The C-terminus of the B-chain of human insulin-like peptide 5 is critical for cognate RXFP4 receptor activity

Nitin A. Patil^{1,2}, Ross A. D. Bathgate^{1,3}, Martina Kocan⁴, Sheng Yu Ang⁴, Julien Tailhades¹, Frances Separovic², Roger Summers⁴, Johannes Grosse⁵, Richard A. Hughes⁶, John D. Wade^{1,2}, Mohammed Akhter Hossain^{1,2*}

¹Florey Institute for Neuroscience & Mental Health, ²School of Chemistry, Bio21 Institute, ³Department of Biochemistry and Molecular Biology, ⁶Department of Pharmacology, University of Melbourne; ⁴Monash Institute of Pharmaceutical Sciences, Parkville, Victoria, Australia; ⁵Takeda Cambridge Ltd., Cambridge, United Kingdom

Address correspondence to

Dr. Mohammad Akhter Hossain

Email: akhter.hossain@unimelb.edu.au

Tel: +61 3 83440414

Professor John Wade

john.wade@florey.edu.au

Howard Florey Research Laboratories

Florey Institute for Neuroscience & Mental Health

University of Melbourne, Melbourne

Victoria, Australia

Conflict of Interest

The authors declare that they have no conflict of interest.

Research involving Human Participants and/or Animals

“This article does not contain any studies with human participants performed by any of the authors.”

“This article does not contain any studies with animals performed by any of the authors.”

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Abstract

Insulin-like peptide 5 (INSL5) is an orexigenic peptide hormone belonging to the relaxin family of peptides. It is expressed primarily in the L-cells of the colon and has a postulated key role in regulating food intake. Its G protein-coupled receptor, RXFP4, is a potential drug target for treating obesity and anorexia. We studied the effect of modification of the C-terminus of the A and B-chains of human INSL5 on RXFP4 binding and activation. Three variants of human INSL5 were prepared using solid phase peptide synthesis and subsequent sequential regioselective disulfide bond formation. The peptides were synthesized as C-terminal acids (both A- and B-chains with free C-termini, i.e. the native form), amides (both chains as the C-terminal amide) and one analogue with the C-terminus of its A-chain as the amide and the C-terminus of the B-chain as the acid. The results showed that C-terminus of the B-chain is more important than that of the A-chain for RXFP4 binding and activity. Amidation of the A-chain C-terminus does not have any effect on the INSL5 activity. The difference in RXFP4 binding and activation between the three peptides is believed to be due to electrostatic interaction of the free carboxylate of INSL5 with a positively charged residue (s), either situated within the INSL5 molecule itself or in the receptor extracellular loops.

Key words: insulin-like peptide 5, INSL5, relaxin, peptide

Introduction

Insulin-like peptide 5 (INSL5) is a peptide hormone member of the insulin superfamily that comprises insulin, insulin-like growth factors I and II (IGFI-II), relaxins 1–3 and insulin like peptides 3–6 (Bathgate et al. 2013). Similar to other family peptides, INSL5 is expressed as a single-chain prohormone that is converted to the mature peptide by formation of three disulfide bonds (one intra-chain and two inter-chains) with two chains (A-chain and B-chain) (Hossain et al. 2008). Its tertiary structure has been determined using solution NMR spectroscopy which shows a high degree of similarity to known structures from other members of the family, particularly within the regions held together by the three disulfide bonds (Haugaard-jönsson et al. 2009). INSL5 binds and activates its cognate receptor known as relaxin-family peptide receptor-4 (RXFP4) (Conklin et al. 1999; Liu et al. 2005). The mRNA of INSL5 is primarily expressed in the colonic L-cells with minor expression in other organs (Grosse et al. 2014). INSL5 has recently been discovered to play an important role in appetite regulation (Grosse et al. 2014). These findings suggest that RXFP4 agonists and antagonists may be prospective therapeutic agents for appetite-related disorders such as cachexia, anorexia and obesity. Considering these important applications, a critical goal is to understand the key aspects of ligand-receptor (INSL5-RXFP4) interaction.

A preliminary structure-activity relationship (SAR) study of mouse INSL5 showed that the carboxyl group at the C-terminus (free C-terminus) of INSL5 plays an important role in the interaction with its receptor (Belgi et al. 2011). Similarly, the free C-terminal (-COOH group) of the B-chain of the related peptide relaxin-3 plays an important role in interacting with its receptor RXFP3, whereas the A-chain C-terminus can be amidated (Shabanpoor et al. 2013a). Since the receptors for both relaxin-3 and INSL5 are similar, we hypothesized that the C-terminus of the INSL5 B-chain, and not that of the INSL5 A-chain, may be crucial for RXFP4 activity. In order to investigate the influence of C-terminus of both chains of human INSL5 (hINSL5), three analogues (Fig. 1) were prepared using solid phase peptide synthesis followed by sequential regioselective folding, and further assayed for RXFP4 binding, cAMP activity and ERK1/2 activity.

Materials and Methods

Materials: All 9-fluorenylmethoxycarbonyl (Fmoc) protected L- α -amino acids and O-(6-chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HCTU) were obtained from GL Biochem (Shanghai, China). Fmoc-Cys(Acm)-Ac-S-TentaGel (0.23 mmol/g) resin was obtained from Rapp Polymere GmbH (Tübingen, Germany). Fmoc-Trp(Boc)-PHB-TentaGel (0.23 mmol/g) and Fmoc-PAL-PEG-PS (0.19 mmol/g) resins were purchased from Applied Biosystems (Melbourne, Australia). All peptide synthesis solvents were obtained from Merck (Melbourne, Australia). 2, 2'-dipyridyl disulfide (DPDS) was from Fluka (Bucha, Switzerland) and Trifluoromethanesulfonic acid (TFMSA) from MP Biomedicals (Sydney, Australia). All other reagents for peptide synthesis and bovine serum albumin (BSA) and 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) were obtained from Sigma-Aldrich (Sydney, Australia).

Peptide synthesis: Linear peptides with a C-terminal amide were assembled on Fmoc-PAL-PEG-PS resin and the A- and B-chains with C-terminal acid were synthesised using Fmoc-Cys)-Ac-S-TentaGel and Fmoc-Trp(Boc)-PHB-TentaGel, respectively. All the B-chain peptides were synthesised using a CEM LibertyTM microwave peptide synthesizer (AI Scientific, Scarborough, Australia), whereas all the A-chain peptides were synthesized by manual stepwise solid phase synthesis. Each peptide was synthesised on a 0.1-mmol scale with excess of Fmoc-protected amino acids, HCTU and DIPEA (5:5:10). Fmoc removal was carried out using 20% piperidine in DMF. The peptides were cleaved from solid support using a cocktail of TFA:anisole:DODT:TIPS (94:3:2:1, v/v) for 2 hr. Combination of the two chains was carried out using sequential regioselective disulfide bond formation as previously described (Shabanpoor et al. 2013b). The crude peptides were analyzed and purified by RP-HPLC on Phenomenex Gemini columns (4.6 $\times$ 250 mm, C18, 5 μ m; 19 $\times$ 150 mm, C18, 5 μ m) using H₂O and a gradient of acetonitrile with 0.1 % TFA as solvent A and B, respectively. The characterization of peptides was carried using MALDI-TOF/TOF mass spectroscopy (Bruker Daltonics, Germany) with sinapinic acid as matrix.

Circular dichroism (CD) spectroscopy: The secondary structural changes of the peptides were measured by recording their CD spectra on JASCO model J815 spectropolarimeter as previously described (Shabanpoor et al. 2013a; Najbar et al. 1997). The CD spectra were recorded in 10 mM phosphate buffered saline (PBS) with peptide concentrations made up to 0.2 μ g/ μ l.

Binding assays: The ability of the peptides to bind to the RXFP4 receptor was measured using Chinese hamster ovary CHO-K1 cells stably expressing human RXFP4 (CHO-K1-RXFP4) (Belgi et al. 2013). The transfected cells were plated out at 50,000 cells per well per 200 μ L in a 96-well ViewPlate. Competition binding

experiments were performed with 5 nM of europium-labeled Eu(A)-mouse INSL5 acid in the presence of increasing amounts of peptides following the protocol described previously (Belgi et al. 2013). Fluorescence was measured at excitation wavelength of 340 nm and emission wavelength of 614 nm on a BMG POLARstar plate reader (BMG Labtech). Each concentration point was measured in triplicate, in three independently performed experiments. GraphPad PRISM 6 (GraphPad Inc., San Diego, CA) was used to analyze the receptor-binding assays data that are expressed as mean $\pm$ SEM.

cAMP activity assays: The receptor activation by the peptides was assessed based on their ability to inhibit cAMP activity in CHO-K1-RXFP4 transfected with a pCRE (cAMP Response Element) β -galactosidase reporter plasmid (Scott et al. 2006; Belgi et al. 2011). CHO-K1-RXFP4 cells were plated out at 8,000 per well per 100 μ L in a Corning cell bind 96-well plate on day 1 and transfected with the pCRE β -galactosidase reporter plasmid on day 2. One day later the cells were stimulated with 1 μ M of forskolin plus or minus increasing concentrations of each peptide as previously described (Belgi et al. 2013). cAMP activity is assessed with a β -galactosidase colorimetric assay measuring absorbance at 570 nm on a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Sydney, Australia). Each concentration point was measured in triplicate, and each experiment was performed independently at least three times. GraphPad PRISM 6 (GraphPad Inc., San Diego, CA) was used to analyze the cAMP inhibition assays data that are expressed as mean $\pm$ SEM.

ERK 1/2 phosphorylation AlphaScreen SureFire assay: CHO-K1-RXFP4 cells were plated out at 50,000 cells per well per 100 μ L in a 96-well plate and grown overnight in complete media (DMEM/F12) at 37°C in a humidified atmosphere of 5% CO₂. Cells were washed twice with PBS and then serum-starved for 6 h followed by stimulation with an increasing concentration of each peptide, serum free DMEM/ F12 as vehicle control, or 10% FBS in DMEM/F12 as positive control for 5 min at 37°C. Following stimulation, the cells were lysed using 100 μ L of lysis buffer and frozen at -20°C according to the assay manufacturer's instructions. For detection of ERK1/2 kinase phosphorylation, the thawed cell lysate, 4 μ L, was transferred to white 384-well microplates (Proxiplates), and 5 μ L of the combination buffer with AlphaScreen donor beads (40 parts reaction buffer, 10 parts activation buffer, and 1 part acceptor beads) was added. The plate was incubated for 2 h at 23°C in the dark on an oscillating platform. Subsequently, 2 μ L of the dilution buffer with AlphaScreen acceptor beads (20 parts dilution buffer and 1 part donor beads) was added. The plate was incubated for 2 h at 23°C in the dark on the oscillating platform one more time. The AlphaScreen signal (counts per second) was measured in 384-well

microplates (Proxiplates) on an EnVision Multilabel Plate Reader (PerkinElmer Life and Analytical Sciences) with excitation at 680 nm and emission at 520 to 620 nm. Results are the mean $\pm$ SEM of three independent experiments.

Result and discussion

To determine the effect of the C-termini of hINSL5 on the receptor binding and activity, three hINSL5 analogues, hINSL5-acid (both A-and B-chains C-termini acid), hINSL5-amide (both A-and B-chains C-termini amide), and hINSL5-B-acid (A-chain with C-terminus amide and B-chain with C-terminus acid) were synthesized using regioselective disulfide bond formation protocol (Hossain et al. 2008). We have previously shown that INSL5 is the most difficult peptide to synthesize among the members of the insulin-relaxin superfamily (Hossain et al. 2008). Considering the challenges in the solid phase peptide synthesis of the A-chain, we carried out the synthesis manually with each coupling performed with 4 equivalent excess of Fmoc-amino acids, HCTU and DIEA. The use of pseudoproline Leu-Ser(ψ^{MeMe} Pro)-OH enabled the successful synthesis of the A-chain (Hossain et al. 2008). The post-synthetic sequential disulfide bond formation was employed as reported in our previous work (Hossain et al. 2006). The successful synthesis of all three analogues was confirmed by MALDI-TOF mass spectrometric and amino acid analysis (Table 1). The overall yields of the hINSL5 analogues in this study (based on the purified B-chain) shown in Table 1, again confirmed that amide peptides (C-termini amides) were easier to assemble compared with acid peptides (C-termini acid).

The binding and cAMP assays showed that hINSL5-amide (both chain amide) has markedly lower affinity for RXFP4 compared with hINSL5-acid (both chain acid) and importantly showed minimal cAMP activity at concentrations up to 1 μ M (Fig 2, Table 2). These results are consistent with our previous study with the murine homologue (Belgi et al. 2011). Activation of RXFP4 also induces ERK1/2 which is another signalling molecule involved in RXFP4-mediated cellular pathways (Belgi et al. 2013). Thus, we also tested all the analogues on human RXFP4 receptor in the ERK1/2 phosphorylation assay (Fig 3). Consistent with cAMP results, hINSL5-acid showed clear dose dependent activation of ERK1/2 phosphorylation whereas the hINSL5-amide demonstrated no activation at concentrations up to 1 μ M (Fig. 3). Finally, to determine the particular effect of the C-termini of individual chains (A-chain or B-chain) we tested binding and activity of hINSL5-B-acid (B acid/A amide). Interestingly, hINSL5-B-acid was found to be almost equipotent to hINSL5-acid both in cAMP

(Fig. 2; Table 2) and pERK assays (Fig. 3) and also demonstrated equivalent binding affinity for RXFP4 (Fig 2, Table 2). This result indicates that a free B-chain C-terminus is important for high RXFP4 binding and activity. A similar trend was observed with human relaxin-3 and its RXFP3 interaction (Shabanpoor et al. 2013a) which reflects the fact that both RXFP3 and 4 have considerable structural similarities. On the other hand, we have recently shown that modifications to the C-terminus of either of the A- or B-chain of human relaxin-2 have no effect on RXFP1 activity (Haugaard-Kedström et al. 2015). However, the RXFP1 receptor has a complex structure with a large ectodomain and the mode of ligand interaction is completely different to RXFP3 and 4 (Hossain et al. 2008; Kong et al. 2010).

We carried out CD spectroscopy analysis of the peptides in order to obtain an insight into their secondary structures. Two of the analogues (INSL5 amide and INSL5-B-acid) were found to aggregate in solution (both in phosphate and TFE buffers). Therefore, it was not possible to compare their secondary structures. However, based on our previous studies with related truncated INSL5 or relaxin-3 analogues (Belgi et al. 2011; Shabanpoor et al. 2013a), we do not expect that a small change at the C-termini (-COOH to -NH₂) would cause dramatic change in overall structural fold. The combined loss of binding and activation in hINSL5 amide suggests that the C-terminal Trp is involved in receptor binding similar to what we have previously demonstrated for mouse INSL5 (Belgi et al. 2011). Furthermore, these data suggest there may be an electrostatic interaction of the free carboxylate of the C-terminal Trp with positively charged residue (s) in the RXFP4 receptor. Indeed, a recent study has demonstrated that Arg^{B23} of the INSL5 B-chain interacts via an electrostatic interaction with both Glu¹⁰⁰ and Asp¹⁰⁴ of RXFP4 (Wang et al. 2014); the role of Trp^{B24} was not investigated.

Conclusion

We have chemically prepared three complex two-chain, three-disulfide-bond hINSL5 peptides possessing either or both C-terminal acids or amides. We observed that the free carboxyl group at the C-terminus of the B chain of hINSL5 is crucial for high RXFP4 affinity and activity. The C-terminal amide peptides are easier to synthesize compared with the acid peptides. Considering the difficulty in synthesis and poor recovery of native INSL5 (INSL5-acid), INSL5-B-acid will be an important template for future structure-function studies.

Acknowledgement

This research was partly funded by NHMRC (Australia) project grants (1023321, 1065481, 1023078) to M. A. H, R. A. D. B and J. D. W and ARC linkage grant (LP120100654) to R.A.H. We are grateful to Tania Ferraro and Sharon Layfield for assistance with biochemical assays. During these studies, M.A.H. was the recipient of Florey Foundation Fellowships. R.A.D.B. is an NHMRC Senior Research Fellow, and J.D.W. is an NHMRC Principal Research Fellow. Studies at the FINMH were supported by the Victorian Government's Operational Infrastructure Support Program.

Abbreviations used

INSL5, insulin-like peptide 5; mINSL5, mouse insulin-like peptide 5; hINSL5, human insulin-like peptide 5; IGFI, insulin-like growth factor I; IGFII, insulin-like growth factor II; INSL3, insulin-like peptide 3; INSL4, insulin-like peptide 4; INSL6, insulin-like peptide 6; RXFP3, relaxin-family peptide receptor 3; RXFP4, relaxin-family peptide receptor 4; Boc, tert-butoxycarbonyl; AcM, acetamidomethyl; cAMP, 3',5'-cyclic adenosine monophosphate; PEG-PS, poly(ethylene glycol)-polystyrene; CD, circular dichroism; CHO, Chinese hamster ovary; DMF, dimethylformamide; DIEA, diisopropylethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; GPCR, G-protein coupled receptor; HCTU, O-(6-chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyl uronium hexafluorophosphate; INSLs, insulin-like peptides; RXFP, relaxin-family peptide; tBu, tert-butyl; TIPS, triisopropylsilane; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; DODT, 3,6-dioxa-1,8-octanedithiol; DPDS, 2,2'-dipyridyldisulfide; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ERK, extracellular-signal-regulated kinase; DMEM, Dulbecco's modified Eagle's medium.

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Table 1: Characterization of hINSL5 variants by MALDI-TOF mass spectrometry and amino acid analysis.

Sr. No.	Peptide	Observed mass (Theoretical mass)	Overall Yield	Peptide Content
1	hINSL5-acid	5047.18 (5044.78)	0.8 %	26.61%
3	hINSL5-B-acid	5046.12 (5043.80)	3.2%	75.39%
4	hINSL5-amide	5043.29 (5042.81)	8%	45.71%

Table 2: Pooled binding affinity (pKi), cAMP activity (pEC₅₀) and ERK1/2 data for hINSL5 variants at RXFP4.

INSL5 variants	Binding (pKi)	N	cAMP activity (pEC ₅₀)	N
hINSL5-acid	7.38 ± 0.04	4	8.34 ± 0.22	3
hINSL5-B-acid	7.31 ± 0.08	5	7.78 ± 0.01*	3
hINSL5-amide	<6	4	<5	3

* p<0.05 vs hInsl5-acid

Figure Legends

Figure 1. Primary structure of human INSL5 analogues: **hINSL5-acid**, **hINSL5-B-acid** and **hINSL5 amide**. C-terminal modifications are highlighted

Figure 2. Comparison of *in vitro* RXFP4 receptor bioassays of hINSL5-B-acid and hINSL5-amide with hINSL5-acid: **(A)** competition binding curve of analogues competing with 5 nM of Eu-(A)-mINSL5; **(B)** cAMP inhibition assay of hINSL5-B-acid, hINSL5-amide and hINSL5-acid. The data are the result of n= 3-4 independent experiments and are expressed as mean $\pm$ SEM.

Figure 3. Comparison of *in vitro* RXFP4 receptor p-ERK1/2 activity of hINSL5-B-acid and hINSL5-amide with hINSL5-acid: Concentration-dependent activation of ERK1/2 phosphorylation: The data are the result of n= 3-4 independent experiments and are expressed as mean $\pm$ SEM.

Figure 1

Figure 2

a) Eu-INSL5 competition binding

b) RXFP4 cAMP activity

Figure 3