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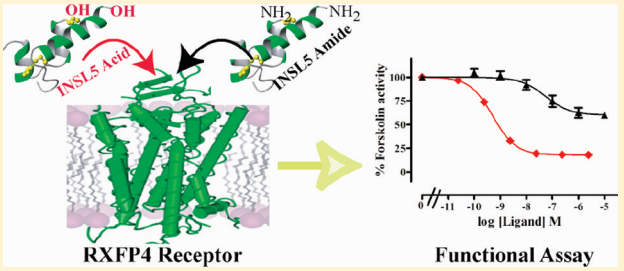
Structure and Function Relationship of Murine Insulin-like Peptide 5 (INSL5): Free C-Terminus Is Essential for RXFP4 Receptor Binding and Activation

Alessia Belgi,[†,‡] Mohammed A. Hossain,*[,†,§] Fazel Shabanpoor,[†,§] Linda Chan,[†,§] Suode Zhang,[†] Ross A. D. Bathgate,[†] Geoffrey W. Tregear,[†,‡] and John D. Wade*[,†,§]

[†]Florey Neuroscience Institutes, [‡]Department of Biochemistry and Molecular Biology, and [§]School of Chemistry, The University of Melbourne, Victoria 3010, Australia

ABSTRACT: Insulin-like peptide 5 (INSL5) is a member of insulin/relaxin superfamily of peptides. It has recently been identified as the cognate ligand for the G-protein-coupled receptor, RXFP4. Although the complete physiological role of this naturally occurring peptide is still under investigation, there is evidence that it acts to both stimulate appetite and activate colon motility. This suggests that both agonists and antagonists of the peptide may have potential therapeutic applications. To further investigate the physiological role of this peptide and because of the ready availability of the mouse as an experimental animal, the preparation of mouse INSL5 was undertaken. Because of its complex structure and the intractable nature of the two constituent chains, different solid phase synthesis strategies were investigated, including the use of a temporary B-chain solubilizing tag. Unfortunately, none provided significantly improved yield of purified mouse INSL5 which reflects the complexity of this peptide. In addition to the native peptide, two mouse INSL5 analogues were also prepared. One had its two chains as C-terminal amides, and the other contained a europium chelate monolabel for use in RXFP4 receptor assays. It was found that the INSL5 amide was substantially less potent than the native acid form. A similar observation was made for the human peptide acid and amide, highlighting the necessity for free C-terminal carboxylates for function. Two additional human INSL5 analogues were prepared to further investigate the necessity of a free C-terminal. The results together provide a first insight into the mechanism whereby INSL5 binds to and activates RXFP4.



The human insulin superfamily consists of insulin-like peptides 3, 4, 5, and 6 (INSL3, -4, -5, and -6), relaxins-1 (H1 relaxin), -2 (H2 relaxin), and -3 (H3 relaxin), insulin, and insulin-like growth factors I and II (IGFI and IGFII).[1] With the exception of IGFs I and II which are single-chain peptides, all members consist of (or are predicted to consist of) two chains, A and B, which are linked by two disulfide bonds with a third intramolecular disulfide within the A-chain in a characteristic insulin arrangement.

INSL5 was first identified in 1999 by an Expressed Sequence Tags (EST) database screening for the presence of the B-chain Cys motif conserved within the insulin superfamily.[2] The gene is highly expressed in the colon, especially in the distal colon, and also in lower levels in thymus, testis, brain (with highest expression in fetal brain compared to adult brain), kidney, prostate, lung, ovary, thyroid, placenta, and pituitary gland.[2,3] The predicted primary structure of INSL5 is a 21-residue A-chain disulfide-linked to a 25-residue B-chain. The sequences of several mammalian INSL5s are now known, and there is considerable homology between species.[4] The tertiary structure of synthetic human INSL5 has been determined by solution NMR spectroscopy and shown to possess a characteristic insulin/relaxin-like fold that consists of three helical segments that enclose a hydrophobic core.[4] The complete physiological role of this natural peptide hormone is still under investigation, but recent evidence outlined in a patent application has suggested it to be an orexigenic peptide and therefore involved in the regulation of appetite.[5] It is also believed to be involved in the activation of colon motility.[5]

INSL5 is the most recent insulin superfamily member for which a receptor has been identified, the G-protein coupled receptor RXFP4, which is specifically coupled to a $G_{i/o}$ protein.[3,6,7] The receptor was originally associated with relaxin-3 as this peptide could bind to both this orphan receptor and to RXFP3. Whereas the latter receptor is predominantly expressed in the brain and both ligand and receptor are highly conserved in different species, RXFP4 expression is primarily restricted to the colon but also to other peripheral tissues such as thyroid, salivary gland, prostate, placenta, thymus, testis, and kidney. It is also expressed in the brain[7] but in low levels and in areas distinct from those of relaxin-3 expression. Genetic analysis revealed RXFP4 is highly conserved within human, monkey, cow, and pig with more than 84% homology, but it has lower homology with mouse RXFP4; in contrast, relaxin-3 genes are

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highly conserved among species, further supporting that the native ligand for RXFP4 differs from relaxin-3.[8] The ability of INSL5 to bind to and activate these receptors was subsequently tested, and it showed high affinity as well as high potency only for RXFP4 and not for other RXFP receptors.[3]

Given the postulated role of INSL5 in feeding and the corresponding potential advantages of both agonists and antagonists as regulators, we have commenced a systematic structure–function relationship study of this peptide. However, an earlier chemical assembly of human INSL5 was unexpectedly difficult due, in part, to the susceptibility to aspartimide formation of an Asp-Gly sequence in the middle of the A-chain and the propensity of the B-chain for resin-bound aggregation.[9] For this reason, we undertook to prepare mouse INSL5 for *in vitro* and *in vivo* studies as it was deemed to be a potentially easier target. It has 71% homology with the human sequence[2] (Figure 1), but the nonconserved residues give the

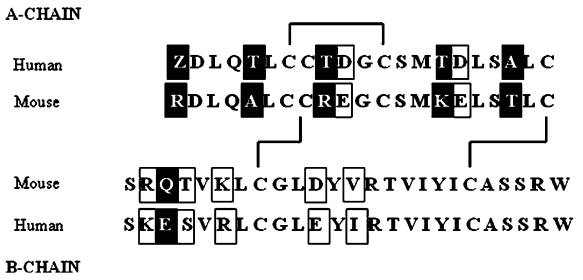

Figure 1. Primary structure of human INSL5 and mouse INSL5. The differences between the two sequences are highlighted with black and white boxes. Black boxes correspond to changes in the nature of the residues, for example from a hydrophobic residue in the human sequence (ThrA9) to a charged residue in the mouse sequence (ArgA9); white boxes are used for conserved mutations. Z in the A-chain of human INSL5 corresponds to pyroglutamic acid.

peptide chains an overall greater positive charge that may improve their solubility and subsequent handling and purification by RP-HPLC. Furthermore, the Asp-Gly sequence within the human A-chain is replaced with a Glu-Gly sequence in the mouse, thus obviating the propensity for aspartimide formation during the assembly of this chain. In addition, *in vivo* experiments are typically conducted using mice which are readily available. Consequently, in the present work we describe the preparation and biological activity of mouse INSL5 as well as of two analogues, one in which the C-terminals of the two chains are amides and the other in which a europium label is introduced for the purpose of development of a nonradioactive binding bioassay.

EXPERIMENTAL PROCEDURES

Materials. 9-Fluoroenylmethoxycarbonyl (Fmoc) protected L-α-amino acids and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU) were purchased from GL Biochem (Shanghai, China). *N*,*N*-Dimethylformamide (DMF), piperidine, trifluoroacetic acid (TFA), and 2-(1*H*-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HATU) were obtained from Auspep (Melbourne, Australia). Fmoc-Trp(Boc)-PAC-PEG-PS and Fmoc-PAL-PEG-PS resins with substitution of *ca*. 0.20 mmol/g were purchased from Applied Biosystems Inc. (Melbourne, Australia); TentaGel S PHB Cys(Acm) Fmoc and TentaGel S PHB Cys(tBu) Fmoc were purchased from Rapp Polymere (Tübingen, Germany). Fmoc-Leu-Ser-($\Psi^{Me,Me}$Pro)-OH, acetonitrile, dichloromethane, diethyl ether, and methanol were from Merck (Melbourne, Australia), and 2-pyridyl disulfide (DPDS) was purchased from Fluka (Buchs, Switzerland). Na_2HPO_4 and $MgSO_4$ were purchased from BDH Chemicals (Sydney, Australia). KH_2PO_4 and $MnCl_2$ were purchased from Ajax Chemicals (Sydney, Australia). $CaCl_2$ was purchased from Mallinckrodt (Melbourne, Australia). Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12), L-glutamine, penicillin/streptomycin, and fetal bovine serum were all purchased from Invitrogen (Melbourne, Australia). Chlorophenol red β-D-galactopyranoside was purchased from Roche (Mannheim, Germany). Diethylenetriaminepentaacetic acid (DTPA)-tetra-(tBu ester) was purchased from Macrocyclics (Dallas, TX). All other reagents were from Sigma-Aldrich (Sydney, Australia).

Methods. *Peptide Synthesis.* The nonsolubilizing tagged peptides were solid phase synthesized on PAC-PEG-PS resin if C-terminal acids or on PAL-PEG-PS if C-terminal amides. Peptides C-terminally linked to a solubilizing tag were assembled on PAL-PEG-PS such that the polycationic tag itself possessed a C-terminal amide. SPPS employed Fmoc chemistry on one of the following instruments: CEM Liberty microwave peptide synthesizer (Ai Scientific, Queensland, Australia), PerSeptive Biosystems Pioneer continuous flow peptide synthesizer (Framingham, MA), or Protein Technologies Tribute batch-wise peptide synthesizer (Tucson, AZ). The side-chain-protecting groups of trifunctional amino acids were TFA-labile, except for *tert*-butyl (tBu)-protected and acetamidomethyl (Acm)-protected cysteine Cys in the A-chain and Cys(Acm) in the B-chain. The peptides were synthesized on a either 0.1 or 0.2 mmol scale using instrument default protocols with either a 4- or 5-fold molar excess of Fmoc-protected amino acids (0.4 or 0.5 mmol for a 0.1 mmol scale; 0.8 or 1.0 mmol for a 0.2 mmol scale) that were activated by using 4- or 5-fold excess of HBTU in the presence of DIEA (10 equiv). N^{α}-Fmoc protecting groups were removed by treating the resin-attached peptide with piperidine (20% v/v) in DMF. Using the microwave synthesizer, the coupling and deprotection were carried out at 75 °C using 25 W microwave power for 5 min and 60 W microwave power for 3 min, respectively. For batch synthesis (on the Tribute) or continuous flow (on the Pioneer), the coupling and deprotection were carried out for 30 and 10 min, respectively.

Preparation of Resin-Bound Base Labile Handle-Linked Solubilizing Tagged Peptide. Fmoc-PAL-PEG-PS (0.2 mmol) was N^{α}-deprotected and then carried through five cycles of acylation with Fmoc-Lys(Boc)-OH and N^{α}-deprotection as described above. The base labile linker, 4-hydroxymethylbenzoic acid (4 equiv), was attached via a 1 h acylation using 4 equiv of HBTU and 6 equiv of DIEA. The C-terminal residue was attached to the resin-bound linker via a double 1 h acylation with the Fmoc-amino acid symmetrical anhydride (5 equiv) in the presence of 0.1 equiv of catalyst, DMAP.

Peptide-Resin Cleavage. On completion of solid phase synthesis, the peptides were cleaved from the solid support by treatment with trifluoroacetic acid (TFA) containing anisole:triisopropylsilane (TIPS):3,6-dioxa-1,8-octanedithiol (DODT):H_2O (93%:2.5%:2%:1.5%:1%, 20 mL) for 2 h. The cleaved products were precipitated in ice-cold diethyl ether and centrifuged at 3000 rpm for 3 min. The pellets were washed by resuspending them in ice-cold diethyl ether and centrifuging them again. This washing process was repeated at least three times.

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Peptide Purification. All the HPLC purifications and analytical reaction monitoring, except for the europium loading on DTPA-(A) mouse INSL5 acid, were performed using a Vydac C18 column, either analytical or preparative (4.6 × 250 mm or 22 × 250 mm, respectively), in a gradient mode with eluant A: 0.1% aqueous TFA and eluant B: 0.1% TFA in acetonitrile. For the europium loading reaction monitoring and purification, a Waters X-Bridge C18 analytical column (4.6 × 250 mm) was used with eluant A: 20 mM triethylammonium acetate (TEAA), pH 7.3, and eluant B: 20 mM TEAA in 90% acetonitrile to prevent Eu(III) liberation from the DTPA chelator as previously described.[10]

General Procedure for the Cleavage of the Solubilizing Tag from Target Peptide. To the purified synthetic solubilizing tag-linked peptides was added 1 mL of ice-cold 0.1 M NaOH. The whole was stirred for 2−5 min, after which the pH was made acidic by the addition of 0.1% aqueous TFA. The solution was then applied directly onto a RP-HPLC column for purification as described below.

Synthesis of Mouse INSL5 Acid. For mouse INSL5 acid the B-chain was assembled with the solubilizing tail, which was previously successfully applied to the synthesis of human insulin glargine A-chain.[11] For the mouse INSL5 A-chain acid intramolecular disulfide bond formation, the conversion of Cys8(tBu) to Cys8(Pyr), and the combination with the B-chain still bearing the solubilizing tail to form the two intermolecular disulfide bonds, the synthetic protocol applied was the same described previously for human INSL5.[9] The target peptide was obtained after treatment with 0.1 M NaOH as described elsewhere in the Methods section.

Synthesis of Mouse INSL5 Amide. Mouse INSL5 amide was assembled following the protocol described previously for the chemical synthesis of human INSL5 achieved in our laboratory.[9] The synthetic peptide was characterized as described below.

Eu(A)-Mouse INSL5 Acid. Solid Phase Peptide Synthesis. The peptides, both the A- and B-chain, were assembled on PAC-PEG-PS resins. In the synthesis of the A-chain, Fmoc-(Dmb)Gly-OH was used in position 11 in the middle of the chain together with a pseudoproline, Fmoc-Leu-Ser-(Ψ^{MeMe}Pro)-OH, close to the C-terminus of the chain to avoid chain aggregation. This approach was adopted in the earlier synthesis of human INSL5 A-chain in our laboratory and shown to be effective.[9]

On-Resin Coupling of the DTPA Chelator to the N-Terminus of Mouse INSL5 A-Chain. The chelator diethylenetriaminepentaacetic acid (DTPA)-tetra(tBu ester) (2 equiv, 0.2 mmol) was coupled to 0.1 mmol of A-chain still anchored to the resin in the presence of HATU (2 equiv, 0.2 mmol) and DIEA (4 equiv, 0.4 mmol). To assess the success of the coupling of DTPA, the TNBSA test[12] was performed which resulted in the absence of free amino groups ($-NH_2$) and hence in the successful coupling of DTPA to the A-chain on-resin.

Mouse DTPA(A)-INSL5 A-Chain Intramolecular Disulfide Bond Formation. Crude DTPA-[Cys7,12 (S-thiol), Cys8 (tBu), Cys21 (Acm)] A-chain (180.0 mg, 62.97 μmol) was dissolved in 150 mL of deionized H_2O/ACN 8/2; a base was added to the mixture to have complete dissolution. To this was added 10 mL of 4 mM 2-pyridyl disulfide (DPDS) in MeOH, and the stirred reaction progress was monitored by analytical RP-HPLC analysis which confirmed that the reaction was completed in 30 min at RT. The solution was loaded onto a preparative RP-HPLC column via the instrument's C-line and purified and freeze-dried to give 60.0 mg of pure Cys7-Cys12 intramolecularly disulfide-bonded DTPA-[Cys8 (tBu), Cys21 (Acm)] A-chain (21.0 μmoles, 33.3% yield). The product was identified by MALDI-TOF MS: m/z observed 2858.6 [$(M + H)^+$] (theory: 2857.6).

Mouse DTPA(A)-INSL5 A-Chain Conversion of Cys8 (tBu) to Cys8 (Pyr). The purified DTPA-[Cys8(tBu), Cys21(Acm)] A-chain (60.0 mg, 21.0 μmol) and 2-DPDS (5 equiv, 23 mg, 104.39 μmol) were dissolved in 1.4 mL of TFA and anisole (9:1 v/v). The solution was chilled on ice before 1.4 mL of trifluoromethanesulfonic acid (TFMSA)/TFA (1:4 v/v) was added. The reaction was allowed to continue for 1 h at 0 °C before the peptide was collected by diethyl ether precipitation followed by centrifugation and purified on a preparative RP-HPLC column. The freeze-dried purified product weighed 32.0 mg (10.99 μmol, 52.3%) and was identified as DTPA-[Cys8(Pyr), Cys21(Acm)] A-chain by MALDI-TOF MS (m/z 2911.4 [$(M + H)^+$]; theory 2911.6).

Combination of Mouse DTPA(A)-INSL5 A-Chain with Mouse INSL5 B-Chain. The DTPA-[Cys8(Pyr), Cys21(Acm)] mouse INSL5 A-chain (24.0 mg, 8.24 μmol) was dissolved in 15 mL of 6 M GnHCl, 0.1 M Gly/NaOH buffer (pH 9.0). The [Cys8(S-thiol), Cys20(Acm)] B-chain (23 mg, 7.69 μmol) was dissolved in deionized water (10 mL) and added slowly to the A-chain solution. The reaction was monitored by analytical HPLC analysis and was found to be complete after 30 min at RT, and the product was identified by MALDI-TOF MS (m/z 5790.8 [$(M + H)^+$], calcd 5789.5). The reaction was stopped by the addition of neat TFA, and the product was purified by preparative RP-HPLC and freeze-dried to give 8.5 mg (1.47 μmol, 19.1% relative to B-chain) of [Cys21A(Acm)/Cys20B-(Acm)]-DTPA-(A)-B.

DTPA-(A) Mouse INSL5 Acid. The DTPA-(A)-B peptide [Cys21A(Acm)/Cys20B(Acm)] (8.5 mg, 1.47 μmol) was dissolved in 5 mL of solvent mixture (4.5 mL glacial acetic acid, 0.5 mL of 60 mM HCl). Then 6 mL of 20 mM iodine/acetic acid was added to the solution. The reaction was monitored by analytical HPLC. After 45 min, the reaction was stopped by precipitation of the peptide following direct addition of ice-cold diethyl ether. DTPA-(A) mouse INSL5 was purified by preparative RP-HPLC and freeze-dried to give 1.75 mg (0.26 μmol, 3.5% relative to B-chain). The peptide was identified by MALDI-TOF MS as a single species (m/z 5646.3 [$(M + H)^+$], calcd 5645.1).

Eu-(A) Mouse INSL5 Acid. The DTPA-(A) mouse INSL5 (1.75 mg, 0.26 μmol) was dissolved in 20 mM TEAA buffer (0.1 mL), pH 7.3. $EuCl_3$ (10 mg) was dissolved in H_2O (1 mL) to have a solution 10 mg/mL; 5 equiv of $EuCl_3$ (1.32 μmol, 34.2 μL of solution 10 mg/mL) was used for the reaction. The DTPA-(A) mouse INSL5 solution was added slowly to the $EuCl_3$ solution, and the reaction was monitored by RP-HPLC using the conditions described elsewhere in the Methods section. After 1 h the reaction was found to be complete, and the product was purified by RP-HPLC and freeze-dried to give 0.5 mg (0.09 μmol, 32.4%). The peptide was identified by MALDI-TOF MS as a single species (m/z 5796.8 [$(M + H)^+$], calcd 5797.1).

Synthesis of Human INSL5 Acid and Human INSL5 A(Asp10Glu)-B Acid. Human INSL5 acid was assembled following the protocol described previously for the chemical synthesis of human INSL5 amide achieved in our laboratory.[9] The A-chain was assembled directly with a Leu-Ser pseudoproline

dipeptide to improve the yield of assembly on resin as discussed previously.[9]

Synthesis of Human INSL5 A(Asp10Glu)acid-B-Solubilizing Tail. For the A-chain of the peptide was used the same one prepared for INSL5 A(Asp10Glu)-B acid; the B-chain was assembled with the same solubilizing tail used for the synthesis of mouse INSL5 acid B-chain. After the formation of all the three disulfide bonds as per the protocol described previously,[9] the solubilizing tail was left on the peptide to block the B-chain from having a free C-terminal acid.

Peptide Characterization. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was carried out on a Bruker Ultraflex II instrument (Bruker Daltonics, Bremen, Germany) and used to characterize the peptides at each intermediate step using sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) as matrix. The matrices were made up in 70% acetonitrile containing 0.1% TFA. The peptide content was determined using vapor-phase acid hydrolysis in 6 M HCl containing 2% phenol at 110 °C for 24 h. The individual amino acids were converted to stable, fluorescent derivatives using a Waters AccQ.Tag kit (Waters, Sydney, Australia). The derivatized amino acids were separated using a Shim-Pak XR-ODS (3 × 75 mm, 2.2 μm) column on Shimadzu RP-HPLC system (Shimadzu, Victoria, Australia).

Circular dichroism (CD) spectroscopy was carried out in a Jasco J815 using the following settings: wavelength range 195−260 nm, scanning speed 50 nm/min, bandwidth 0.1 nm, cell length 1 mm at room temperature. The peptide samples were prepared at 0.1 μg/μL in phosphate buffered saline (PBS: 10 mM potassium phosphate buffer with 137 mM NaCl, pH 7.4). The raw data from the spectra in millidegree of ellipticity (θ) were converted to mean residual weight ellipticity (MRE).

Functional Assays. The lyophilized peptides obtained from the synthetic assembly were generally dissolved in 20% DMSO/H_2O as a stock solution at a concentration of 1 μg/μL. Aliquots were removed and then diluted to the required concentrations with the appropriate buffer used for the functional assays described below.

Binding Assays. Chinese hamster ovary CHO-K1 cells transfected with human RXFP4 were plated out at 50 000 cells per well per 200 μL in a 96-well ViewPlate with clear bottom and white walls precoated with poly-L-lysine. 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.[10] Fluorescent measurements were carried out at excitation of 340 nm and emission of 614 nm on a Victor Plate Reader (Perkin-Elmer). Each concentration point was measured in triplicate, and each experiment was performed independently for a total of three times. GraphPad PRISM 5 (GraphPad Inc., San Diego, CA) was used to analyze the receptor-binding assays data that are expressed as mean ± SEM.

cAMP Activity Assays. Agonist Assays. The peptides were tested for their ability to inhibit cAMP activity in CHO-K1 cells cotransfected with human RXFP4 and a pCRE (cAMP Response Element) β-galactosidase reporter plasmid. The cells, already transfected with RXFP4, were plated out at 8000 per well per 100 μL in a Corning cell bind 96-well plate on day 1, transfected with the pCRE β-galactosidase reporter plasmid on day 2, and stimulated with 1 μM of forskolin and incubated with increasing concentrations of each peptide on day 3 as previously described.[13] cAMP concentration is assessed with a β-galactosidase colorimetric assay measuring absorbance at 570 nm on a Benchmark Plus microplate spectrophotometer (BIO RAD). Each concentration point was measured in triplicate, and each experiment was performed independently for a total of three times. GraphPad PRISM 5 (GrapdPad Inc., San Diego, CA) was used to analyze the cAMP inhibition assays data that are expressed as mean ± SEM.

Antagonist Assays. The peptides were tested for their ability to compete with INSL5 and rescue forskolin induced cAMP that was reduced to ∼50% by the addition of INSL5. The assays are performed as described above for day 1 and day 2. On day 3 the cells are stimulated with 1 μM forskolin and incubated with solutions containing 10 nM of INSL5 and increasing concentrations of the tested peptides. The data analysis was performed as per the agonist assays described above.

Pertussis Toxin Assay. The ability of the peptides to inhibit cAMP accumulation via a $G_{i/o}$ protein mediated mechanism was verified using pertussis toxin. RXFP4 cells were transfected with pCRE β-galactosidase reporter plasmid as above (agonist assays) and 5 h after the transfection the cells were treated with pertussis toxin (100 ng/mL as previously described[14]) for 18 h (day 3). The pertussis toxin solution was then removed by aspiration, and the cells were stimulated with forskolin (1 μM) and incubated for 6 h with increasing concentrations of the peptides. Measurement of cAMP concentration and analysis of the assays were performed as per cAMP activity assay described above.

RESULTS

Synthesis and Characterization of the Peptides. Following the first chemical synthesis of human INSL5 (Figure 1),[9] the mouse peptide was successfully assembled via a similar synthesis protocol optimized in our laboratory. Conventional SPPS using Fmoc chemistry was first used for the synthesis of the A- and B-chains each having C-terminal amides. The two chains were consequently purified, and the three disulfide bonds were formed regioselectively and consecutively following our optimized protocol. However, the synthesis of the mouse INSL5 amide was, in fact, found to be exceptionally challenging and required several attempts. Its subsequent purification was also very difficult (Figure 2). Conventional Fmoc solid phase synthesis resulted in several side products that were identified as truncated or deleted sequences. Consequently, other syntheses were performed in which conditions were modified including double coupling cycles, microwave-assisted deprotection and coupling, use of pseudoproline dipeptides, DBU in place of piperidine as a stronger deprotection base, or a combination of these conditions together. Curiously, no significant improvement could be obtained. This suggested that the sequence of the peptide is unusually resistant to ready assembly. The crude cleaved peptide was also difficult to purify by RP-HPLC. It eluted as a very broad peak despite several different elution buffers and conditions being examined to try and effect a reasonable separation. The yield of resulting purified mouse INSL5 B-chain amide was therefore very low as was the final overall yield of synthetic two-chain mouse INSL5 amide (2.2%). The synthesis of the mouse INSL5 in which the A- and B-chains were in the C-terminal acid form was then undertaken. Given the earlier difficulties with the synthesis of mouse B-chain amide, the use of a pentalysine tag linked at the C-terminus of the peptide via a base-labile linker as a solubilizing tail for the peptide was employed.[11] Such a "tail",

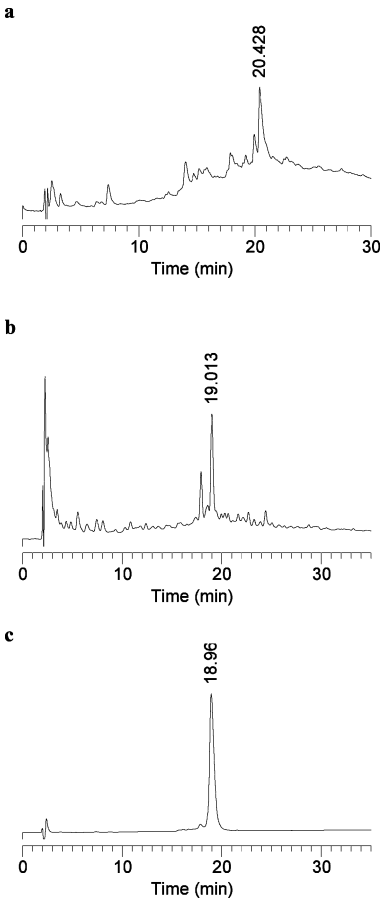

Figure 2. RP-HPLC of crude synthetic mouse INSL5 B-chain amide after standard solid phase synthesis (a). Conditions: Vydac C18 column (4.6 × 250 mm, pore size 300 Å, particle size 5 μm); buffer A, 0.1% aqueous TFA; buffer B, 0.1% TFA in CH3CN. Gradient = 20−50% B over 30 min. RP-HPLC of crude synthetic mouse INSL5 B-chain with the C-terminal penta-Lys tail (b) and after purification (c). Conditions: Vydac C18 column (4.6 × 250 mm, pore size 300 Å, particle size 5 μm); buffer A, 0.1% aqueous TFA; buffer B, 0.1% TFA in CH3CN. Gradient = 20−50% B over 30 min for (b) and gradient = 25−40% B over 30 min for (c).

while predominantly used as a solubilization aid, increases the overall hydrophilicity of the peptide, which in turn can improve its recovery during conventional HPLC. It was subsequently found that the synthesis of the mouse INSL5 B-chain with solubilizing tail was straightforward, suggesting that the tail acted as an effective spacer to prevent or minimize the negative influence of the solid support during the assembly. The handling and purification of the peptide were also significantly improved compared to the B-chain amide. As shown in Figure 2b,c, the assembly of mouse INSL5 B-chain with the tail showed a major chromatographic peak that was identified by MALDI-TOF MS as being the target peptide, with few side products. The crude peptide was easily dissolved and subjected to preparative HPLC to provide the highly purified mouse INSL5 B-chain linked to the tail (Figure 2c) whereas in the case of the B-chain amide most of the peptide could not be recovered from the purification step. After combining the peptide with the A-chain by regioselective disulfide bond formation and subsequent removal of the tail by saponification, the mouse INSL5 acid was obtained in overall yield of 1.9%.

Conformation of the Peptides. The CD spectra of synthetic mouse INSL5 acid and amide were measured in PBS (pH 7.4) (Figure 3). The studies revealed that both peptides possess a significant degree of α-helical conformation (with pronounced double minimum at ∼208 and ∼222 nm) along

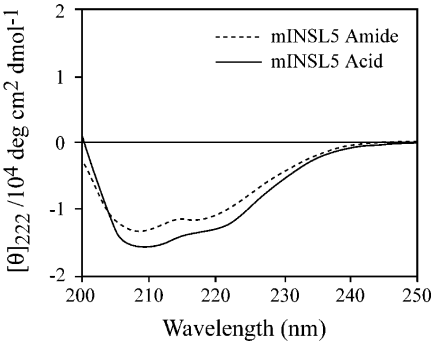

Figure 3. CD spectra of mouse INSL5 acid (black line) and amide (dot line) measured at room temperature at a concentration of 0.1 μg/μL in PBS.

with β-sheet and/random-coiled structure. The α-helical content of INSL5 acid, which was calculated from the mean residual weight ellipticity (MRE) at 222 nm, $[\theta]_{222}$,[15] was found to be 35% ($[\theta]_{222}$ = 12 347.0). The α-helical content of INSL5 amide, calculated in the same way ($[\theta]_{222}$ = 9488.0, α-helix content = 27%), was found to be lower compared with INSL5 acid. The difference in the MRE/helix content between these two peptides suggests that the acid form of INSL5 is more structured compared with its amide form.

Binding Assays. The synthesis of Eu-(A) mouse INSL5 acid enabled the development of competition binding assays (Figure 4). In order to use Eu-(A) mouse INSL5 acid in these assays, the peptide was tested as described below. Several competition binding assays with different concentrations of Eu-(A) mouse INSL5 were performed to determine the minimum concentration of europium labeled peptide to give sufficient specific binding for accurate fluorescent measurements (Figure 5). The best result was provided by a concentration of 5 nM of Eu-(A) mouse INSL5, which was subsequently used in all the competition binding assays with the synthetic mouse INSL5 peptides. To determine the time the fluorescent signal of Eu-(A) mouse INSL5 (generated by the liberation of Eu^{3+} from the DTPA cage) took to reach its maximum, the release of Eu^{3+} from DTPA was monitored over time. The fluorescence of 10 μL of 5 nM Eu-(A) mouse INSL5 with 100 μL of enhancement solution was measured in triplicate at 5 min intervals for 70 min (Figure 6) as described previously.[10] After 20 min, the signal reached its maximum, stabilized, and then began to decrease slightly. Therefore, this time was used in all assays as the end point for europium liberation before measurement of the fluorescence. The relative affinities of the developed peptides for RXFP4 could then be tested in competition binding assays using Eu-(A) mouse INSL5 acid as labeled ligand. For the mouse INSL5 peptides the measured affinity, expressed as pIC_{50} ± SEM was 8.47 ± 0.03 for the acid and 7.07 ± 0.12 for the amide (Figure 7a, Table 1). For the human peptides pIC_{50} values were 7.59 ± 0.14 for INSL5 acid and 6.94 ± 0.14 for the amide. Importantly, both sets of peptides were able to achieve full displacement and were best fitted with a single site binding algorithm.

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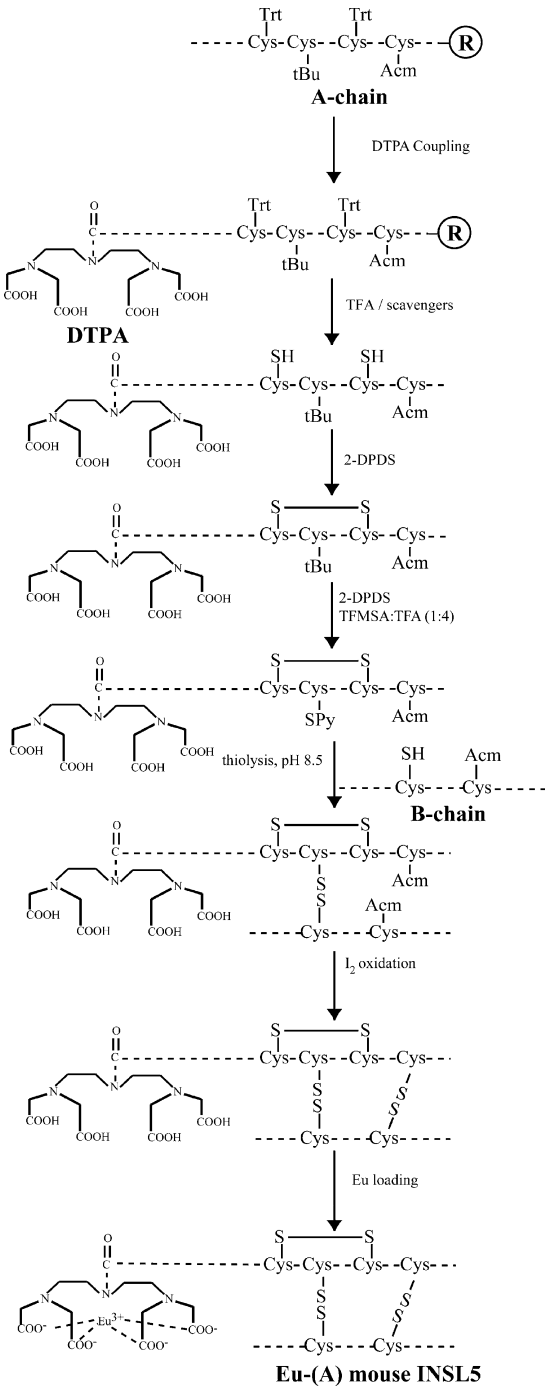

Figure 4. Scheme of the synthesis protocol for Eu-(A) mouse INSL5: The two chains are assembled via Fmoc SPPS using orthogonal protecting groups for the cysteines to enable the regioselective sequential disulfide bond formation: Trt (acid-labile), Pyr (derived from *t*-Bu conversion; thiolysis-labile), and Acm (iodine-labile). The DTPA chelator is coupled on-resin to the N-terminus of the A-chain which is subsequently cleaved from the resin by acid treatment. The DTPA-A chain is oxidized to form the intramolecular disulfide bond between Cys7 and Cys12. DTPA-[Cys8*t*-Bu, Cys21Acm] A-chain is then converted into DTPA-[Cys8Pyr, Cys21Acm] and combined with the B-chain [Cys8SH, Cys20Acm] to form the interchain bond CysA8-CysB8 via thiolysis. Iodine oxidation is used to form the last disulfide bond CysA21-CysB20 and give DTPA-(A) mouse INSL5 acid; Eu is eventually loaded to the DTPA cage to give the target peptide Eu-(A) mouse INSL5 acid.

cAMP Inhibition Assays. The ability of the peptides to inhibit forskolin-stimulated cAMP production was determined as a measure of the ability of the peptides to activate the RXFP4 receptor which is coupled to $G_{i/o}$ proteins. The data are represented as dose response curves in Figure 7b,c, and the potencies expressed as pEC_{50} and E_{max} (mean ± SEM) are outlined in Table 1. All the peptides demonstrated a capacity to activate RXFP4 but with different potencies (pEC_{50}) and maximum activation capacity (E_{max}). Mouse and human INSL5 acid demonstrated high potency (pEC_{50} 9.29 ± 0.09 and 8.62 ± 0.11, respectively, Figure 7b) with maximum activities reflecting full agonist activity. In contrast, mouse and human INSL5 amide demonstrated lower potency (pEC_{50} is 6.85 ± 0.50 and 7.48 ± 0.18, respectively, Figure 7b) as well as decreased maximum activity similar to partial agonist activity. The human A(Asp10Glu)-B acid analogue demonstrated very similar activity to the human INSL5 acid (pEC_{50} = 8.74 ± 0.10), whereas the same analogue with the B-chain solubilizing tail intact, A(Asp10Glu)-B-solubilizing tail analogue, demonstrated lower potency and maximum activity (pEC_{50} 6.00 ± 0.68, Figure 7c).

Given that mouse INSL5 amide demonstrated similarities in activity to a partial agonist, it was also tested for its ability to antagonize the activity of INSL5 acid at RXFP4. Therefore, human INSL5 was used to lower the level of cAMP to about 50% of the forskolin-stimulated level, and increasing amounts of mouse INSL5 amide were added to test if the peptide was able to antagonize the action of the human INSL5 acid peptide. An increase in the level of cAMP from 50% to about 68% was

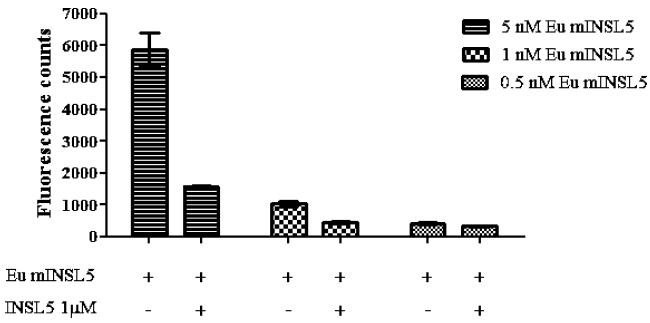

Figure 5. Determination of the range of specific binding: three different concentrations of Eu-(A) mouse INSL5, 0.1−1 and 5 nM, have been tested on RXFP4 expressing cells without and with 1 μM of INSL5 to determine the concentration of the labeled peptide that generates the largest window of specific binding calculated as the difference between the top and the bottom of the competition binding curve. Values are expressed as mean ± SEM.

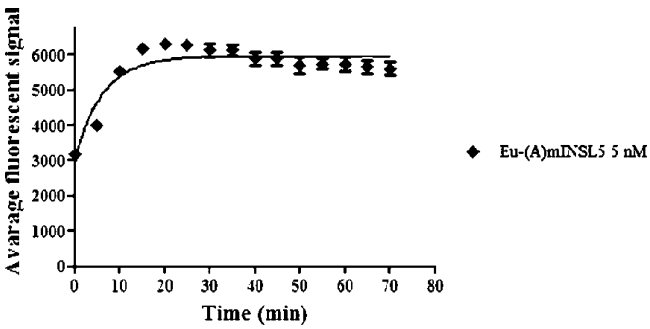

Figure 6. Release of Eu^{3+} over time. 5 nM Eu-(A) mouse INSL5 (10 μL) was added to enhancement solution (100 μL) in triplicate, and the fluorescence was measured every 5 min for 70 min. The maximum of fluorescence signal was reached at about 20 min.

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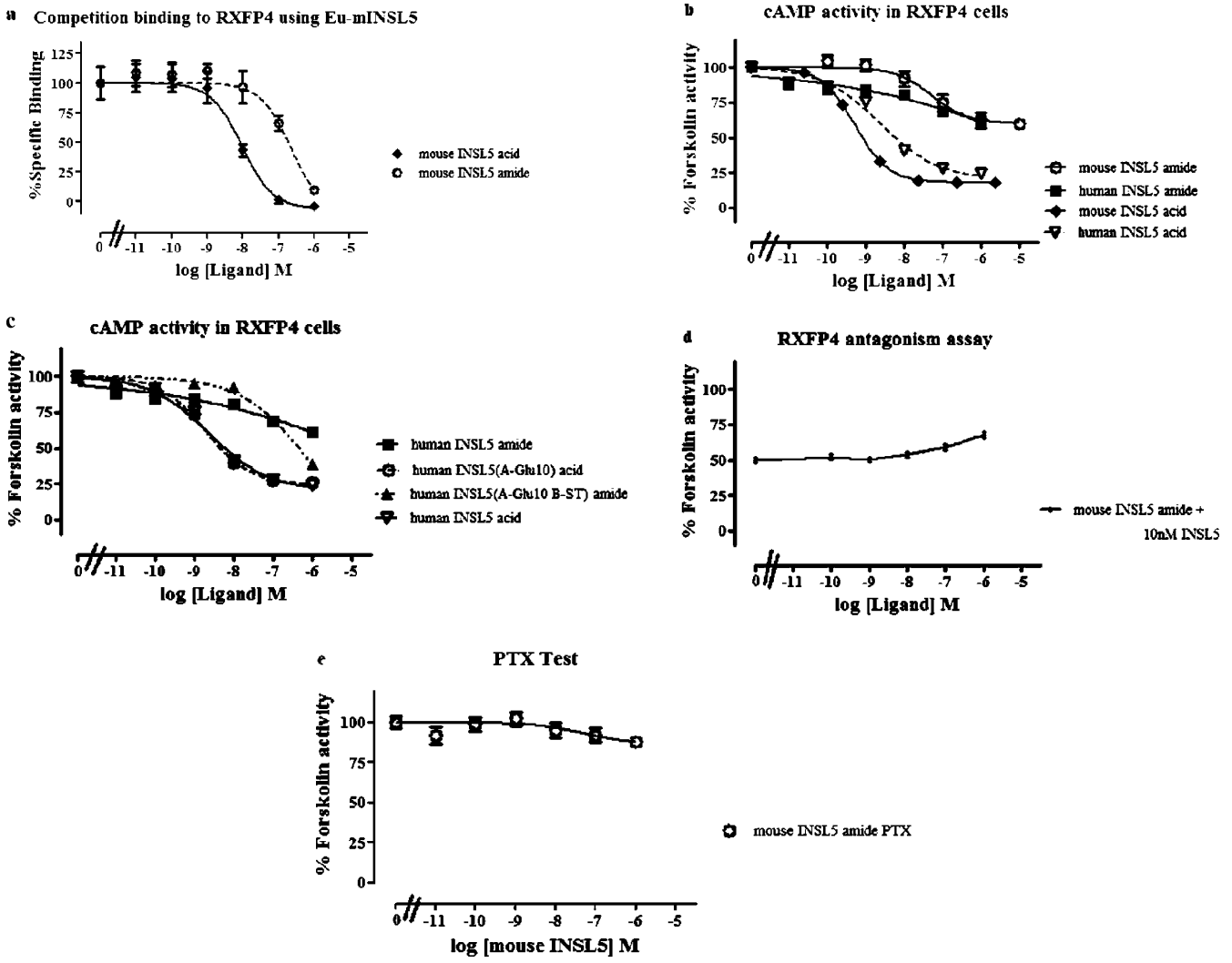

Figure 7. (a) Competition binding curve of mouse INSL5 acid and amide on RXFP4 expressing cells using Eu-(A) mouse INSL5 acid. The pIC_{50} of the peptides were determined to be 8.47 ± 0.03 and 7.07 ± 0.12 (expressed as mean ± SEM; n = 3) for the acid and amide, respectively. (b) cAMP inhibition assay: RXFP4 expressing cells stimulated with 1 μM of forskolin were incubated for 6 h with increasing amounts of peptides. Mouse INSL5 acid and amide are compared to human INSL5 acid and amide. (c) cAMP inhibition assay of the human INSL5 analogues A(Asp10Glu)-B and A(Asp10Glu)-B-solubilizing tail compared to native human INSL5 acid and amide. (d) Antagonist assay of mouse INSL5 amide: RXFP4 expressing cells are incubated with a constant concentration of INSL5 (10 nM) to reduce the cAMP level at about 50% and increasing amounts of mouse INSL5 amide to test the ability of the peptide to rescue cAMP production. The peptide increased the level of cAMP from 50% to about 68%, less than 35%. (e) Pertussis toxin experiment: RXFP4 expressing cells were pretreated for 18 h with pertussis toxin (100 ng/mL), then the toxin solution was removed, and the cells were stimulated with 1 μM of forskolin and increasing amounts of mouse INSL5 amide.

Table 1. Receptor Binding Affinity (pIC_{50}) and Activation (pEC_{50}) on RXFP4 Receptor[a]

peptides	pIC_{50}	pEC_{50}	E_{max} (%)	% α-helix
mouse INSL5 acid	8.47 ± 0.03	9.29 ± 0.09	82.09 ± 2.40	34.9
mouse INSL5 amide	7.07 ± 0.12	6.85 ± 0.50	50.52 ± 7.58	26.9
human INSL5 acid	7.59 ± 0.14	8.62 ± 0.11	80.46 ± 1.83	ND
human INSL5 amide	6.94 ± 0.14	7.48 ± 0.18	58.30 ± 3.18	ND
human INSL5 GluA10	ND	8.74 ± 0.10	76.77 ± 2.31	ND
human INSL5 GluA10, B-STail	ND	6.00 ± 0.68	ND	ND

[a]Data expressed as mean ± SEM of n = 3 separate experiments. E_{max} (%): efficacy maximum of the peptide in the cAMP assay relative to forskolin as 100%. % α-helix: percentage of α-helicity. B-STail = B-chain with C-terminal solubilizing tag.

observed at the highest concentration used (1 μM) (Figure 7d), showing the peptide had a very weak antagonist effect.

The different shapes of the cAMP dose response curves for INSL5 amide and the lack of maximal activation can also be explained by potential $G_{i/o}$ independent actions. Therefore, pertussis toxin was used to test whether mouse INSL5 amide was reducing forskolin-stimulated cAMP production via a $G_{i/o}$-meditated mechanism. When mouse INSL5 amide was used on RXFP4 expressing cells pretreated with pertussis toxin, no cAMP inhibition was observed, implying the peptide does indeed act

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through $G_{i/o}$ proteins (Figure 7e). Collectively, the data support that mouse INSL5 amide is a partial agonist with a $G_{i/o}$-meditated mechanism of activation.

DISCUSSION

The predicted native mouse INSL5 peptide (Figure 1) was assembled via conventional SPPS of the two individual A- and B-chains followed by stepwise regioselective disulfide bond formation as per the protocol optimized in our laboratory.[9] The peptide was initially assembled with both chains in the amide form as previous studies on the related relaxin-3 showed that this modification was tolerated,[16] and our previous studies showed that a human INSL5 amide peptide showed activity at RXFP4.[9] The assembly of mouse INSL5 B-chain in the amide form was unexpectedly difficult, and several synthetic and purification conditions were investigated. Nevertheless, no significant improvements were found in term of final yield. For the synthesis of the B-chain of mouse INSL5 in the acid form a pentalysine tag at the C-terminus was used as a mean to increase the overall hydrophilicity of the peptide. The synthesis of the B-chain with the solubilizing tail was found to be improved and the HPLC purification easier compared to mouse B-chain amide (Figure 2). Nevertheless, the overall yield of the final two-chain peptide following removal of the solubilizing tag was not improved.

The synthesis of Eu(A)-mouse INSL5 was a significant achievement not only due to the complexity of its assembly but also because it allowed the development of a competition binding assay using the native labeled peptide (Figure 4). Previously, the only labeled peptide available for use in an RXFP4 receptor binding assay was europium- or iodine-labeled relaxin-3 that can also bind to the RXFP4 receptor but which is not the cognate ligand and therefore may interact with the receptor in a different manner. Europium-labeled INSL5 as probe for binding assays is a very valuable tool as it has high fluorescent emission peak and hence a high sensitivity and also enables safer handling compared to the radioligand previously used in these bioassays.[10]

The two mouse INSL5 peptides, one C-terminal amide and the other C-terminal acid, were each tested for binding and activation on cell lines expressing RXFP4. Interestingly, the acid form was shown to possess about 25-fold greater affinity for RXFP4 than the amide (Figure 7, Table 1). This reduced affinity was correlated with a reduced ability to inhibit forskolin stimulated cAMP production. Additionally, mouse INSL5 amide was not able to decrease forskolin stimulated cAMP activity to the same degree as mouse INSL5 acid and displayed activity like a partial agonist (Table 1, Figure 7).

These interesting results redirected our attention to the human peptide to assess if the findings described above were a unique feature of the mouse peptide or they occurred for both forms. A similar trend but with a less dramatic difference between amide and acid form was in fact subsequently observed in the cAMP assays of human INSL5 peptides (which were also synthesized and tested within our laboratories) (Figure 7b). Furthermore, the cAMP curves of human and mouse INSL5 amide have a very similar profile which resembles that of a partial agonist (Figure 7b). This suggests the mechanism of their activation of RXFP4 is different from that of a full agonist with its characteristic sigmoidal shape.

The mouse INSL5 amide was therefore tested in two separate experiments to further test its mechanism of action. First, pertussis toxin was used to test if the peptide was decreasing forskolin stimulated cAMP via a $G_{i/o}$ protein-mediated mechanism. The results clearly demonstrated that pertussis toxin blocked the effects of the amide; hence, it is indeed activating RXFP4 via a $G_{i/o}$ protein-mediated mechanism (Figure 7e). Next, the ability of mouse INSL5 amide to antagonize INSL5 acid cAMP inhibition was tested to determine if the amide was indeed acting as a partial agonist. The results suggest that the INSL5 amide does indeed have a small antagonistic effect at high concentration, suggesting that it is in fact acting as a partial agonist (Figure 7d).

The data strongly suggest the necessity for a C-terminal acid for optimum RXFP4 activity. This necessity was also supported by the cAMP inhibition assay results of two human INSL5 analogues, one in which both chains possess a C-terminal acid and the other having a solubilizing tail at the C-terminus of the B-chain (Figure 7c). The peptide "capped" with the solubilizing tail also showed a decreased ability to activate the receptor having a curve more similar to the one of human INSL5 amide. These analogues were also assembled with a mutation within the A-chain of Asp10A into Glu to avoid aspartimide formation issues during the chain synthesis. The C-terminal acid analogue that lacks the solubilizing tail had a cAMP inhibition profile resembling the one of human INSL5 acid, suggesting that the Asp-Glu mutation is well tolerated and has no effect on the peptide's bioactivity. This mutation is present in the native sequence of mouse INSL5, as mentioned above, suggesting that this difference between the mouse and human native sequences is not responsible for any different effect on the RXFP4 receptor. However, what is clear from the data is that the C-terminus of the B-chain needs to be free, considering the loss of activity associated with the B-chain linked to a solubilizing tag and that the acid form of the peptides has a higher affinity and full agonist activity on the receptor.

Such dependence on either a free acid or amide is not unusual. Oxytocin is only active as its C-terminal amide, whereas H2 relaxin and INSL3 are equally active in either C-terminal forms.[17,18] In the case of INSL5, the requirement for a free C-terminal carboxylate may reflect the need for the presence of a positive charge in the receptor close to the domains involved in binding and activation which results in subsequent favorable electrostatic interaction with the acid form. Indeed, positively charged amino acids are present in the exoloops of the RXFP4[6] and therefore are candidates for interaction with the peptide. However, the precise identification of the residue(s) in these domains that are involved in the ligand−receptor interaction(s) are unknown and will require mapping studies. However, this supposed more favorable interaction between the peptide acid and the positive charge within the receptor may well be responsible of locking the peptide in a conformation that results in an effective binding to the receptor and consequently on its most effective activation.

Within the INSL peptide family, INSL5 is the first report where native (acid) and amidated C-termini showed dramatic differences in their activity. The data presented are so far the first gained in the elucidation of the mechanism of interaction between INSL5 and RXFP4 and suggest the necessity of free C-termini acid and particularly of a free B-chain C-terminus acid as shown in the decreased activity of a human INSL5 analogue "capped" with a solubilizing tail at the C-terminus of the B-chain or having a B-chain C-terminal amide. This correlates with the mechanism of activation of relaxin-3 of both RXFP3 and RXFP4 receptors[19] where the C-terminal Trp residue on the B-chain needs to be free for the peptide to signal through the

receptors; in fact, the addition of an extra Ser residue after the C-terminal Trp led to the loss of relaxin-3 agonist function on both RXFP3 and RXFP4.[19] As relaxin-3 is a member of the same superfamily and it is coupled to the same class of receptor as INSL5, the hypothesis advanced is that INSL5 interacts with RXFP4 in a similar fashion to relaxin-3 with its cognate receptor RXFP3, and therefore the conserved Trp at the end of the INSL5 B-chain may play an important role in the system INSL5/RXFP4, too. Our studies on INSL5 provide evidence that the C-terminus needs to be in the acid form. Further investigations on the role of C-terminus Trp are already underway in our laboratory. The availability of a sensitive europium-labeled analogue for fluorescent measurement of binding interactions between INSL5 and its RXFP4 receptor will facilitate the further structure–function relationship study of this ancient member of the insulin superfamily and help provide further insight into its role in feeding as well as enable the development of agonists and antagonists of the peptide that may be useful therapeutics in the treatment of disorders of appetite as well as of colonic motility alterations.

AUTHOR INFORMATION

Corresponding Author

*Ph: +61 3 83447285. Fax: +61 3 93481707. E-mail: john.wade@florey.edu.au (J.D.W.); akhter.hossain@florey.edu.au (M.A.H.).

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ABBREVIATIONS

Acm, acetamidomethyl; CD, circular dichroism; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DODT, 3,6-dioxa-1,8-octanedithiol; DPDS, 2-pyridyl disulfide; DTPA, diethylenetriaminepentaacetic acid; ETS, expressed sequence tags; Fmoc, 9-fluor enylmethoxycarbonyl; HATU, 2-(1*H*-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate; INSL3, -4, -5, or -6, insulin-like peptide 3, 4, 5, or 6; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; ND, not determined; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; PTX, pertussis toxin; Pyr, pyridinyl; RP-HPLC, reversed-phase high-pressure liquid chromatography; RXFP 3–4, relaxin family peptide receptor 3–4; SPPS, solid phase peptide synthesis; tBu, *tert*-butyl; TEAA, triethylammonium acetate; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; TIPS, triisopropylsilane; TNBSA, 2,4,6-trinitrobenzenesulfonic acid; Z, pyroglutamic acid.

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