Development of nanobodies targeting the relaxin receptor, RXFP1

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Background and rationale

The relaxin family peptide receptor 1 (RXFP1) is a class A GPCR that mediates the physiological effects of the hormone relaxin-2[1], an important regulator during pregnancy. However, it is the non-reproductive actions, namely in reducing inflammation and fibrosis, that have made this axis an attractive therapeutic target for cardiovascular and fibrotic diseases[2]. Still, rational structure-based drug design has remained challenging due to limited structural and mechanistic insight into the mode[3-6] in which relaxin-2 binds and activates the receptor.

Unlike canonical class A GPCR activation, relaxin-2 binds the large extracellular domain (ectodomain) of RXFP1 that is composed of multiple leucine-rich-repeats (LRRs)[7]. Central to the complex mode of RXFP1 activation is the low-density lipoprotein A (LDLa) module[8], which is appended N-terminally to the LRRs and connected via a linker. Though, the mechanism in which the LDLa module and linker communicate activation from the ectodomain to the transmembrane domain (TMD) remains poorly defined. Recently, the active-state structure was determined using cryo-EM[6], though the ectodomain, especially the LDLa module and linker, remains conformationally heterogenous even when bound to relaxin-2 and, therefore, difficult to structurally capture. Achieving higher resolution in the ectodomain could contribute meaningfully to a fuller view of the framework that bridges relaxin-2 binding and RXFP1 activation.

Hypothesis/Aims

To aid the structural study of GPCRs, camelid heavy-chain only antibody fragments, also known as nanobodies, are used as auxiliary binding partners to stabilise dynamic regions[9]. Increasingly, nanobodies targeting the extracellular domain of GPCRs have assisted structural imaging[10] and have enabled pharmacological modulation of GPCRs[11] as agonists, antagonists, or allosteric modulators. My research aims to identify RXFP1 ectodomain binding nanobodies to function as a structural tool for ectodomain stabilization, as a pharmacological tool either by directly modulating receptor function or as nanobody conjugates, and as a research tool to better assess disease contexts where relaxin-2 is overexpressed.

Methodology

A library of nanobodies targeting RXFP1 was generated through immunization of alpacas with purified RXFP1 protein and RXFP1 expressing cells. To enrich high-affinity nanobody clones, we used phage display technology and iterative magnetic-based selection against a truncated RXFP1 ECD containing only the LDLa module and linker. Individual nanobody clones with high affinity

for RXFP1 were identified with a phage ELISA and tested for preferential binding in a flow cytometry binding assay on RXFP1-expressing and non-expressing cells. The lead nanobody was extensively characterized with binding assays including biolayer interferometry, competition binding, and NanoBRET binding kinetics, and functional assays including reporter gene and BRET cAMP sensors in recombinant and endogenously expressing cell-lines. To further elucidate the molecular mechanism, a structural model of the LDLa-linker in complex with the nanobody was generated, and flow cytometry was performed on RXFP1 chimeras and single alanine mutants.

Results

Our selection method yielded a single, highly conserved group of nanobodies that preferentially bound RXFP1. The lead nanobody, TL1, possessed low-nanomolar affinity for the isolated LDLa-linker and RXFP1-ectodomain and was able to displace Europium labelled with high affinity (pK_i = 9.14 ± 0.040). However, TL1, when tested against a RXFP1 construct without the TMD, does not displace relaxin-2 which indicates the nanobody likely binds to a topographically different, non-overlapping site. In functional assays, TL1 does not stimulate cAMP signaling, but specifically inhibits relaxin-2 mediated RXFP1 signaling (pIC_{50} = 7.54 ± 0.06). Increasing concentrations of the nanobody shifts the dose response of relaxin-2 rightwards while also reducing the maximum efficacy of relaxin-2, thus the data was fit to an allosteric model (pK_B = 8.74 ± 0.13, α = 0.0552 ± 0.029, β = 0.0982 ± 0.045). Epitope mapping and structural modelling indicates that TL1 binds non-conserved residues within the linker.

Conclusion

In the search for antagonists of RXFP1, relaxin-2 peptide analogues[12] and small molecules agents have been thus far unsuccessful due to an incomplete understanding of the mode of binding and activation. Novel GPCR ligands, such as nanobodies and mini-binders, have emerged as effective functional modulators that can be rationally developed through *in silico* design or bespoke selection strategies. Herein, we discover a nanobody that binds RXFP1 with high affinity and inhibits relaxin-2 binding and induced cAMP signalling through a novel allosteric mode of binding.

Statement of the scientific innovation and significance of the study

A better understanding of the RXFP1 receptor, especially its structure and method of activation, could lay the foundation for therapeutics that act on the receptor to treat cardiovascular and fibrotic diseases. The nanobodies themselves may modulate receptor function or could be further engineered to serve as theranostic tools to detect RXFP1 overexpression as exemplified in some ovarian cancer models[13,14].

Statement on the nominating student's specific role in the study

The nominated student conceived and performed the selection strategy for enriching RXFP1-binding nanobodies. They also performed assays including flow cytometry, biolayer interferometry, cAMP kinetics, and NanoBRET competition binding as well as analyzing the data.

Declaration

"We declare that this document was prepared primarily by the nominating student and has undergone minimal corrections by the supervisor or any other author on the accompanying abstract."

Student's name:…Tim Lkhagvajargal…Student's signature:…  .Date:05/05/26…..

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