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Creating new specific ligand–receptor pairs for transgene regulation

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The creation of specifically matched ligand–receptor pairs that are orthogonal to naturally present interacting pairs is essential for the development of small molecule-regulated gene expression systems for biotechnological applications. However, for many years this task has represented a significant challenge for synthetic chemists and protein engineers. Recently, Doyle and colleagues demonstrated that highly specific ligand–receptor pairs can be engineered in a rapid fashion by creating large libraries of protein variants and applying a selection scheme to identify variants with improved activation by the target synthetic ligand.

Introduction

Numerous unique ligand–protein interactions exist in nature, each with its own highly specialized function. The specificity of these interactions is such that were a ligand designed by nature for interaction with one protein to activate or inhibit another, the consequences might be severe. The engineering of highly specific ligand–protein pairs that are orthogonal to existing natural ligand–protein pairs therefore represents an important challenge that would allow us to selectively regulate gene expression for applications such as the study of gene function, gene therapy, tissue engineering and metabolic engineering [1,2]. The ability to engineer new specific ligand–protein pairs would also be useful in the selective regulation of cellular processes for the study of phenomena such as apoptosis, genetic recombination, signal transduction and motor protein function [3].

In recent years, much effort has been focused on creating orthogonal ligand–protein pairs based on the

class of naturally occurring transcription factors known as nuclear hormone receptors (NHRs). The main reasons for this inclination towards NHRs are their rapid and dose-dependent induction properties [4–6] and ease of manipulation [7,8]. Ligand–receptor pairs based on NHRs that are fully orthogonal to host regulatory pathways have already been found, including a truncated progesterone receptor (PR) mutant that is selectively activated by the PR antagonist RU486 [9], and the insect ecdysone receptor–ponasterone A pair [10]. However, these systems were not created by design. Numerous strategies for engineering orthogonal ligand–receptor pairs have been developed with varying degrees of effectiveness in terms of generality of approach, as well as degree of specificity of interaction achieved. Two broad approaches have been applied (Figure 1): (i) identify a mutant receptor with weakened response to the natural ligand and create various synthetic ligands to rematch the interaction with the target mutant receptor; and (ii) identify a synthetic ligand that poorly activates the natural receptor and create variant receptors that are strongly activated by the target ligand. Of these two approaches the second has been particularly effective in engineering orthogonal ligand–receptor pairs, as demonstrated by the work of Doyle and co-workers [11]. Their approach distinguishes itself from other methods for creating orthogonal ligand–receptor pairs, both in its ‘user-friendliness’ and its ability to generate highly specific ligand–receptor pairs.

The synthetic chemists’ approach

Not surprisingly, the approach taken by synthetic chemists and protein engineers to create orthogonal ligand–receptor pairs has differed. Synthetic chemists

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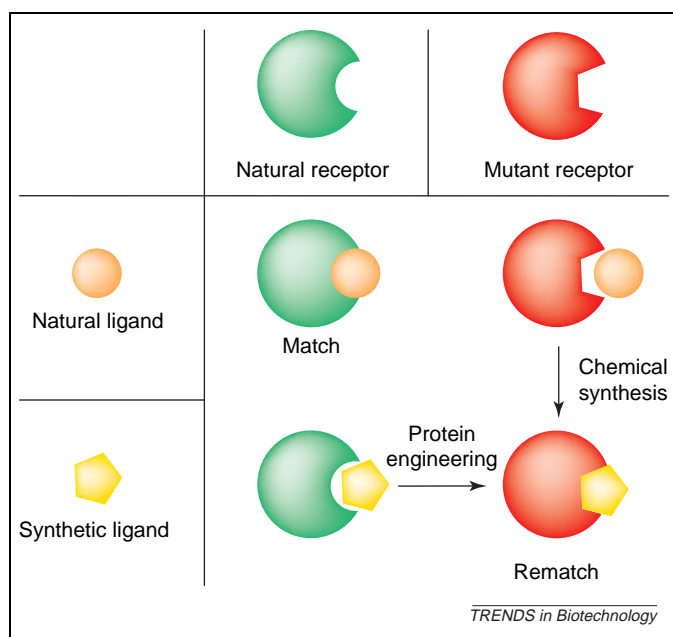


Figure 1. Scheme illustrating the required rematch of a synthetic ligand with an engineered mutant receptor to create an orthogonal ligand-receptor pair. Two broad approaches can be used to achieve the desired rematch: (i) chemical synthesis – synthesize various ligands to rematch a given target mutant receptor displaying weakened activation by the natural ligand; or (ii) protein engineering – sift through a variety of receptor mutants to identify variants with enhanced specificity for a target synthetic ligand that does not activate the natural receptor.

have tended to spend minimal time searching for mutant receptor variants that are weakly activated by the natural ligand, focusing the bulk of their efforts on synthesizing ligands that rematch interaction with the chosen mutant receptor(s). Protein engineers, however, have focused their efforts on creating protein mutants based on a wild-type NHR in an attempt to identify variants that are specifically activated by one or more selected ligands that fail to activate the wild-type receptor.

The chemical manipulation of ligands based on rational structure–function considerations to rematch interaction with a given mutant receptor of human estrogen receptor α (hER α) or hER β has been demonstrated by several studies [12–14]. Despite these advances, the degree of specificity shift towards the target mutant receptor achieved in even the best cases was only moderate. In addition, the general application of this approach is hindered by two factors: (i) our ability to modify ligands to create optimized variants to rematch a mutant receptor is limited to the changes allowable by existing chemical synthesis methods; and (ii) the synthesized ligand modifications are mostly based on rational predictions of complementary interaction with the protein ligand-binding pocket, and such predictions are often inaccurate.

The protein engineers' approach

It is ironic that although they are far more structurally complex on a molecular level than small molecules, proteins are in some sense easier to manipulate structurally. For example, substitution of one or more protein residues with amino acids of other identities is routinely carried out using any of several simple procedures for DNA-based site-directed mutagenesis and large

quantities of the protein with the substituted amino acid can be expressed and purified in a matter of days. It comes as no surprise, therefore, that numerous attempts have been made to create protein variants based on NHRs that preferentially respond to a selected synthetic ligand over the natural ligand. Doyle *et al.* [15] previously made certain amino acid substitutions in the ligand-binding domain (LBD) of the retinoid X receptor (RXR) based purely on the consideration that the mutant protein is likely to remain functional with the introduced substitutions. Remarkably, some of the resultant functional mutant proteins displayed increased specificity for a synthetic ligand compared with the natural ligand. In other work, Miller and Whelan [16,17] performed multiple rounds of random point mutagenesis on the DNA sequence encoding the LBD of hER α and subjected the resulting protein library to a selection scheme to identify variants with increased response to a target synthetic ligand. Using this approach, several mutants with moderately increased specificity for the target ligand compared with the natural ligand were found.

The methodology for engineering ligand–receptor pairs recently published by Doyle and colleagues [11] represents an advancement over other techniques (such as those described above) for creating new specific ligand–receptor pairs in two key respects. First, the degree of specificity shift achieved in favor of the target synthetic ligand compared with the natural ligand is significantly higher than that achieved by other reported methods. Second, the described engineering approach is rapid and extendable to other ligand–receptor systems. To demonstrate their method, the authors applied a combinatorial codon randomization approach, whereby specific residue positions within the RXR-LBD were simultaneously randomized on a genetic level to any of a rationally selected subset of amino acids, so as to generate protein variants likely to bind to the selected synthetic retinoid-like compound LG335, but not to the natural ligand 9-*cis* retinoic acid (9cRA). In particular, six ligand-contacting sites were combinatorially randomized; at three sites, four possible amino acid substitutions were allowed, whereas the remaining three sites were allowed to randomize to any of eight possible amino acids. The use of a yeast two-hybrid based screening system, which couples the strength of mutant receptor–ligand interactions within host cells to their survival and growth on media lacking the essential nutrient adenine, allows the rapid selection of variant receptors that respond strongly to a selected synthetic ligand within a large library. Thus, by using a combinatorial codon randomization approach to generate a library of RXR variants, coupled with a yeast two-hybrid system to select for LG335-specific variants within the library, the authors rapidly sifted through a large library of RXR LBD variants (32 768 amino acid combinations generated by a gene library of $\sim 3 \times 10^6$ codon combinations) to identify 11 variants with increased specificity for LG335 compared with 9cRA. The two most LG335-selective variants each contained four amino acid substitutions relative to the wild-type RXR and had improved ligand response potencies toward LG335 of ~ 300 - and ~ 10 -fold, with accompanying drops in 9cRA ligand

activation by >7-fold and >45-fold, respectively in mammalian HEK 293 cells.

It should be noted that an alternative protein engineering method based on directed evolution was recently developed to engineer highly specific ligand–receptor pairs with great success [18].

Potential for application in biotechnology

The methodology presented by Doyle and colleagues represents an important tool in a protein engineer's toolbox, for engineering new receptors for target ligands as well as (potentially) enzymes for new substrates. This technology will certainly make it easier for biotechnologists to create highly specific protein–ligand pairs for important applications such as human therapy and functional genomics. However, certain advances would significantly enhance the ability of the described technology to impact these fields. The use of screening and selection systems that allow the rapid screening of much larger libraries of protein variants (larger than the $\sim 4 \times 10^5$ generated), for example, would allow us to generate more diverse libraries in which more sites in the ligand-binding domain are simultaneously randomized, thus allowing the identification of ligand–receptor pairs with even higher specificity. Such an expansion of screening power could also potentially allow the randomization of selected protein sites to all possible 20 amino acids, eliminating the need to select a subset of amino acids for randomization, based on potentially flawed rational considerations. The incorporation of a counter selection or screening scheme to simultaneously allow positive selection for the target ligand and negative selection against the natural ligand might also broaden the applicability of the technology because increased strength of response towards the target ligand need not always be accompanied by weakened activation by the natural ligand, as has been observed previously [19,20].

Conclusions

Of the two broad approaches to engineering new highly specific ligand–receptor pairs for transgene regulation applications, the re-engineering of receptors to rematch interaction with a given synthetic ligand has proven more effective and user friendly than the synthesis of various small molecule ligands to complement a given mutant receptor. The value of the protein engineering approach has been highlighted by the recent work of Doyle and colleagues, whose protein library generation based on combinatorial codon randomization, coupled with yeast two-hybrid selection of protein variants with increased specificity for a target ligand, rapidly produced highly specific ligand–receptor pairs. It is expected that further optimization of the described technology, as well as the development of other similar combinatorial protein engineering approaches, will increasingly contribute to the creation of ligand–receptor pairs for transgene regulation in biomedical applications.

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