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Cell-free protein synthesis as a tool to study RXFP3-Relaxin-3 protein interactions

Patrick J. Shilling[1,2], Ross A.D. Bathgate[1,3], Paul R. Gooley[1,2,*]

[1] Department of Biochemistry and Molecular Biology, The University of Melbourne, Victoria, Australia.
[2] Bio21 Biotechnology and Molecular Sciences Institute, The University of Melbourne, Victoria.
[3] Florey Institute for Neuroscience and Mental Health, The University of Melbourne, Victoria, Australia.

Summary

With the discovery of the relaxin family peptide receptors there is interest in obtaining a clearer understanding of the structure of these proteins and the molecular mechanism of receptor-ligand interaction. As G-protein coupled receptors, obtaining milligram quantities for structural investigations is hampered by the inherent instability of these integral membrane proteins. In the current context, understanding of GPCR structural biology has increased dramatically with crystal structures of several inactive and now active forms solved. In addition, the first nuclear magnetic resonance structure of a GPCR was obtained which is of crucial importance to studying these receptors in a more "biologically relevant" setting. However despite this expansion in the field, most structures have been solved on modified systems so as to increase stability and are not necessarily representative of the native receptors. In relation to the relaxin family peptide receptors, we chose to investigate relaxin-family peptide receptor-3 expressed by cell-free protein synthesis. In contrast to *in-vivo* expression, cell-free was capable of producing large amounts of native receptor which makes it amenable to demanding structural studies.

Key words

Cell-free protein synthesis, GPCR, RXFP3

Report

We report the development of a cell-free protein synthesis (CFPS) system for the production of the relaxin-family peptide receptor-3 (RXFP3), the cognate receptor for the neuropeptide relaxin-3. CFPS is a method whereby proteins are produced in an *in-vitro* environment by using cell extracts (S30 extract) derived from organisms such as *Escherichia coli*. S30 extracts contain all the necessary components required for mRNA and protein production. The benefit of this technique for protein synthesis is that it can overcome difficulties associated with producing hard to express proteins *in-vivo*, including integral membrane proteins. In addition, advantages to this system include the ability to alter the environmental conditions within the reaction which is not possible in *in vivo* systems. For example, surfactants, chaperones or cofactors, necessary for the overexpression and correct folding of some proteins, can be added directly to the reaction. To aid mechanistic studies proteins may be labelled at any site through specific labelling with unnatural amino acids. Cell-free (CF) reactions are also notably fast, with reactions as short as 8 hours producing up to 1 mg/mL

* Corresponding Author: Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Victoria, Australia, 30 Flemington Road, Parkville, Victoria, 301; Email: prg@unimelb.edu.au.

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of expressed GPCR (Ishihara et al., 2005)there are critical difficulties in synthesizing membrane proteins, such as the low protein expression levels and the formation of insoluble aggregates. However, structure determinations by X-ray crystallography require the purification of milligram quantities of membrane proteins. In this study, we tried to solve these problems by using cell-free protein expression with an E coli S30 extract, with G protein coupled receptors (GPCRs.

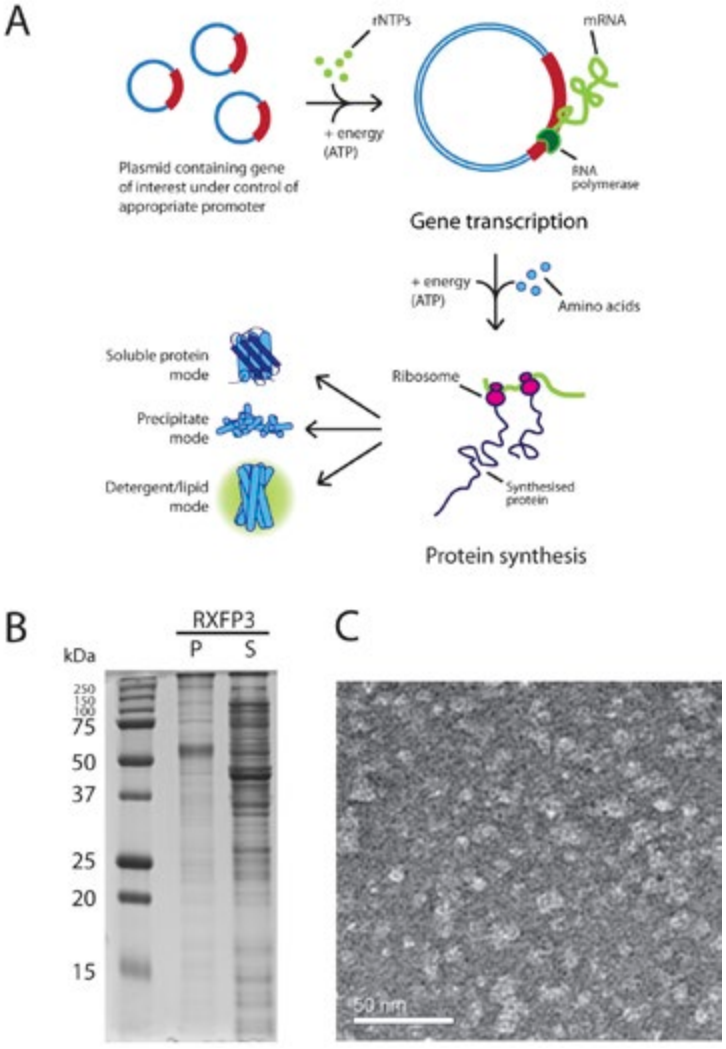

Figure 1. Cell-free system and RXFP3 expression. A: Breakdown of CFPS. B: Expression of RXFP3 in the precipitate mode. RXFP3 (52kDa) is seen in the Pellet (P) versus in solution (S). C: Negatively stained electron micrograph of purified RXFP3 in DDM.

Preparation of the *E. coli* S30 extract was performed with minor modifications to published methods using the BL21 (DE3) strain which allowed for the induction of T7-RNA polymerase (Apponyi et al., 2008). Full length RXFP3 was expressed either as an N-terminal or C-terminal His-tag fusion in the presence or absence of specific detergents Polyoxyethylene-(23)-lauryl-ether or Polyoxyethylene-(20)-cetyl-ether (Brij35 or Brij58 respectively) (Figure 1A). Expression was performed overnight at 30 °C with moderate shaking (170 RPM) utilising the continuous exchange CF method, which separates the reaction chamber from a solute feeding chamber at a 1:14 ratio via a dialysis membrane.

Purification made use of two features: The solubility state of RXFP3 in either the presence or absence of detergents, and the His-tag coupled to either the N- or C-terminus. In the presence of detergent, RXFP3 was capable of direct expression into micelles, maintaining protein solubility and allowing direct application of the CF reaction mix onto a Ni-NTA column. If the protein was expressed in the absence of solubilising detergent, the protein precipitated, as expected, during translation of the polypeptide chain. Upon completion of the CF reaction the end product was isolated simply by centrifugation to produce a sample of RXFP3 that is approximately 70 % pure (Figure 1B). In addition, the precipitated form of RXFP3 was readily refolded into the detergent 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)] (LMPG) and further purified on Ni-NTA. While direct expression into detergents is a more favoured approach, as it is expected to produce a folded protein, less RXFP3 is expressed compared to expression in the absence of detergent. Structural integrity has been demonstrated for GPCRs refolded into detergents following CF expression, although the choice of detergent is critical (Klammt et al., 2007; Junge et al., 2010). This also appears to be the case for RXFP3 (Figure 1C).

Presented here is an alternative method for the production of the RXFPs. This technique allows for a high expression of milligram quantities of pure protein for receptor-ligand binding studies that is not easily produced recombinantly. In addition, CFPS is relatively open to manipulation and can produce protein in a short time frame.

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