Original Article

MOLECULAR METABOLISM

A viral insulin-like peptide is a natural competitive antagonist of the human IGF-1 receptor

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ABSTRACT

Objective: Natural sources of molecular diversity remain of utmost importance as a reservoir of proteins and peptides with unique biological functions. We recently identified such a family of viral insulin-like peptides (VILPs). We sought to advance the chemical methods in synthesis to explore the structure-function relationship within these VILPs, and the molecular basis for differential biological activities relative to human IGF-1 and insulin.

Methods: Optimized chemical methods in synthesis were established for a set of VILPs and related analogs. These modified forms included the substitution of select VILP chains with those derived from human insulin and IGF-1. Each peptide was assessed *in vitro* for agonism and antagonism at the human insulin and the human insulin-like growth factor 1 receptor (IGF-1R).

Results: We report here that one of these VILPs, lymphocystis disease virus-1 (LCDV1)-VILP, has the unique property to be a potent and full antagonist of the IGF-1R. We demonstrate the coordinated importance of the B- and C-chains of the VILP in regulating this activity. Moreover, mutation of the glycine following the first cysteine in the B-chain of IGF-1 to serine, in concert with substitution to the connecting peptide of LCDV1-VILP, converted native IGF-1 to a high potency antagonist.

Conclusions: The results reveal novel aspects in ligand—receptor interactions at the IGF-1 receptor and identify a set of antagonists of potential medicinal importance.

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Keywords Insulin; Insulin-like growth factor 1; IGF-1; Peptide synthesis; Receptor antagonism; Virus-derived peptides

1. INTRODUCTION

Insulin and insulin-like growth factors-1 and -2 (IGF-1 and IGF-2) are of central importance in the physiological regulation of growth and metabolism [1,2]. These hormones share striking sequence homology with the most notable difference being the proteolytic processing of the insulin precursor proinsulin to generate a high potency, two-chain hormone [3,4]. This contrasts with the single-chain IGF-1 sequence which possesses a twelve-residue connecting peptide that is vital to its biological potency and selectivity [4,5]. These peptide hormones signal through two tyrosine kinase receptors that are also homologous in structure and mechanism of action — the insulin receptor (IR) and the IGF-1 receptor (IGF-1R) [6,7]. Each hormone displays a nanomolar affinity for its cognate receptor with cross-activation at reduced potency by nearly a hundredfold at the homologous receptor [4—7]. Whether the low—affinity interaction at each other's receptor has physiological function is a debated question, but it constitutes a safety concern when insulin plasma concentrations are appreciably elevated either because of endogenous hypersecretion or exogenous therapeutic administration [8, 9]. However, insulin analogs that have increased IGF-1 activity presented safety uncertainties regarding the potential to accelerate microvascular mitogenesis, and one has demonstrated increased carcinogenicity in preclinical testing that terminated its clinical development [10,11].

Conversely, the development of IGF-1R antagonists has received much attention considering their potential as therapeutics for various cancers and vascular complications of diabetes [12,13]. A two residue C-peptide mutation of native IGF-1 (R36E/R37E) has been reported to function as an antagonist of IGF signaling [14,15]. Increased medicinal interest has been drawn to neutralizing antibodies against IGF-1 or its receptor and small molecule-based intracellular tyrosine kinase inhibitors. However, these antagonists have failed in late-phase clinical trials usually because of undesired inhibition of the insulin receptor or other receptor tyrosine kinases [16,17]. Therefore, whether a high potency, selective IGF-1 antagonist might be employed to treat diseases of enhanced growth such as proliferative retinopathy [18,19] or integrated with insulin agonism to lessen chronic low-level IGF-1 agonism in vascular tissues of diabetic patients, remains a question of high biological and therapeutic significance.

As a pharmacological class, receptor antagonism is also of great importance to decipher biological function and form the basis of innovative drugs. Unfortunately, there is no single proven method for

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the identification of receptor antagonists; their discovery is often a matter of brute force or experimental serendipity [20]. The emergence of high throughput diversity screening has provided an important tool in the search for antagonists [21], and using this technique, peptide-based insulin antagonists have been identified [22]. Nonetheless, natural sources still constitute an important cradle of molecular diversity that exceeds the practical limit in synthetic libraries [23]. In this regard, a recent comprehensive bioinformatics search has identified four members of the Iridoviridae family of DNA viruses that encode peptides with significant sequence homology to hormones of the insulin/IGF-1 family. Among them, three viral insulin-like peptides (VILPs) were chemically synthesized, and their biological properties were characterized [24]. These peptides competed for binding to the insulin and IGF-1 receptors and possessed varying levels of insulin and IGF-1 agonist activity, deepening the prospect for a potential role of VILPs in human diseases.

Here, we report discoverey that one of the VILPs has the unique property of serving as a potent and selective antagonist of IGF-1 receptor signaling. The structural basis of the inhibition is a combination of a single amino acid change in B-chain sequence, together with a unique connecting peptide that serves to enhance receptor-negative cooperativity. The introduction of select elements from the IGF-1 sequence to LCDV1 restores high potency IGF-1R agonism, while the inverse substitution of LCDV1-VILP sequence to an IGF-1 backbone generates receptor antagonism. Furthermore, the IGF-1R antagonism can be selectively introduced to a peptide that maintains full insulin receptor agonism. The synthesis of two-chain analogs of three VILPs devoid of their connecting peptides (GIV, SGIV, and LCDV1) confirms the difference in the biological activity of the full-length linear peptides; especially the antagonism displayed by LCDV1. These novel findings provide a fresh perspective to enrich our understanding of the structural basis for biological signaling at the homologous insulin and IGF-1 receptors, and provide direction to create ligands that selectively silence endogenous IGF-1 bioactivity, while maintaining insulin receptor agonism.

2. METHODS

Methods, including statements of data availability and any associated accession codes and references, are provided in supplemental materials.

3. RESULTS

3.1. Synthesis of insulin-like peptides

We previously reported the synthesis of three VILPs through air-mediated simultaneous oxidation of the three native disulfide bonds [24]. This approach is the most synthetically straightforward, but leads to peptides that are less suitable for subsequent study. This is because the poor physical properties of the unfolded precursors render them incompatible with purification before disulfide formation and poorly soluble in solvents commonly employed for native folding. The stepwise selective formation of three disulfide bonds, while more laborious and challenging, is a certain approach to synthesize high purity peptides of a single disulfide configuration. Consequently, we employed selective protection with trityl (Trt) at CysA6-A11, acetamidomethyl (Acm) at CysB19-A20, and *tert*-butyl (*t*Bu) CysB7-CysA7 in solid-phase peptide assembly [2, 4,25], (Figure 1A).

The most synthetically challenging of the three VILPs (LCDV1, SGIV, and GIV) proved to be LCDV1-VILP. To increase the quality of peptide assembly and foster intermediate chromatography, two isoacyl-dipeptides were inserted at Ile1-Thr2 and Ala43-Thr44. Sequential synthesis of the first two disulfide bonds, A6-A11 and B19-A20 were iodine-mediated in aq. AcOH with the former occurring at an accelerated speed and followed by the latter without change in reaction condition. The B7-A7 disulfide bond was subsequently introduced without alteration to the two previously formed disulfide bonds in DMSO/TFA, and the two isoacyl-bonds were then rapidly converted to the native amides by aqueous treatment at pH 8, before purification by high-performance chromatography. Each of the three synthetic VILPs was qualitatively compared to those prepared by air oxidation, but in each instance, the new method of synthesis resulted in a higher yield with a much-sharpened chromatographic peak and enhanced MS-analysis (Figure 1B, S1-3, Table S1), signifying greater final quality.

The integrity of the disulfides was confirmed by a combination of treatment with TCEP and peptide mapping. The treatment of LCDV1-VILP yielded a single entity with a mass of six additional units to confirm the presence of three disulfides (Figs. S1 and 4). We synthesized all three possible LCDV1-VILP tryptic fragment disulfide isomers of B (1—17)-A (6—19) by an orthogonal method to confirm the B7-A7 and A6-A11 crosslinks (Figs. S5—10). The third disulfide bond B19-A21 was assigned as a unique tryptic fragment by LCMS (Fig. S5). For comparative purposes, the two-chain insulin-like analogs of each VILP was synthesized using a stepwise formation of the three disulfides (Fig. S11, Table S2). The B7 cysteine was activated by 2,2′-dithiodipyridine and chain combination with unprotected CysA7 formed the A-B heterodimer in high yield. Iodine oxidation followed by DMSO-TFA treatment provided each of the two-chain VILPs (Figs. S12—14).

3.2. LCDV1-VILP is a potent, full antagonist at IGF-1R

The *in vitro* bioactivity of the VILPs and their two chain-analogs were assessed at the human IGF-1R, the insulin receptors B and A (IR-B and IR-A) (Figure 2A, B, S15), in engineered cells overexpress each receptor as previously described [4]. The single-chain form of the peptides obtained by directed disulfide synthesis confirmed previously reported results [24], but with somewhat enhanced potency consistent with their superior purity. Each VILP is a low potency insulin agonist with a reduced ability to stimulate kinase activity than native human IGF-1 at each of the two insulin receptor isoforms (Figure 2B and S15). The Grouper Iridovirus (GIV)-VILP and Singapore Grouper Iridovirus (SGIV)-VILP sequences gained potency on the insulin receptor when synthesized as two-chain peptides without a C-peptide. This is comparable to what occurs for insulin as assessed relative to its single-chain precursor proinsulin [26], and IGF-1 without its C-peptide [4,5,27]. In contrast, LCDV1-VILP synthesized in a two-chain form uniquely showed reduced potency at IR-A and IR-B, compared to single-chain LCFV1-VILP (Figure 2D and S15). Surprisingly, the two-chain form of LCDV1-VILP acted as an agonist on the IGF-1R, while the single-chain form exhibited very low-level agonism below 10 nM concentration consistent with a prior report [24]; but at higher concentrations, caused a reduction in basal receptor autophosphorylation, which suggests antagonistic activity (Figure 2C).

To further investigate this competitive antagonism, we assessed the activity of the single and two-chain LCDV1-VILP in the presence of human IGF-1 at a near-maximal stimulatory (EC_{80}) concentration, (Supplemental Methods). Under these conditions, single-chain LCDV1-VILP proved to be a potent, fully functional IGF-1 antagonist. This effect was dependent on the connecting peptide linking the A and B chains as such activity was not observed in the two-chain LCDV1-VILP

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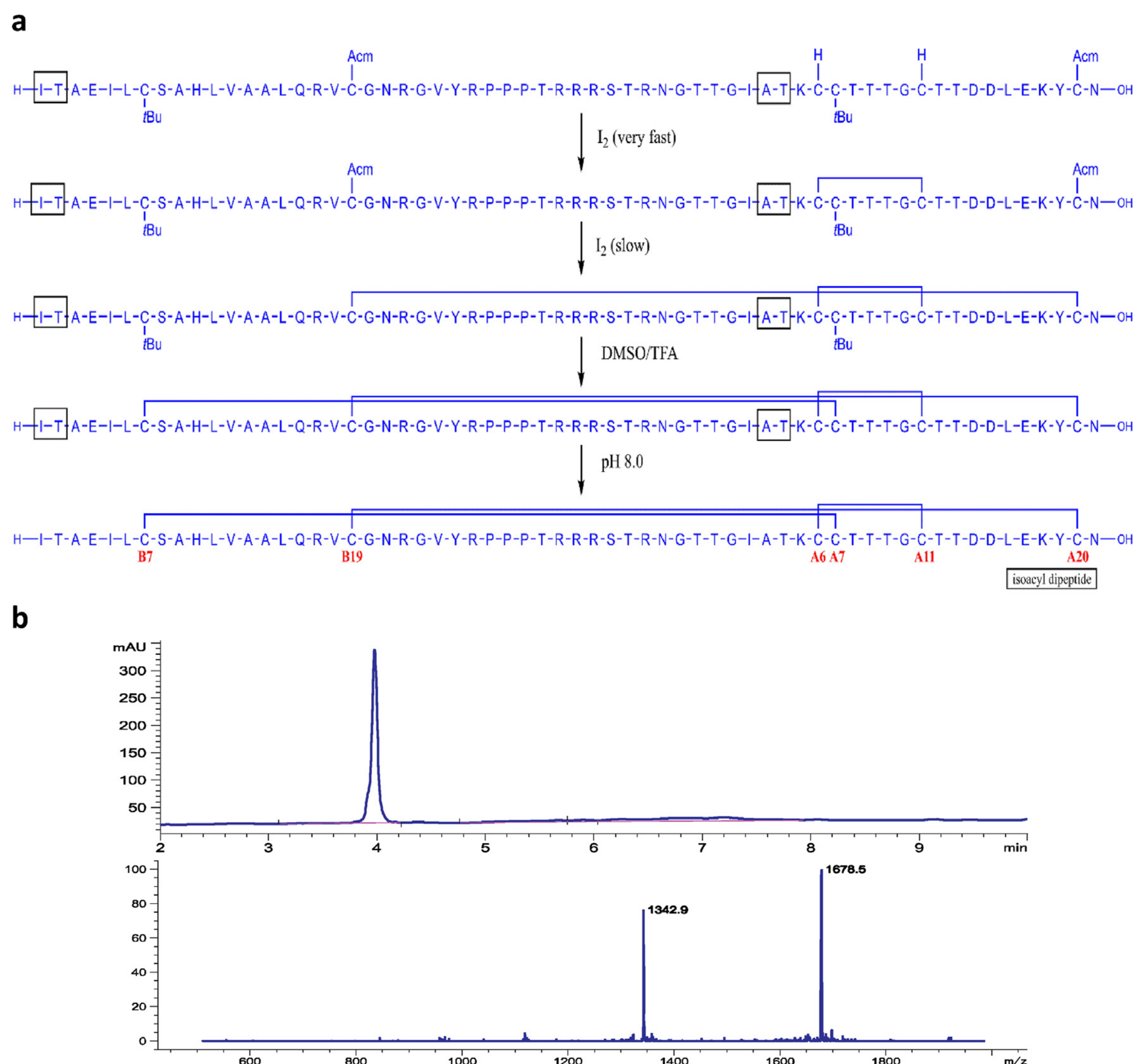

Figure 1: Synthesis of LCDV1-VILP. A: stepwise directed synthesis of the three disulfides in which iodine oxidation rapidly forms the A6-A11 bond, and subsequently, Acm-removal of the B19-A20 disulfide is formed. Insulin residue numbering is employed (shown in red) with each cysteine identified in LCDV1. The isoacyl dipeptides are enclosed in a box. The A6-A11 disulfide numbering is retained despite the insertion of an additional amino acid in LCDV1 and for analogs that include this A-chain [1,4,and7]], for comparative purposes. Following chromatographic purification and conversion of the isoacyl bonds to amides, at pH 8 with aqueous treatment, the final disulfide (B7-A7) is formed by DMSO treatment in anhydrous TFA. **B:** LC-MS analysis of the synthetic LCDV1-VILP with a single sharp peak at 3.9 min, and the +4 and + 5 ions demonstrating a final mass of 6710 Da.

(Figure 2E). The antagonism was specific to IGF-1R. Thus, both single-chain and two-chain LCDV1-VILPs behaved as partial, low-level agonists at IR-B and IR-A (Figure 2D and S15).

In summary, the VILPs segregate to two groups. GIV and SGIV behave much like native IGF-1 with relatively high potency and specificity for IGF-1R, compared to their activity at the two insulin receptor isoforms. In both cases, the IGF-1R activity is further reduced and the insulin receptor activity is enhanced when the connecting peptide is removed. However, LCDV1-VILP is unique in demonstrating selective IGF-1R antagonism and low potency insulin receptor agonism. The two-chain form is devoid of IGF-1R antagonism and like the other VILPs, activates the insulin receptor, although it remains less potent than GIV-VILP and SGIV-VILP (Figure 2B,D). When comparing LCDV1-VILP as an IGF-1 antagonist to the only previously reported antagonist derived by the double anionic mutation in the C-peptide of native IGF-1 (**14, 15**), single-chain LCDV1-VILP proved to be dramatically more potent and fully suppressive (Figs. S16 and 17).

3.3. LCDV1-VILP B and C chains govern IGF-1R antagonism

To investigate the structural basis of the LCDV1-VILP-mediated IGF1-R antagonism, six chimeric analogs of human IGF-1 and LCDV1-VILP were prepared, in which an individual A, B, or C-chain was substituted in each human ligand sequence (Figure 3A, Table S1). The three analogs with LCDV1-VILP A chain [1, 5, and 6]] were successfully synthesized using the same method as used for the single-chain VILPs (Figs. S18—20). Each analog with IGF-1 A chain failed by this approach and required an intermediate assembly step that included chemical ligation of two peptide fragments [28] to generate high-quality linear peptide precursors suitable for stepwise disulfide formation (Figs. S21—25). Using the same protocol for disulfide bond formation, a Cys-isomer to the desired product was observed that consisted of an internal A-chain (A7-20) and B-chain (B7-19) disulfide, instead of the native intermolecular disulfides (A7-B7 and A20-B19) (Fig. S21). The isomers were separable by preparative chromatography. Additionally, the human IGF-1 A-chain

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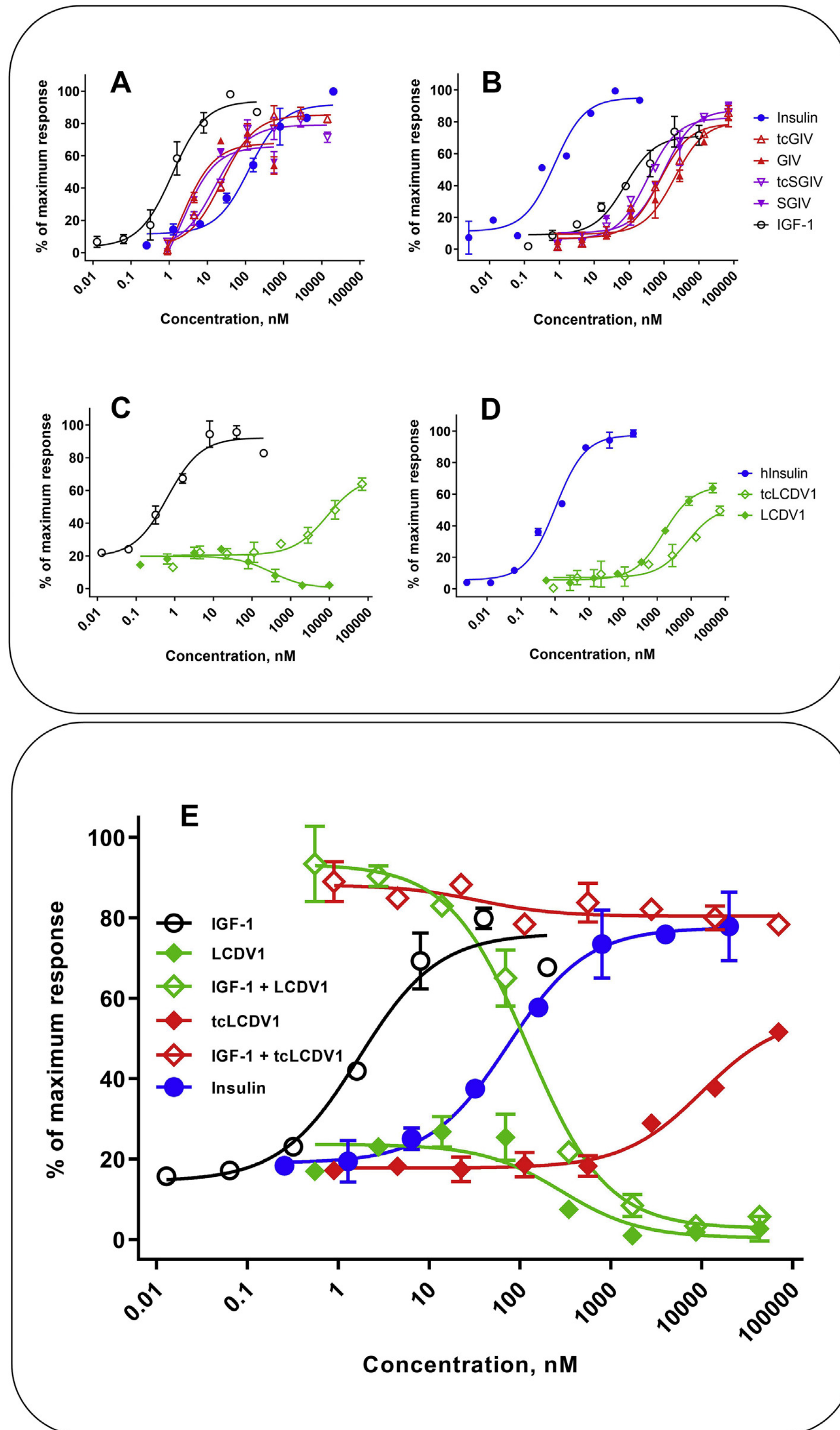

Figure 2: Bioactivity of VILPs. A & C: Stimulation of autophosphorylation of IGF-1R with single-chain and two-chain forms of VILPs derived from three viral sources (GIV, SGIV, and LCDV1). **B & D**: Stimulation of IR-B with single-chain and two-chain forms of VILPs. **E:** Stimulation of IGF-1R autophosphorylation with single-chain and two-chain forms of LCDV1-VILP (solid symbols) and antagonism of full IGF-1 mediated agonism with LCDV1-VILP peptides (open symbols). The presence of a constant EC_{80} concentration of IGF-1 (10 nM) was used in the measurements of antagonism. The black line illustrates dose-dependent agonism with IGF-1 and the blue line with insulin.

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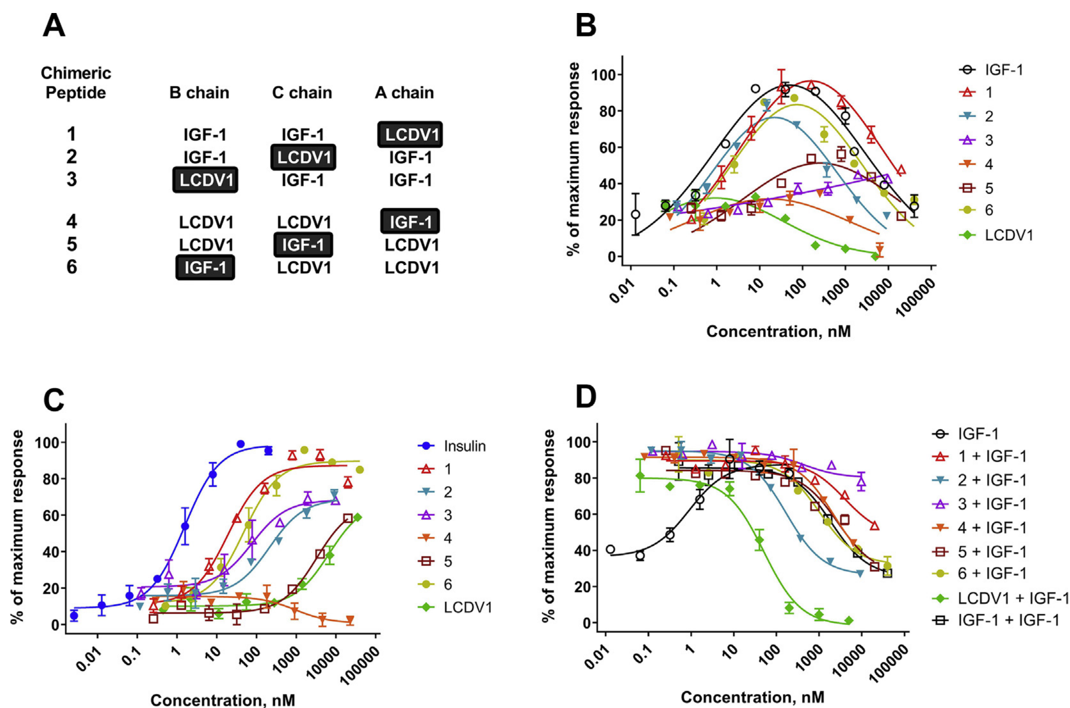

Figure 3: Bioactivity of six chimeric VLIP-IGF-1 peptides. A: Domain swap of LDCV in the IGF-1 sequence (**1**—**3**) and IGF-1 in the LCDV1-VILP sequence (**4**—**6**) with the substituted domain highlighted in black. **B:** Assessment of IGF-1R agonism relative to IGF-1 and LCDV1-VILP. **C:** Assessment of IR-B agonism relative to insulin and LCDV1-VILP. **D:** Assessment of IGF-1R antagonism relative to LCDV1-VILP with the black circles illustrating dose-dependent IGF-1 agonism and all other peptides tested at an increasing concentration in the presence of an EC_{80} concentration of IGF-1 (10 nM).

contained a methionine that is absent in LCDV1-VILP, and it partially oxidizes to sulfoxide during DMSO treatment. Thus, both peptide forms were synthesized. The presence of Met(O) was determined to be of no apparent consequence to the bioactivity of the ligand on either IGF-1R (Figs. S22, 23, and 26) or IR-B.

The biological activity of all six chimeric ligands was assessed for IGF-1R agonism (Figure 3B), IR-B agonism (Figure 3C), and IGF-1R antagonism (Figure 3D) across a broad concentration range. IGF-1 at concentrations approximately a thousand-fold greater than where full activity is observed exhibits lower maximal activity, and this progressively continues with each increase in concentration, reflective of negative receptor cooperativity [5,32]. Each of the chimeric peptides that contain an IGF-1 B chain [1,2,6] were potent IGF-1R agonists, with peptides **1** and **6** being comparable and of increased efficacy relative to peptide **2,** which consists of a human IGF-1 sequence with an LCDV1-VILP C-peptide. The remaining three peptides [3—5] have B-chain sequences from LCDV1-VILP and demonstrated weak, partial IGF-1R agonism consistent with LCDV1-VILP (Figure 3B). Analog **4** displayed no activity at insulin receptor, with slight basal antagonism at the highest concentrations (Figure 3C).

Peptide **6** differs from LCDV1-VILP by having just the N-terminus changed to IGF-1 sequence (Figure 3A), and this substitution dramatically bestowed IGF-1R (Figure 3B) and much enhanced insulin receptor agonism (Figure 3C). There was no other single domain swap in LCDV1-VILP (analogs **4** and **5**) that imposed such a change in bioactivity. Peptide **6** and the two other peptides with an IGF-1 based B-chain [1,2] constituted a differentiated set of three potent IGF-1R agonists, suggesting that the B-chain is a primary source for the diminished activity of

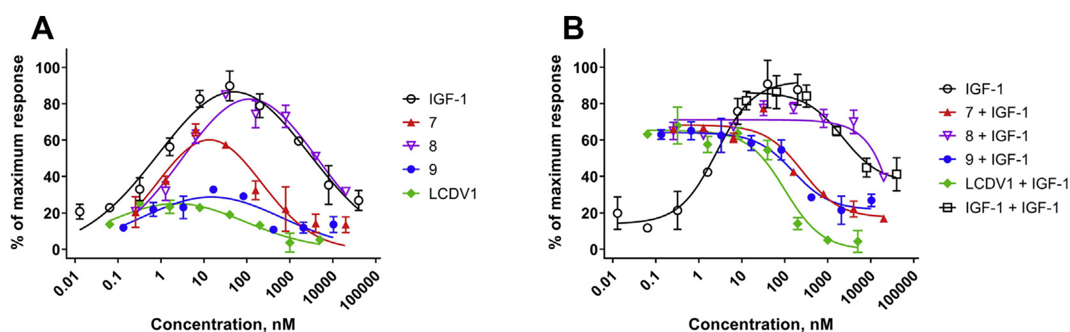

Figure 4: Bioactivity of three VLIP-IGF-1 chimeric peptides. A: Assessment of IGF-1R agonism relative to IGF-1 and LCDV1-VILP; peptide **7** LCDV1-VILP with B8Gly, peptide **8** IGF-1 with B8Ser, peptide **9** is analog 8 with the LCDV1-VILP C-peptide. **B:** Assessment of IGF-1R antagonism relative to IGF-1 and LCDV1-VILP. All peptides were tested at increasing concentration in the presence of an EC_{80} concentration of IGF-1 (10 nM).

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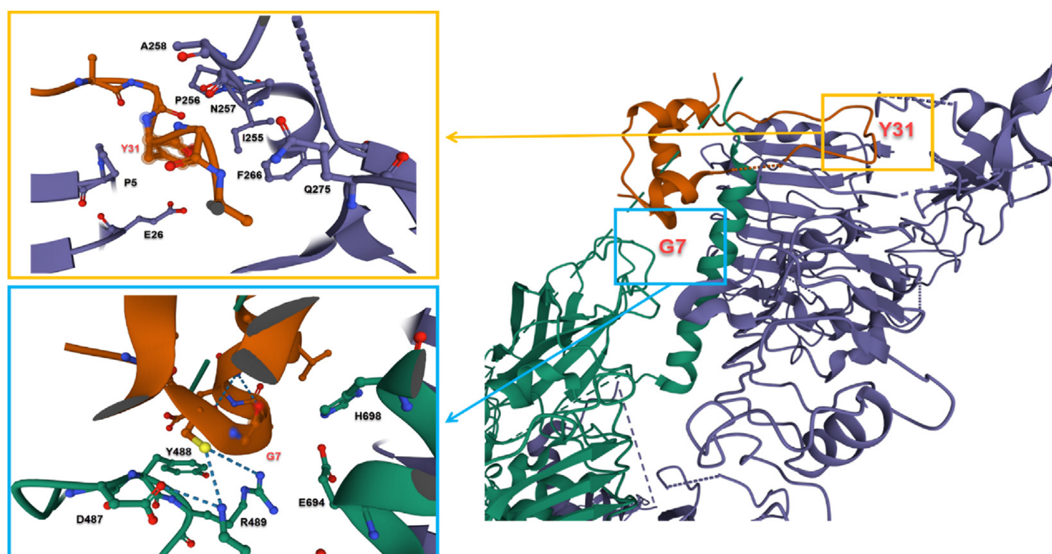

Figure 5: 3D image of IGF-1 bound to the IGF-1R highlighting the relative topographic positions of G7 and Y31 (IGF-1 numbering, which confers to B8 and C2). Zoom boxes magnify the IGF-1R environment that surrounds G7 and Y31. Image from RCSB PDB (rcsb.org), PDB ID 6PYH [36].

LCDV1-VILP at the IGF-1 receptor. Peptide **5** is LCDV1-VILP with an IGF-1 C-peptide (Figure 3A). It is much reduced in IGF-1 receptor activity relative to native IGF-1 with partial agonism that distinguishes it from LCDV1-VILP (Figure 3B), but no apparent difference in activity at the insulin receptor (Figure 3C). Peptide **4** represents the selective substitution in LCDV1-VILP at the A-chain to IGF-1 (Figure 3A), and it shows little activity at either of the two receptors (Figure 3B,C).

3.4. A single B-chain residue and the C-chain are central to the activity profile.

Having determined the critical importance of B-chain sequence to the differences in LCDV1-VILP and IGF-1 bioactivity, we identified by inspection that in LCDV1-VILP the serine that follows the first cysteine in the B-chain (residue B8, Table S1 employing insulin residue numbering) represents a unique residue in this position and is without precedent in any other insulin-related peptide, except a single mutant of proinsulin observed in a patient with neonatal diabetes [29]. Chemical mutagenesis studies have shown that this is a site of extreme importance to insulin structure and receptor binding affinity [30,31]. To assess the relative importance of this site for IGF-1R antagonism, we prepared LCDV1-VILP with a mutation of the B-chain serine to glycine (peptide **7**), (Fig. S27 and Table S1). The inverse change of glycine to serine in IGF-1 was assessed alone (peptide **8**) and in concert with a substitution to the LCDV1-VILP connecting peptide (analog **9**), (Figs. S28 and 29). As above, LCDV1-VILP remains notable in its ability to fully antagonize IGF-1 activity with an IC_{50} of approximately 100 nM (Figure 4B). IGF-1, at a very high concentration, can also serve as a self-antagonist, presumably owing to the negative cooperativity observed in IGF-1 binding [5,32]; however, this is observed at concentrations approximately one hundredfold greater than those of LCDV1-VILP and is incomplete even at 50 μM.
The single substitution of the serine with glycine in LCDV1-VILP (peptide **7**) lessens its ability to antagonize IGF-1R (Figure 4B) and significantly increases its agonism (Figure 4A). This same substitution, in concert with substitution to the IGF-1 C-peptide, provides an LCDV1-VILP-based analog (peptide **8**) that is nearly equivalent to native IGF-1 in activity profile. As shown above, substitution in LCDV1-VILP with the IGF-1 connecting peptide (peptide **5**) yielded a weak IGF-1R agonist that is reduced in antagonistic potency by more than tenfold relative to LCDV1-VILP (Figure 3B,D). The inverse substitution pattern in peptide **8,** where the glycine in IGF-1 is changed to serine and the C-peptide is converted to LCDV1-VILP sequence provides a peptide (peptide **9**) that is much suppressed in its inherent IGF-1R agonism and nearly as effective as LCDV1-VILP in its IGF-1R antagonism (Figure 4A,B).

4. DISCUSSION

The recent discovery of viral insulin-like peptides has expanded our view of the evolutionary relationship within this important family of peptides [24,33]. Here, we report much improved methods for the chemical synthesis of three viral insulin-like peptides, and more importantly, demonstrate the unique structure—function relationships of LCDV1-VILP which define the structural basis of its behavior as a potent, full and selective competitive antagonist of the IGF-1R.
The optimized chemical synthesis we describe employs stepwise formation of the three disulfide bonds using an orthogonal protection scheme of Trt, Acm, and *t*Bu cysteine in peptide assembly. Isoacyl-dipeptides were used to improve chain assembly and the biophysical properties of synthetic intermediates. Chimeric peptides of LCDV1-VILP and IGF-1 presented an extra synthetic complexity that was successfully addressed with an intermediate fragment ligation. Two-chain VILPs were obtained using a comparable synthetic strategy employed in the synthesis of single-chain VILPs. Peptides with only one disulfide pairing pattern were obtained, and the integrity of the final products was confirmed by LC-MS and peptide mapping. The synthetic yield within this set of peptides varied fivefold in a range of 0.3—1.5%, based on initial resin loading. The single-chain peptide precursors assembled on resin were at the higher end of this range and those requiring intermediate fragment ligament at the lower end with LCDV1 were obtained at 0.7% total yield. The two-chain analogs were of comparable yield to the single-chain analogs prepared by ligation, with two-chain LCDV1 obtained in 0.3% yield. Attempts to prepare these peptides by biosynthesis in *Escherichia coli* proved unsuccessful where the physical

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properties of the unfolded linear peptides proved to limit the oxidative formation of the three disulfides in aqueous alkaline buffers.

The biological assessment of the single-chain VILPs aligns with our previous study, but shows enhanced potency reflecting the improved methods of synthesis that provide enhanced purity. Much like native IGF-1, GIV-VILP and SGIV-VILP are potent agonists at IGF-1R, with appreciable selectivity relative to IR-B (Figure 2A,B). The removal of the C-peptide reduces potency at IGF-1R but increases it at both insulin receptor isoforms. This change in relative activity at the insulin and IGF-1 receptors is directionally comparable to that reported for human IGF-1 [4,27]. The insulin receptor potency of the two-chain analogs of GIV-VILP and SGIV-VILP has been separately observed to approach the IR activity of IGF-1 [34]. However, LCDV1-VILP is different from the two other viral insulin-like peptides. It has a primary sequence that is atypical with a unique connecting peptide and devoid of the tyrosine at the second position of the IGF-1 C-peptide that confers high potency at IGF-1R [4,35]. The relative location of the C-peptide in the three-dimensional structure of IGF-1, when bound to the receptor complex is shown in Figure 5 [36]. The aromatic side chain of the C2 tyrosine (Y31) of IGF1 sits between two proline residues residing in the L1 and CRD domains, respectively. It is further bordered by other conserved hydrophobic receptor residues. LCDV1-VILP also notably differs in its insulin-like A- and B-chains, where the intramolecular A-chain disulfide is spaced by an atypical, additional fifth amino acid (A6-A12), and the B-chain is different at sites that are of high homology in the insulin family and of recognized importance to biological structure and activity [1—5,28—31].

LCDV1-VILP is a weak and partial insulin receptor agonist that loses potency when its C-peptide is removed, which is contrary to what is observed for IGF-1, GIV-VILP, and SGIV-VILP. As a single-chain peptide, it displays low-level IGF-1R agonism in a dose range of 0.1—10 nM and potent, but full IGF-1R antagonism at higher concentrations. The two-chain form of LCDV1-VILP is distinctly different and displays agonism at the IGF-1R, indicating that the C-chain is important for antagonism. It is also a low potency agonist at both insulin receptor isoforms, something that has not been reported when these peptides were prepared by a different procedure and studied at lower concentrations [34]. Through synthetic shuffling of the A, B, and C chains of LCDV1-VILP and IGF-1, the central importance of the B-chain to the differences in LCDV1-VILP and IGF-1 bioactivities was identified. Surprisingly, the A-chain, where the atypical intramolecular disulfide resides, was of little consequence in determining its unique bioactivity; but it may still serve an undetermined function in biosynthesis or biophysical stability. All three analogs that contain an IGF-1 B chain displayed full and potent agonism of IGF-1R, which stands in direct contrast to the three analogs that did not (Figure 3B).

The notable difference in the IGF-1 potency of the chimeric peptides that contain the IGF-1 B-chain [1,2,6] from those with lCDV1-VLIP sequence [3—5] focused attention on structural changes that might singularly justify the variance in bioactivity. The change at the first residue that follows the first B-chain cysteine, which is glycine in human IGF-1 and serine in LCDV1-VILP is observed in only one mutant, proinsulin, among all reported insulin-like peptides sequences [29]. This includes GIV-VILP, SGIV-VILP, and the more distant insulin-related peptides, such as relaxin that signal through GPCRs. In insulin itself, changes at this position have been demonstrated to dramatically destroy insulin receptor binding affinity when substituted by alanine, D-alanine, or D-serine; but surprisingly, L-serine substitution retains high receptor affinity, although bioactivity was not measured [30,31]. It is believed to be an anchoring amino acid for the turn at the initiation of the B-chain central helix that is of seminal importance to insulin structure and function. The single mutation of the B8 glycine to serine constitutes the basis of neonatal diabetes that has been ascribed to poor folding in the proinsulin precursor that serves to illustrate the pathological impact of this single mutation on structure in a related single-chain insulin-like peptide [29—31].

The relative location of B8Gly in IGF-1 as reported for the receptor complex is shown in Figure 5 [36]. The inverse change of serine to glycine in LCDV1-VILP introduces a very significant increase in IGF-1R agonism and the simultaneous inclusion of the IGF-1 C-peptide nearly restores full biological activity (Figure 4A,B). Strikingly, the same two reciprocal changes in IGF-1 convert it to a ligand that behaves similarly to LCDV1-VILP. Consequently, the serine in LCDV1-VILP serves to suppress IGF-1R agonism that is otherwise possible with glycine (peptides **1, 2, 6**) (Fig 3B), and the LCDV1-VILP C-peptide further suppresses the activity relative to the IGF-1 C-peptide (peptide **9**) to silence agonism (Figure 4A). The relative location of the C-peptide in IGF-1 is also described for the receptor complex in Figure 5 and recognized to be of importance to full bioactivity. The IGF-1R active conformation is reported to be asymmetric and to bind a single ligand, with the unoccupied receptor alpha-CT restrained and disfavoring the binding of a second ligand [36]. The binding of a second ligand is favored at increased ligand concentration and serves to decrease functional activity. Our results reflect this negative cooperativity in the bell-shaped biological response to IGF-1, where concentrations much higher than required for full activity reduce receptor activation (Figure 3B). The change in the C-peptide sequence to that possessed by LCDV1 derived insulin-like peptide in concert with the B8Ser change was observed to strengthen negative cooperativity relative to that which we observed in IGF-1 (Figure 4). Tyr31, the second residue in the connecting peptide of IGF-1, is well recognized for its importance in IGF-1 agonism [5,36]. This residue is absent in LCDV1, as a repeating triple arginine sequence (RRR) initiates the connecting peptide sequence of LCDV1. In continuing studies, we have observed that when LCDV1 is selectively substituted at residue 31 with tyrosine, the peptide retains high potency antagonism. Consequently, we conclude that the absence of this tyrosine in LCDV1 connecting peptides is not the basis for antagonism. The substitution of the RRR with GYG, as it appears in IGF-1, significantly weakens antagonism (results not presented) indicating that the two arginine residues are necessary for functional antagonism.

The discovery of IGF-1R antagonism in LCDV1-VILP initiates a new chapter in the rich history associated with the determination of the structural basis for insulin and IGF-1 receptor action [1—7]. Without high potency, a full antagonist has emerged from chemical modifications of insulin or IGF-1. The single synthetic analog that does not exist naturally constitutes a two amino acid-modified IGF-1 that we observed to be a weak, partial agonist, when compared to LCDV1-VILP (Fig. S17). While IGF-1R antagonism can be achieved by antibodies and kinase inhibitors, they are fundamentally different in their biodistribution and selectivity. Consequently, LCDV1-VILP represents an important alternative approach to IGF-1R silencing. Whether these results can be extended through further structural modification to identify antagonism at the homologous insulin receptor, remains to be determined. However, ligands that demonstrate insulin agonism devoid of activity at the IGF-1R (peptide **3**) or are suppressive of endogenous IGF-1 activity might eventually constitute to be less mitogenic, and thus, a safer form of insulin therapy. Collectively, the results demonstrate that natural forms of structural diversity such as these peptides derived from viruses continue to constitute a vital

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source in the discovery of novel bioactivity that complements high throughput synthetic methods that have more recently transformed protein optimization [23,24,33].

AUTHOR CONTRIBUTIONS

FZ designed, performed, and analyzed the peptide synthesis. RDD designed and analyzed the peptide synthesis and *in vitro* studies. VG designed, performed, and analyzed the *in vitro* studies. EA and CRK interpreted the results. All authors contributed to the writing and editing of the article.

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CONFLICT OF INTEREST

VG is presently employed by Novo Nordisk.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2021.101316.

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