Development of a scaffold displaying exoloops of RXFP1

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Summary

Relaxin family peptide receptor 1 (RXFP1), the cognate receptor for relaxin, is a G-protein coupled receptor (GPCR) possessing a unique extracellular region consisting of a domain of 10 leucine rich repeats (LRRs) linked to an N-terminal low density lipoprotein Class A module. Relaxin binds to its receptor primarily by a high affinity interaction with the LRRs. An additional low-affinity interaction has been proposed to occur between relaxin and the the exoloops (ELs) of the transmembrane domain, however the molecular detail of this interaction remains undefined.

While site directed mutagenesis and subsequent functional characterisation of these mutants traditionally allows identification of residues contributing to receptor function, in this case results are complicated by the presence of the high affinity binding site in the LRRs. To create a tool to investigate the low-affinity interaction, a protein scaffold system displaying exoloops 1 and 2 from RXFP1 was designed. This was achieved by inserting RXFP1 exoloops 1 and 2 into the native loops of a thermostabilised 6 kDa GB1 protein creating EL1/EL2-GB1. This protein has been expressed and purified in milligram quantities and used in conjunction with biophysical techniques such as NMR to explore relaxin binding to the exoloops of RXFP1.

Key words

Protein engineering, GPCR, Relaxin, Protein expression

RXFP1 is a unique class-A G protein-coupled receptor (GPCR) belonging to the leucine rich repeat (LRR) family defined by an extracellular domain containing 10 LRRs linked to an N-terminal low-density lipoprotein class-A module. The LRRs form the primary high-affinity binding site for relaxin (Bathgate, 2006., Bullesbach, 2005). Furthermore a low-affinity binding site involving EL2 within the transmembrane domain has been suggested (Halls, 2005., Sudo, 2003). Molecular characterisation of relaxin binding to the low-affinity site in the full length receptor is complicated and masked by the presence of the high-affinity site within the LRRs, requiring an alternate approach for investigation (Kong, 2010).

Consequently, a soluble protein scaffold containing EL1 and EL2 from RXFP1 was designed to probe relaxin binding to the exoloops in isolation from the high-affinity binding site. The design, expression and purification of this engineered protein are described below.

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Design of a scaffold containing exoloops of RXFP1 (EL1/EL2-GB1)

A thermostabilised variant of streptococcal protein G (GB1) was chosen to use as the scaffold to display EL1 and EL2 from RXFP1, based on previous success (Datta, 2003). The scaffold has two sites introduced into juxtaposed loops for sequence insertion in a GPCR-like orientation (Figure 1).

RXFP1 loop sequences were determined using transmembrane prediction software (http://www.cbs.dtu.dk/services/TMHMM) and incorporated into tGB1 flanked by Gly linkers to generate EL1/EL2-GB1. The linkers add flexibility within the loops to enable formation of native conformations. One key structural feature of these loops is a disulphide bond connecting the C-terminal of EL1 with the middle of EL2 which mimics the native disulphide linkage between the ELs in the native RXFP1 (Hsu, 2002).

Expression and Purification of EL1/EL2-GB1

EL1/EL2-GB1 was expressed as an N-terminal His$_6$-tag fusion in BL21 (DE3) Escherichia coli after induction with 0.5 mM IPTG, 16°C for 24 hours. Protein was extracted from inclusion bodies after cell lysis and repeat washes (50 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM DTT pH 8) in addition to a detergent-free wash before solubilisation in 8 M urea at 4°C. Protein was then purified on Ni-Sepharose Fast Flow resin (GE Healthcare) under denaturing conditions (8 M urea, 1 M NaH$_2$PO$_4$, 10 mM Tris) and eluted at pH 4. The protein was refolded in 3
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M urea (500 mM NaCl, 50 mM Tris, 50 mM glycine, 100 mM glucose, 4 mM reduced glutathione, 0.4 mM oxidised glutathione) then 1 M urea (500 mM NaCl, 50 mM Tris, 50 mM glycine, 100 mM glucose, 4 mM reduced glutathione, 0.4 mM oxidised glutathione) at room temperature prior to overnight dialysis in 20 mM Tris pH 8. Protein was further purified on DEAE beads (GE Healthcare) using a NaCl gradient with EL1/EL2-GB1 elution at 100-200 mM NaCl prior to final purification on a Hiload 16/60 Superdex 75 gel filtration column (GE Healthcare). Yields of 5 mg/L culture are routinely obtained and protein identity and disulphide bond formation was confirmed by ESI-TOFF mass spectrometry.

Results/Discussion:

The scaffold was engineered to display defined RXFP1 EL sequences accounting for the flexibility required within the loops and the disulphide bond between EL1 and EL2 conserved in most GPCRs. A method for the adequate expression and purification of EL1/EL2-GB1 has been established and the identity of the purified protein, including confirmation of the formation of the critical disulphide bond, was determined by mass spectrometry.

A new tool has been produced for investigating the secondary ligand binding site within RXFP1. Being a small soluble protein, ligand interactions with the loops on this scaffold can be assessed by a number of biophysical techniques such as Nuclear Magnetic Resonance spectroscopy to allow identification of interacting residues. Ultimately, this will enhance our understanding of RXFP1 activation which is important for drug design at this receptor.

References


