (53%) than on IR-A (85%) affinity.

The effects of additions of C<sub>II</sub> domain amino acids to HI on its binding to IR-B were rather surprising. Except analogue 5, with only 33% HI binding affinity, the other C<sub>II</sub> domain-derived analogues have affinities of IR-B similar to (4 and 6), or slightly higher than (7), that of HI. Interestingly, the most potent analogue, 7, contains the longest, four extra amino acids, modification from the C<sub>II</sub> domain ([Table 1](#), [Figure 4B](#), and [Figure S2](#)).

**IGF-1R Binding Affinities.** All analogues exhibited very low binding affinity for IGF-1R compared to the affinity of IGF-1 for this receptor. In general, the analogues have binding potencies similar to that of human insulin. The exceptions are analogue 1, the IGF-1R binding affinity of which is 3 times lower, and analogue 3, which binds IGF-1R 2 times stronger than HI ([Table 1](#), [Figure 4C](#), and [Figure S3](#)).

**Autophosphorylation of IR and IGF-1R.** Activation of IR-A, IR-B, and IGF-1R was measured in R<sup>-</sup>/IR-A, R<sup>-</sup>/IR-B, and R<sup>+39</sup> cells, respectively, and by the detection of the

autophosphorylation of IR Tyr1158 (IR-B numbering) or the analogous IGF-1R Tyr1131. Cells were stimulated either by the natural ligand (human IGF-1, IGF-2, or insulin) or by an insulin analogue for 10 min, at a ligand concentration of 10 nM each. The results are shown in Figure 4 in correlation with binding affinities of analogues. Representative immunoblots are shown in Figure S4.

Generally, IR-A autophosphorylation abilities of the analogues (Figure 4A) followed their IR-A binding trends. All analogues exhibited a slightly reduced capability to activate IR-A compared to that of human insulin; they were within IGF-2 activation range and were enhanced in relation to IGF-1.

The IR-B stimulation abilities of analogues 1–3 (with the D<sub>I</sub> and D<sub>II</sub> domains) (Figure 4B) were comparable with that of human insulin. However, “C<sub>II</sub> domain analogues” 4–6 showed significantly higher (2–3 times higher than that of HI) levels of autophosphorylation of the IR-B. Interestingly, analogue 7, with enhanced (150%) binding potency for IR-B, stimulated IR-B like HI. Therefore, the IR-B receptor autophosphorylation abilities of insulin analogues modified by fragments of the C<sub>II</sub> domain are not really correlated with their binding potencies for this receptor: analogues 4 and 6, strong activators of IR-B, are equipotent with HI in IR-B binding, while analogue 5 (also a good stimulant of IR-B) has only 33% of the IR-B binding potency of HI.

The addition of sequences derived from D<sub>I</sub>, D<sub>II</sub>, and C<sub>II</sub> domains to human insulin did not significantly influence the ability of these ligands to stimulate the autophosphorylation of IGF-1R. All analogues 1–7 stimulated this receptor with an efficiency comparable to that of human insulin (Figure 4C).

## DISCUSSION

The C and D domains of IGF-1 and IGF-2 represent the major structural differences between these growth factors and insulin (Figure 1). However, it was proposed that the A and B domains of IGFs are the main determinants of their specific binding and activation of IGF-1R.<sup>16,20</sup> This has been recently supported by a similar mode of binding of insulin and IGF-1 to IR/IGF-1R constructs observed in the respective crystal structures.<sup>17–19</sup> Therefore, we can envisage that the roles of the C and D domains are limited to more subtle modulation of binding of IGF-1 and IGF-2 to IR-A, IR-B, and IGF-1R, and subsequent tuning of their activation.<sup>24,51</sup>

Initial studies of insulin:D domain hybrids were reported in the 1980s.<sup>33,34,52</sup> Although they provided valuable data, insight into binding affinities of these analogues for individual “isolated” IR isoforms and IGF-1R was not gained at that time. Moreover, the understanding of the role of the IGF-2 C<sub>II</sub> domain in the receptor selectivity of this hormone is still limited.

These structural and functional ambiguities prompted us to investigate whether insulin analogues, which carry D<sub>I</sub>, D<sub>II</sub>, and C<sub>II</sub> domains, could be molecular probes for testing the functionalities of these structural segments.

The sensitivity of interactions of insulin with IR-A and IR-B and the feasibility of the chemical synthesis of insulin-based analogues (in comparison to IGF-based scaffolds) were the decisive factors in selection of this hormone as a working template in this study.

The addition of amino acids from the D<sub>I</sub> domain (analogues 1 and 2) had a visible negative effect on insulin IR-A and IR-B binding affinities [25–30% of that of HI (Table 1)]. This confirmed the previously proposed unfavorable interference of

the D<sub>I</sub> domain with binding to IR<sup>34</sup> and agrees with the observation that deletion of the D<sub>I</sub> domain in IGF-1 doubles its IR binding affinity.<sup>25,26</sup> Moreover, the D<sub>II</sub> → D<sub>I</sub> swap in IGF-2 also decreased its IR-A affinity to 37%.<sup>24</sup> Here, we have shown that addition of the first three amino acids of the D<sub>I</sub> domain (P-L-K, analogue 1) to insulin A21 site is sufficient to lower its level of IR-A binding. Unfortunately, we have not succeeded in synthesizing the A<sup>A21</sup>-P-L<sup>A23</sup> analogue with the short, initial D<sub>I</sub> sequence. This analogue would help in understanding better the functional significance of these particular two amino acids of the D<sub>I</sub> domain, as they represent a unique IGF-1 D domain insert (in comparison with the shorter D<sub>II</sub> domain) (Figure 1). Nevertheless, it seems that not the length of the D<sub>I</sub> domain but its particular P-L-K sequence is the determining factor for the different IR-A and IR-B binding abilities of IGF-1 and IGF-2. This underlines further the presence and role of the usually structurally significant proline residue at position 62 in the D<sub>I</sub> domain, which may have a specific effect on the conformation of the D domain. It would amplify the interference of the D<sub>I</sub> domain with the hormone's binding site at IR, as its receptor-bound conformation is likely different from the D<sub>II</sub>-specific fold.

In contrast to that of the D<sub>I</sub> domain, the addition of the D<sub>II</sub> domain to the insulin molecule (analogue 3) has a marginal effect (85%) on its IR-A binding and a relatively small negative effect (53%) on its affinity for IR-B (Table 1). Although the peculiar role of N<sup>A21</sup> → A<sup>A21</sup> substitution in analogues 1–3 cannot be ignored (this single mutation reduces the level of insulin IR-A binding to ~65%<sup>53,54</sup>), we assume that the IR binding trends of these analogues are meaningful, as all of them contain the Ala<sup>A21</sup> mutation. Therefore, the different binding affinities of analogue 3 for IR isoforms, compared to that of HI, can result from the presence of the whole additional D<sub>II</sub> domain, rather than from the impact of the Ala<sup>A21</sup> mutation. The relatively more important decrease in the IR-B binding affinity of analogue 3 compared to its effect on IR-A could also indicate that (i) the D<sub>II</sub> domain is, at least partly, responsible for a lower affinity of IGF-2 for IR-B and (ii) the D<sub>II</sub> domain does not play a major role in the interaction with IR-A. Because only the 12 additional amino acids at the C-terminus of the  $\alpha$  subunit (encoded by exon 11) are the difference between IR-A and IR-B, some specific, unfavorable interaction of the D<sub>II</sub> domain with the  $\alpha$ CT segment of IR-B could be behind the mechanism of the “D<sub>II</sub>-mediated” lower affinity for IR-B. This could also mean that the D<sub>II</sub> domain does not interact, or does so in an only marginal fashion, with IR-A.

It may be expected that the addition of D<sub>I</sub> and D<sub>II</sub> domains to insulin will, somehow, increase the IGF-1R affinity of these analogues. However, this effect was only partially noted in the D<sub>II</sub> domain-containing analogue 3. Its binding affinity for IGF-1R is >2 times higher than that of HI (Table 1 and Figure 4). The nondeleterious effect of the D<sub>II</sub> domain for IGF-1R was observed also by Denley et al.,<sup>24</sup> who found that the IGF-1 analogue with the added D<sub>II</sub> domain had a similar binding affinity for native IGF-1. It was rather surprising that the binding affinity for IGF-1R was not increased in the analogues containing the whole D<sub>I</sub> domain (or its fragment, analogue 2 or 1, respectively), and that their binding affinities were similar to or significantly lower than the affinity of HI [analogue 2, 107%; analogue 1, 33% (Table 1)]. Denley et al.<sup>24</sup> observed that addition of the D<sub>I</sub> domain to IGF-2 enhanced 3-fold the IGF-1R binding affinity of the hybrid in comparison with that of native IGF-2. Hence, it is possible that the role of the D<sub>I</sub>

domain in binding to IGF-1R can differ in the context of insulin and IGF molecules.

All three (1–3) D<sub>I</sub> and D<sub>II</sub> analogues show a similar ability to activate IR-A (Figure 4A and Figure S4), despite their relatively different IR-A binding affinities [25–84% (Table 1)]; these IR-A stimulation properties are also lower than those of HI and IGF-2. Moreover, the autophosphorylation of IR-A by analogues 1–3 does not simply, and fully, correlate with their binding affinities, and the insertion of D<sub>I</sub> and D<sub>II</sub> domains into insulin has a less negative impact on IR-A autophosphorylation than on binding. The activations of IR-B by analogues 1–3 are also similar; however, in comparison with the autophosphorylation of IR-A, their effect is less pronounced here, and these analogues are also able to activate IR-B better than IGF-2 (Figure 4B and Figure S4). This suggests that the core of the hormone(s) (insulin or IGF) plays a more important role in these processes than the D domains. Similar effects can be seen in the autophosphorylation of IGF-1R, where analogues 1–3 stimulate the receptor at the HI level, and much more weakly than both IGFs (Figure 4C and Figure S4).

The second part of our work here concerned whether the incorporation of the elements of the C<sub>II</sub> domain into insulins template (analogues 4–7) would allow us to monitor their impact on hormone:receptor specificity. It must be stressed that the reports concerning the significance of the C<sub>I</sub> and C<sub>II</sub> domains (or particular amino acids) are infrequent, and studies of the C<sub>II</sub> domain are especially limited.<sup>24–28,55,56</sup> This results likely from the methodological barrier, i.e., difficult synthesis of this particular amino acid sequence. For example, the high content of arginine in the C<sub>II</sub> domain decreases significantly the solubility of these peptides. Hence, this was also the main reason behind our unsuccessful synthesis of the insulin analogue with the entire C<sub>II</sub> domain. Furthermore, the Arg residues interfere also with the trypsin-catalyzed semisynthesis of the analogue, as the Arg-involving peptide bonds are digested even under the tryptic-digest unfavorable conditions (e.g., pH 7 and organic solvent). Therefore, we had to include Arg → Lys substitutions in the C<sub>II</sub> domain, which is similar to the approach of the previous study concerning the C<sub>I</sub> domain of IGF-1.<sup>27</sup> Here, we also tried to use the Boc protection of Lys to prepare an analogue containing the entire C<sub>II</sub>-like domain (G<sup>B23</sup>FFY-TPKTSKVS<sup>B38</sup>) by trypsin-catalyzed semisynthesis. Unfortunately, the 16-amino acid peptide precursor composed of the B23–B30 C-terminal segment of insulin that was followed by the eight amino acids of the C<sub>II</sub> domain was insoluble in solvents necessary for enzymatic semisynthesis. Therefore, we focused here on the systematic enzymatic semisynthesis of insulin analogues with the C-terminus of B chain extended by one, two, three, and four residues from the C<sub>II</sub> domain; they also contained Arg → Lys substitutions, with lysine side chains temporarily N<sup>ε</sup>-protected by the Pac groups. This approach was more successful, and the Pac Lys protection was removed enzymatically with penicillin amidohydrolase after the semisynthesis.<sup>43,46</sup> However, some proteolytic side reactions were also experienced here, probably because of a prolonged Pac cleavage time that was required for the removal of these multiple protective groups. Hence, the use of a cocktail of protease inhibitors was needed because of the poor yield of these reactions (in the range of 1.5–5%), as well.

Despite these synthetic difficulties, we made four (4–7) new hybrid analogues of human insulin containing one to four C<sub>II</sub> domain residues (with Arg → Lys substitutions); they were subsequently characterized with all three types of receptors. We

expected that the addition of fragments of the C<sub>II</sub> domain to insulin will enhance its binding to the “mitogenic” IR-A and IGF-1R receptors, weakening simultaneously its interaction with the “metabolic” IR-B isoform. Although only moderate, and nonsignificant, increases in the binding affinities of 4–7 for IGF-1R were observed (Table 1) [paralleled by similar small IGF-1R autophosphorylation effects (Figure 4C)], the binding affinities and autophosphorylation ability of these analogues for IR-A and IR-B were unexpected. Analogues 4–7 have moderately decreased binding affinities [from 43 to 88% (Table 1)] for IR-A compared to HI, paralleled by their similar ability to activate this receptor (Figure 4A). More surprising were the equipotent, or slightly increased, IR-B binding affinities of analogues 4, 6, and 7, with only analogue 5 being a significantly weaker IR-B binder [33% (Table 1 and Figure 4B)]. Its lower binding affinity for IR-B [33%, and to some extent also for IR-A, as well (43%)] could be caused by a negative effect of the C-terminal LysB32. This effect is similar to the impact of the C-terminal arginine residues in insulin glargine, which has two extra arginines at the C-terminus of the B chain, and its IR-B binding affinity is also lower than that of HI.<sup>57–59</sup> Some C domain-related reports suggest that the C<sub>I</sub> domain (but not the C<sub>II</sub> domain) should be considered as an important factor in hormone–IGF-1R binding, but with negligible effect on their IR binding.<sup>25</sup> In contrast, the C<sub>II</sub> domain was supposed to be critical for signaling through IR-A.<sup>51</sup> However, a slightly positive effect of the C<sub>II</sub> domain on IR-B binding was observed for the IGF-1 analogue with this whole IGF-2 segment, as well.<sup>24</sup>

Interestingly, the IR-B autophosphorylation abilities of analogues 4–6 do not fully follow their IR-B binding affinities as they are significantly enhanced. Here, only analogue 7 has its IR-B autophosphorylation ability proportional to its IR-B binding affinity (Figure 4B). The preferential activation of IR-B by analogues 4–6 is interesting, as it was already indicated that a relatively moderate IR isoform specificity of insulin analogues may have a significant impact on their biological effects.<sup>12</sup> Hence, IR-B-specific analogues could indeed present important applications in assuring a more physiological profile of clinical insulins *in vivo*, with an enhanced hepatic mode of action.<sup>60</sup>

It should be stressed that the extra amino acids added to the C-terminus of the B chain in analogues 4–7 might not represent the true C<sub>II</sub> mimics, as their selection was significantly driven here by the semisynthetic yields. Our modifications should be thus rather considered as new (but IGF-derived), artificial structural motifs that add new properties to the insulin molecule; this is different from the “natural”, biological impact of the C<sub>II</sub> domain. Nevertheless, they showed that the use of even significantly constrained/modified sequences within the C<sub>II</sub> domain could result in protein probes that provide valuable insight into the hormone’s functionality. Moreover, they could open a new path into the design and creation of novel and important analogues with enhanced IR-B specificity, needed for clinical applications.

The preferential binding/autophosphorylation of IR-B by analogues 4–7 evokes also questions about a direct interaction of their B chain C-terminal “extra” residues (B31–B34) with  $\alpha$ -CT peptide of IR-B. The position of 12 amino acids of IR-B exon 11 in the receptor structure is still unknown. However, it can be assumed that the conformation of insulin B21–B27 residues (visible in the insulin:IR-A complex) on the L1 domain may be similar upon binding to both IR isoforms. If this is indeed the case, then a direct interaction between the C-

terminus of the insulin B chain and exon 11-encoded additional IR-B residues cannot be excluded. Therefore, our results concerning analogues 4–7, together with the recently published evidence of the increased hormone's IR-B sensitivity obtained through modification of the C-terminal part of the insulin B chain,<sup>12,49,61</sup> underline and indicate the importance of this region for achieving IR-B-specific hormone analogues. This opens new venues for a rational manipulation of the insulin B26-onward part of the hormone, which was usually thought to be unimportant for its functionality. We show here that a careful extension of the B chain beyond the B30 site can introduce new and exciting properties into the insulin molecule.

In summary, our insulin-based hybrid hormonal probes with elements of IGF-1 and IGF-2 presented here suggest that (i) the D<sub>I</sub> domain plays a more negative role in binding to IR than the D<sub>II</sub> domain does, (ii) D<sub>I</sub> domain P-L-K residues are a determining factor for a different IR-A and IR-B binding affinity of IGF-1 and IGF-2, and (iii) the addition of amino acids “mimicking” the C<sub>II</sub> domain to the C-terminus of the insulin B chain may result in an unexpected, specifically deepened, autophosphorylation of “metabolic” IR-B. Our research evidence underlines also the sophistication and complexity of the insulin/IGF/IR/IGF-1R signaling system, in which hormone:receptor binding and receptor activation strengths are frequently not fully correlated and are, likely, modulated further by the half-life of these complexes and their endocytotic fate.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00140.

Supplementary Figures S1–S4 (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

\*E-mail: zakova@uochb.cas.cz.

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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

C<sub>I</sub>, IGF-1 C domain; C<sub>II</sub>, IGF-2 C domain; D<sub>I</sub>, IGF-1 D domain; D<sub>II</sub>, IGF-2 D domain; DIPEA, *N,N*-diisopropylethylamine; EDT, 1,2-ethanedithiol; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HI, human insulin; IR, insulin receptor; HOBt, 1-hydroxybenzotriazole; IGF, insulin-like growth factor; IGF-1R, insulin-like growth factor 1 receptor; Pac, phenylacetyl; PDB, Protein Data Bank; TIS, triisopropylsilane.

## ■ REFERENCES

(1) Vigneri, P., Frasca, F., Sciacca, L., Pandini, G., and Vigneri, R. (2009) Diabetes and cancer. *Endocr.-Relat. Cancer* 16, 1103–1123.

(2) Cohen, D. H., and LeRoith, D. (2012) Obesity, type 2 diabetes, and cancer: the insulin and IGF connection. *Endocr.-Relat. Cancer* 19, F27–F45.

(3) Murray-Rust, J., McLeod, A. N., Blundell, T. L., and Wood, S. P. (1992) Structure and evolution of insulins: implications for receptor binding. *BioEssays* 14, 325–331.

(4) Gallagher, E. J., and LeRoith, D. (2011) Minireview: IGF, Insulin, and Cancer. *Endocrinology* 152, 2546–2551.

(5) LeRoith, D. (2006) Insulin-like growth factors. *Seminars in Medicine of the Beth Israel Deaconess Medical Center* 336, 633–640.

(6) Denley, A., Cosgrove, L. J., Booker, G. W., Wallace, J. C., and Forbes, B. E. (2005) Molecular interactions of the IGF system. *Cytokine Growth Factor Rev.* 16, 421–439.

(7) Adams, T. E., Epa, V. C., Garrett, T. P., and Ward, C. W. (2000) Structure and function of the type 1 insulin-like growth factor receptor. *Cell. Mol. Life Sci.* 57, 1050–1093.

(8) De Meyts, P. (2004) Insulin and its receptor: structure, function and evolution. *BioEssays* 26, 1351–1362.

(9) De Meyts, P., and Whittaker, J. (2002) Structural biology of insulin and IGF1 receptors: implications for drug design. *Nat. Rev. Drug Discovery* 1, 769–783.

(10) Moller, D. E., Yokota, A., Caro, J. F., and Flier, J. S. (1989) Tissue-specific expression of two alternatively spliced insulin receptor mRNAs in man. *Mol. Endocrinol.* 3, 1263–1269.

(11) Seino, S., and Bell, G. I. (1989) Alternative splicing of human insulin receptor messenger RNA. *Biochem. Biophys. Res. Commun.* 159, 312–316.

(12) Vienberg, S. G., Bouman, S. D., Sorensen, H., Stidsen, C. E., Kjeldsen, T., Glendorf, T., Sorensen, A. R., Olsen, G. S., Andersen, B., and Nishimura, E. (2011) Receptor-isoform-selective insulin analogues give tissue-preferential effects. *Biochem. J.* 440, 301–308.

(13) Morcavallo, A., Genua, M., Palumbo, A., Kletvikova, E., Jiracek, J., Brzozowski, A. M., Iozzo, R. V., Belfiore, A., and Morriore, A. (2012) Insulin and Insulin-like Growth Factor II Differentially Regulate Endocytic Sorting and Stability of Insulin Receptor Isoform A. *J. Biol. Chem.* 287, 11422–11436.

(14) Frasca, F., Pandini, G., Sciacca, L., Mineo, R., Costantino, A., Goldfine, I. D., Belfiore, A., and Vigneri, R. (1999) Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. *Mol. Cell. Biol.* 19, 3278–3288.

(15) Ziegler, A. N., Chidambaram, S., Forbes, B. E., Wood, T. L., and Levison, S. W. (2014) Insulin-like Growth Factor-II (IGF- II) and IGF- II. Analogs with Enhanced Insulin Receptor- a Binding Affinity Promote Neural Stem Cell Expansion. *J. Biol. Chem.* 289, 4626–4633.

(16) De Meyts, P. (2015) Insulin/receptor binding: The last piece of the puzzle? *BioEssays* 37, 389–397.

(17) Menting, J. G., Whittaker, J., Margetts, M. B., Whittaker, L. J., Kong, G. K. W., Smith, B. J., Watson, C. J., Zakova, L., Kletvikova, E., Jiracek, J., Chan, S. J., Steiner, D. F., Dodson, G. G., Brzozowski, A. M., Weiss, M. A., Ward, C. W., and Lawrence, M. C. (2013) How insulin engages its primary binding site on the insulin receptor. *Nature* 493, 241–245.

(18) Menting, J. G., Yang, Y. W., Chan, S. J., Phillips, N. B., Smith, B. J., Whittaker, J., Wickramasinghe, N. P., Whittaker, L. J., Pandeyarajan, V., Wan, Z. L., Yadav, S. P., Carroll, J. M., Strokes, N., Roberts, C. T., Ismail-Beigi, F., Milewski, W., Steiner, D. F., Chauhan, V. S., Ward, C. W., Weiss, M. A., and Lawrence, M. C. (2014) Protective hinge in insulin opens to enable its receptor engagement. *Proc. Natl. Acad. Sci. U. S. A.* 111, E3395–E3404.

(19) Menting, J. G., Lawrence, C. F., Kong, G. K., Margetts, M. B., Ward, C. W., and Lawrence, M. C. (2015) Structural Congruency of Ligand Binding to the Insulin and Insulin/Type 1 Insulin-like Growth Factor Hybrid Receptors. *Structure* 23, 1271–1282.

(20) Gauguin, L., Delaine, C., Alvino, C. L., McNeil, K. A., Wallace, J. C., Forbes, B. E., and De Meyts, P. (2008) Alanine scanning of a putative receptor binding surface of insulin-like growth factor-I. *J. Biol. Chem.* 283, 20821–20829.

- (21) Gauguin, L., Klaproth, B., Sajid, W., Andersen, A. S., McNeil, K. A., Forbes, B. E., and De Meyts, P. (2008) Structural basis for the lower affinity of the insulin-like growth factors for the insulin receptor. *J. Biol. Chem.* 283, 2604–2613.
- (22) Renteria, M. E., Gandhi, N. S., Vinuesa, P., Helmerhorst, E., and Mancera, R. L. (2008) A Comparative Structural Bioinformatics Analysis of the Insulin Receptor Family Ectodomain Based on Phylogenetic Information. *PLoS One* 3 (11), e3667.
- (23) Alvino, C. L., McNeil, K. A., Ong, S. C., Delaine, C., Booker, G. W., Wallace, J. C., Whittaker, J., and Forbes, B. E. (2009) A Novel Approach to Identify Two Distinct Receptor Binding Surfaces of Insulin-like Growth Factor II. *J. Biol. Chem.* 284, 7656–7664.
- (24) Denley, A., Bonython, E. R., Booker, G. W., Cosgrove, L. J., Forbes, B. E., Ward, C. W., and Wallace, J. C. (2004) Structural determinants for high-affinity binding of insulin-like growth factor II to insulin receptor (IR)-A, the exon 11 minus isoform of the IR. *Mol. Endocrinol.* 18, 2502–2512.
- (25) Bayne, M. L., Applebaum, J., Underwood, D., Chicchi, G. G., Green, B. G., Hayes, N. S., and Cascieri, M. A. (1989) The C region of human insulin-like growth factor (IGF) I is required for high affinity binding to the type I IGF receptor. *J. Biol. Chem.* 264, 11004–11008.
- (26) Kristensen, C., Andersen, A. S., Hach, M., Wiberg, F. C., Schaffer, L., and Kjeldsen, T. (1995) A Single-Chain Insulin-Like Growth-Factor-I Insulin Hybrid Binds with High-Affinity to the Insulin-Receptor. *Biochem. J.* 305, 981–986.
- (27) Cara, J. F., Mirmira, R. G., Nakagawa, S. H., and Tager, H. S. (1990) An Insulin-Like Growth Factor-I Insulin Hybrid Exhibiting High Potency for Interaction with the Type-I Insulin-Like Growth-Factor and Insulin-Receptors of Placental Plasma-Membranes. *J. Biol. Chem.* 265, 17820–17825.
- (28) Gill, R., Wallach, B., Verma, C., Urso, B., DeWolf, E., Grotzinger, J., MurrayRust, J., Pitts, J., Wollmer, A., DeMeyts, P., and Wood, S. (1996) Engineering the C-region of human insulin-like growth factor-I: Implications for receptor binding. *Protein Eng., Des. Sel.* 9, 1011–1019.
- (29) Keyhanfar, M., Booker, G. W., Whittaker, J., Wallace, J. C., and Forbes, B. E. (2007) Precise mapping of an IGF-I-binding site on the IGF-1R. *Biochem. J.* 401, 269–277.
- (30) Whittaker, J., Groth, A. V., Mynarcik, D. C., Pluzek, L., Gadsboll, V. L., and Whittaker, L. J. (2001) Alanine scanning mutagenesis of a type I insulin-like growth factor receptor ligand binding site. *J. Biol. Chem.* 276, 43980–43986.
- (31) Henderson, S. T., Brierley, G. V. B., Surinya, K. H., Priebe, I. K., Catcheside, D. E. A., Wallace, J. C., Forbes, B. E., and Cosgrove, L. J. (2015) Delineation of the IGF-II C domain elements involved in binding and activation of the IR-A, IR-B and IGF-IR. *Growth Horm. IGF Res.* 25, 20–27.
- (32) Sorensen, H., Whittaker, L., Hinrichsen, J., Groth, A., and Whittaker, J. (2004) Mapping of the insulin-like growth factor II binding site of the type I insulin-like growth factor receptor by alanine scanning mutagenesis. *FEBS Lett.* 565, 19–22.
- (33) King, G. L., Kahn, C. R., Samuels, B., Danho, W., Bullesbach, E. E., and Gattner, H. G. (1982) Synthesis and Characterization of Molecular Hybrids of Insulin and Insulin-Like Growth Factor-I - the Role of the A-Chain Extension Peptide. *J. Biol. Chem.* 257, 869–873.
- (34) Devroede, M. A., Rechler, M. M., Nissley, S. P., Ogawa, H., Joshi, S., Burke, G. T., and Katsoyannis, P. G. (1986) Mitogenic Activity and Receptor Reactivity of Hybrid Molecules Containing Portions of the Insulin-Like Growth Factor-I (Igf-I), Igf-II, and Insulin Molecules. *Diabetes* 35, 355–361.
- (35) Sakano, K. I., Enjoh, T., Numata, F., Fujiwara, H., Marumoto, Y., Higashihashi, N., Sato, Y., Perdue, J. F., and Fujitayamaguchi, Y. (1991) The Design, Expression, and Characterization of Human Insulin-Like Growth Factor-II (Igf-II) Mutants Specific for Either the IGF-II Cation-Independent Mannose 6-Phosphate Receptor Or IGF-I Receptor. *J. Biol. Chem.* 266, 20626–20635.
- (36) Morrione, A., Valentini, B., Xu, S. Q., Yumet, G., Louvi, A., Efstratiadis, A., and Baserga, R. (1997) Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. *Proc. Natl. Acad. Sci. U. S. A.* 94, 3777–3782.
- (37) Versteijhe, S., Klaproth, B., Borup, R., Palsgaard, J., Jensen, M., Gray, S. G., and De Meyts, P. (2013) IGF-I, IGF-II, and Insulin Stimulate Different Gene Expression Responses through Binding to the IGF-I Receptor. *Front. Endocrinol.* 4, 98.
- (38) Slaaby, R. (2015) Specific insulin/IGF1 hybrid receptor activation assay reveals IGF1 as a more potent ligand than insulin. *Sci. Rep.* 5, 7911.
- (39) Belfiore, A., Frasca, F., Pandini, G., Sciacca, L., and Vigneri, R. (2009) Insulin Receptor Isoforms and Insulin Receptor/Insulin-Like Growth Factor Receptor Hybrids in Physiology and Disease. *Endocr. Rev.* 30, 586–623.
- (40) Kosinova, L., Veverka, V., Novotna, P., Collinsova, M., Urbanova, M., Moody, N. R., Turkenburg, J. P., Jiracek, J., Brzozowski, A. M., and Zakova, L. (2014) Insight into the Structural and Biological Relevance of the T/R Transition of the N-Terminus of the B-Chain in Human Insulin. *Biochemistry* 53, 3392–3402.
- (41) Krizkova, K., Veverka, V., Maletinska, L., Hexnerova, R., Brzozowski, A. M., Jiracek, J., and Zakova, L. (2014) Structural and Functional Study of the GlnB22-Insulin Mutant Responsible for Maturity-Onset Diabetes of the Young. *PLoS One* 9 (11), e112883.
- (42) Chance, R. E., Hoffmann, J. A., Kroeff, E. P., Johnson, M. G., Schirmer, E. W., and Bromer, W. W. (1981) The production of human insulin using recombinant DNA technology and a new chain combination procedure. In *Proceedings of the 7th American Peptide Symposium* (Rich, D. H., and Gross, E., Eds.) pp 721–728.
- (43) Zakova, L., Zyka, D., Jezek, J., Hanclova, I., Sanda, M., Brzozowski, A. M., and Jiracek, J. (2007) The use of Fmoc-Lys(Pac)-OH and penicillin G acylase in the preparation of novel semisynthetic insulin analogs. *J. Pept. Sci.* 13, 334–341.
- (44) Zakova, L., Kazdova, L., Hanclova, I., Protivinska, E., Sanda, M., Budesinsky, M., and Jiracek, J. (2008) Insulin analogues with modifications at position B26. Divergence of binding affinity and biological activity. *Biochemistry* 47, 5858–5868.
- (45) Nakagawa, S. H., and Tager, H. S. (1992) Importance of aliphatic side-chain structure at positions 2 and 3 of the insulin A chain in insulin-receptor interactions. *Biochemistry* 31, 3204–3214.
- (46) Antolikova, E., Zakova, L., Turkenburg, J. P., Watson, C. J., Hanclova, I., Sanda, M., Cooper, A., Kraus, T., Brzozowski, A. M., and Jiracek, J. (2011) Non-equivalent Role of Inter- and Intramolecular Hydrogen Bonds in the Insulin Dimer Interface. *J. Biol. Chem.* 286, 36968–36977.
- (47) Sell, C., Dumenil, G., Deveaud, C., Miura, M., Coppola, D., Deangelis, T., Rubin, R., Efstratiadis, A., and Baserga, R. (1994) Effect of a Null Mutation of the Insulin-Like Growth-Factor-I Receptor Gene on Growth and Transformation of Mouse Embryo Fibroblasts. *Mol. Cell. Biol.* 14, 3604–3612.
- (48) Miura, M., Surmacz, E., Burgaud, J. L., and Baserga, R. (1995) Different Effects on Mitogenesis and Transformation of a Mutation at Tyrosine-1251 of the Insulin-Like Growth-Factor-I Receptor. *J. Biol. Chem.* 270, 22639–22644.
- (49) Zakova, L., Kletvikova, E., Lepsik, M., Collinsova, M., Watson, C. J., Turkenburg, J. P., Jiracek, J., and Brzozowski, A. M. (2014) Human insulin analogues modified at the B26 site reveal a hormone conformation that is undetected in the receptor complex. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* 70, 2765–2774.
- (50) Slaaby, R., Andersen, A. S., and Brandt, J. (2008) IGF-I binding to the IGF-I receptor is affected by contaminants in commercial BSA: The contaminants are proteins with IGF-I binding properties. *Growth Horm. IGF Res.* 18, 267–274.
- (51) Denley, A., Brierley, G. V., Carroll, J. M., Lindenberg, A., Booker, G. W., Cosgrove, L. J., Wallace, J. C., Forbes, B. E., and Roberts, C. T. (2006) Differential activation of insulin receptor isoforms by insulin-like growth factors is determined by the C domain. *Endocrinology* 147, 1029–1036.
- (52) Devroede, M. A., Rechler, M. M., Nissley, S. P., Joshi, S., Burke, G. T., and Katsoyannis, P. G. (1985) Hybrid Molecules Containing the B-Domain of Insulin-Like Growth Factor-I Are Recognized by Carrier

Proteins of the Growth-Factor. *Proc. Natl. Acad. Sci. U. S. A.* 82, 3010–3014.

(53) Kristensen, C., Kjeldsen, T., Wiberg, F. C., Schaffer, L., Hach, M., Havelund, S., Bass, J., Steiner, D. F., and Andersen, A. S. (1997) Alanine scanning mutagenesis of insulin. *J. Biol. Chem.* 272, 12978–12983.

(54) Jensen, A. M. (2000) Analysis of structure-function relationships of the insulin molecule by alanine-scanning mutagenesis. Master's Thesis, University of Copenhagen, Copenhagen.

(55) Forbes, B. E., Hartfield, P. J., McNeil, K. A., Surinya, K. H., Milner, S. J., Cosgrove, L. J., and Wallace, J. C. (2002) Characteristics of binding of insulin-like growth factor (IGF)-I and IGF-II analogues to the type 1 IGF receptor determined by BIAcore analysis - Correlation of binding affinity with ability to prevent apoptosis. *Eur. J. Biochem.* 269, 961–968.

(56) Wang, P., Cai, R. R., Feng, Y. M., and Zhang, Y. S. (2000) Studies on insulin/IGF-1 hybrid and IGF-1 growth-promoting functional region. *IUBMB Life* 49, 321–325.

(57) Werner, U., Korn, M., Schmidt, R., Wendrich, T. M., and Tennagels, N. (2014) Metabolic effect and receptor signalling profile of a non-metabolisable insulin glargine analogue. *Arch. Physiol. Biochem.* 120, 158–165.

(58) Le Roith, D. (2007) Insulin glargine and receptor-mediated signalling: Clinical implications in treating type 2 diabetes. *Diabetes/Metab. Res. Rev.* 23, 593–599.

(59) Kurtzhals, P., Schaffer, L., Sorensen, A., Kristensen, C., Jonassen, I., Schmid, C., and Trub, T. (2000) Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. *Diabetes* 49, 999–1005.

(60) Herring, R., Jones, R. H., and Russell-Jones, D. L. (2014) Hepatoselectivity and the evolution of insulin. *Diabetes, Obes. Metab.* 16, 1–8.

(61) Vikova, J., Collinsova, M., Kletvikova, E., Budesinsky, M., Kaplan, V., Zakova, L., Veverka, V., Hexnerova, R., Avino, R. J., Strakova, J., Selicharova, I., Vanek, V., Wright, D. W., Watson, C. J., Turkenburg, J. P., Brzozowski, A. M., and Jiracek, J. (2016) Rational steering of insulin binding specificity by intra-chain chemical crosslinking. *Sci. Rep.* 6, 19431.