Developing therapeutic proteins by engineering ligand–receptor interactions

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Ligand–receptor interactions govern myriad cell signaling pathways that regulate homeostasis and ensure that cells respond properly to stimuli. Growth factors, cytokines and other regulatory elements use these interactions to mediate cell responses, including proliferation, migration, angiogenesis, immune responses and cell death. Proteins that inhibit these processes have potential as therapeutics for cancer and autoimmune disorders, whereas proteins that stimulate these processes offer promise in regenerative medicine. Although much of the focus in this area over the past decade has been on monoclonal antibodies, recently there has been increased interest in the use of non-antibody proteins as therapeutic agents. Here, we review recent advances and accomplishments in the use of rational and combinatorial protein engineering approaches to developing ligands and receptors as agonists and antagonists against clinically important targets.

Introduction

Ligand–receptor interactions are tightly controlled to regulate signaling pathways involved in either maintaining cellular homeostasis or directing a particular biological program, such as embryonic development, an immune response or a response to injury. Dysregulation of these pathways can result in a broad array of diseases, including cancer, autoimmune disorders and regenerative disorders. The remarkable specificity of protein–protein interactions combined with the success of protein-based therapeutics has demonstrated the potential to combat a range of disorders by targeting specific ligand–receptor interactions [1]. For example, two biopharmaceuticals that selectively target dysregulated ligand–receptor interactions associated with cancer or autoimmune disorders, respectively, are bevacizumab, a monoclonal antibody that disrupts binding of vascular endothelial growth factor (VEGF) to its receptor, and etanercept, a recombinant protein that inhibits the activity of tumor necrosis factor (TNF) [1].

Given the success of these and other biopharmaceuticals, interest in protein-based therapeutics has surged in recent years. In the USA, biopharmaceuticals now account for one in four submissions for Food and Drug Administration (FDA) approval [2]. Furthermore, there are more than 180 FDA-approved protein products, which generate approximately US$50 billion in revenues annually, a number that is growing rapidly [3]. Much of this success has come from monoclonal antibodies [4], which carry out their biological activities either through immune-related effector functions or by inhibiting dysregulated ligand–receptor interactions. Despite their significant clinical success, antibodies have several well-known limitations: expensive manufacturing and handling facilities are often required; they are large in size (~150 kDa), which can limit their penetration into tumors or tissues; they are often associated with potentially undesired effector functions; and various intellectual property barriers exist. As a result, researchers are turning to non-antibody protein therapeutics to find ways to overcome these limitations.

Protein antagonists, polypeptides that prevent a receptor from being activated by its native ligand, have applications as therapeutics for cancer and autoimmune disorders. Conversely, protein agonists, polypeptides that bind to receptors to stimulate a biological response, would be useful in regenerative medicine applications. For example, consider the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase that regulates biological functions such as cell migration and proliferation. An antagonist of EGFR would offer therapeutic value by inhibiting uncontrolled proliferation in EGFR-overexpressing cancer cells [5], whereas an agonist would be useful for stimulating cell migration and proliferation in wound healing [6].

Rational and combinatorial approaches have been used to engineer proteins with desired properties such as altered binding affinity, or increased stability and levels of recombinant expression. A description of these approaches is presented in Box 1. This review discusses protein engineering strategies aimed at altering natural ligand–receptor interactions to modulate biological responses. We will not address the engineering of antibodies [7,8], non-antibody scaffolds [9] or specific directed evolution platforms [10–13], because these topics have been reviewed recently elsewhere. Rather, we will evaluate advances that use rational and combinatorial engineering approaches to generate ligand- and receptor-based agonists or antagonists with therapeutic potential.
Pearce and colleagues [14] studied mutant hGH proteins and problems that can arise from affinity-based screening. Increased cell proliferation offer a poignant example of the process by which the cell regulates its response to external stimuli. In general, receptor internalization occurs at a low basal level for inactive receptors and is upregulated upon receptor activation. Many therapeutically important signaling proteins, such as the ligands for receptor tyrosine kinases and G-protein-coupled receptors (GPCRs), are co-internalized with their receptors by vesicles that fuse with endosomes [17]. As shown in Figure 1, the ligand can be either recycled out of the cell intact, which enables continued positive signaling, or sent to the lysosome for degradation, which leads to signal depletion. Whether the ligand is recycled or degraded is largely determined by its affinity for the receptor in the endosomal compartment; in general, ligand–receptor complexes that remain bound and active are favored for degradation, whereas those that readily dissociate are favored for recycling [18]. This presents an obvious problem for those trying to develop effective agonists, because an engineered ligand with very high affinity might be degraded rapidly, thereby diminishing its potential activity.

Sarkar, Lauffenburger and colleagues [19] addressed this problem for granulocyte colony-stimulating factor (GCSF), a cytokine that increases white blood cell production and is used to aid recovery after chemotherapy. They engineered an improved GCSF variant by leveraging the pH differential in the extracellular space (pH 7) versus endosomal compartments (pH 5–6). A ligand that binds very tightly to the receptor on the cell surface but binds very weakly in the endosome would improve the extracellular half-life through recycling, thereby enhancing potency. Histidine side-chains (pKa ~6.4) will be neutral in the extracellular medium but protonated in the endosome. Using computational models, six positions on GCSF that have excessive negative charge density relative to their site of interaction on the receptor were individually substituted with neutral histidine or positively charged histidine, and the free energies of binding were computed for each mutant. The in silico model revealed two histidine mutations that showed the desired properties for being a better agonist: stronger binding relative to wild type with neutral histidine (in the extracellular space); and weaker binding relative to wild type with charged histidine (in the endosomes). Each of these mutations resulted in a ten-fold longer half-life and an enhancement of up to two-fold in cell proliferation assays versus wild type GCSF [19].

Another example of engineering ligand–receptor trafficking involves efforts to improve the agonistic effects of interleukin 2 (IL2). The recombinant form of this cytokine is approved for use in treating malignant melanomas [20]. Stimulation of T cells by IL2 is initiated when it binds to its α, β and γ receptor subunits (IL2RA, IL2RB and IL2RG, respectively). Upon binding, the ligand–receptor complex is internalized within endosomes. Here, the α subunit of the receptor dissociates and is recycled back to the cell surface, whereas the β and γ subunits are transported to lysosomes for degradation [21]. Therefore, mutations that disrupt the binding of IL2 to IL2RB and IL2RG or that increase binding to IL2RA within the endosomal compartment are most likely to increase ligand recycling, and hence increase potency. Rao, Wittrup and colleagues [20] applied directed evolution to engineer IL2 so that it bound...
with picomolar affinity to the IL2RA subunit. This method produced an IL2 mutant with enhanced agonistic activity due to increased recycling. Notably, the IL2 mutants exhibited receptor binding affinity and stimulatory effects on T cells similar to those of IL15. IL15 has a similar mode of activation to IL2; however, it binds to its own private IL15RA subunit with a 1000-fold greater affinity than that of wild-type IL2 and its α receptor [22]. Therefore, it was suggested that the engineered IL2 mutants could be used to stimulate responses modulated by IL15 for cancer immunotherapy.

**Engineering agonists on the basis of sequence variation**

Another approach to engineering agonists involves introducing mutations found in families of natural protein variants that are similar in structure or sequence. The reasoning behind this method is that nature has already sampled which amino acid residues are well suited for binding interactions, folding, expression and/or biological activity. This strategy has been used to engineer more-stable variants of human fibroblast growth factor 1 (FGF1), a protein that has clinical relevance for wound healing and regenerative medicine. Wild type FGF1 ligand possesses low thermodynamic and proteolytic stability, and as a result has a short *in vivo* half-life [23]. Using homology models with the assumption that residues conserved across most homologs give the highest contribution to stability, the analysis of consensus residues for an FGF1 alignment of 140 sequences followed by stability calculations on potential sites for substitution gave a series of point mutations that could increase the stability of this ligand [24]. The resulting mutants had thermal denaturing temperatures up to 27 °C higher than those of wild type FGF1, stimulated approximately ten-fold greater cell proliferation, and exhibited improved proteolytic resistance [25].

Likewise, protein variants with orthologous mutations—amino acid substitutions found in other species—are often isolated from directed evolution experiments, even when libraries of variants are generated by random mutagenesis. The sequences of previously reported engineered mutants of EGFR [26], IL2 [20] and the protease subtilisin [27] were analyzed, and it was found that mutations that led to gain of function were biased towards residues found in orthologs of the parent protein, whereas mutations that led to loss of function occurred primarily in highly conserved positions [28].

To generate enhanced EGF agonists by directed evolution, orthologous mutations were introduced into yeast-displayed libraries of EGF mutants, which were screened for clones that had increased binding to a soluble form of the EGFR extracellular domain. EGF mutants with up to a 30-fold increase in binding affinity for EGFR were identified [28] and were shown to significantly enhance fibroblast cell migration relative to wild type EGF (S.E. Beck, J.R. Cochran, et al., unpublished). In other studies, Chang and coworkers [29] and Brideau-Andersen and coworkers [30] shuffled the DNA of 20 human interferon-α (IFN-α)
Engineering protein-based antagonists

Altering a natural ligand or receptor to function as an antagonist is an effective strategy for creating protein-based biological inhibitors. The following examples have used rational engineering to develop the initial antagonist; however, directed evolution either has or could be applied to optimize properties such as target protein binding affinity, stability and recombinant protein expression levels.

Engineering ligands to bind to and antagonize receptors

An early example wherein structural and functional information was used to rationally engineer an activating ligand into a receptor antagonist is demonstrated in the development of pegvisomant, an engineered form of hGH that has been approved for the treatment of acromegaly. The hGH ligand, which is monovalent, induces dimerization and activation of the hGH receptor (hGHR) through two distinct receptor binding sites (Figure 2a, left). Fuh, Wells and coworkers [31] rationally introduced a single point mutation into hGH to abolish one of its receptor binding sites; a small amino acid (glycine) at the secondary binding interface was mutated to a large amino acid (arginine) to sterically block the binding site, while the primary binding site was left unmodified. This hGH mutant binds to but does not activate hGHR, and it inhibits hGH-induced cell proliferation, thus acting as an hGHR antagonist (Figure 2a, right) [31]. Eight additional amino acid mutations, identified by directed evolution, improved the binding affinity to hGHR and resulted in a more potent, high-affinity hGHR antagonist [32].

Other rational engineering approaches have been used to transform agonists into antagonists for ligand–receptor systems with different modes of activation. VEGF (also known as VEGFA) functions as a homodimer, with two VEGFA receptor binding sites per ligand. Amino acids involved in receptor binding are located in loops or turns at opposite ends of the homodimer. An antagonistic VEGFA heterodimer was rationally designed by abolishing one binding interface but leaving the other interface unmodified [33]. To ensure disruption of the binding interface, while maintaining the overall structure of the VEGFA mutant proteins, loops at one of the binding interfaces were substituted with the corresponding loops for platelet-derived growth factor B (PDGFB), a cytokine that has the same overall fold as VEGFA but which has no binding activity towards VEGF receptors. The resulting protein bound to soluble forms of the VEGF receptor with similar affinity to that of wild type VEGFA. This led to the inhibition of VEGFA-induced endothelial cell proliferation, suggesting that this approach could be useful for targeting tumor angiogenesis. In another approach, a peptide linker was used to covalently join two mutant VEGFA monomers. The mutant monomers were designed to form only one functional binding interface by altering the loop–turn length and amino acid content of one of the binding sites [34]. This single-chain VEGFA molecule still activated the receptor, albeit much more weakly than the wild type ligand did; therefore, further rational engineering would be necessary to develop a full antagonist.

In another approach, the NK1 fragment (the N-terminal domain and first kringle domain) of hepatocyte growth factor (HGF; also referred to as scatter factor) was transformed from an agonist to an antagonist of the HGF receptor (MET) tyrosine kinase, an oncogenic protein involved in the invasion and metastasis of many human cancers. MET is activated by homodimers of NK1 (Figure 2b, left) [35,36]. The crystal structure of NK1 homodimers revealed individual or combinations of amino acids that would be expected to stabilize the dimer interface. These amino acids were mutated to alanine to abolish the stabilizing interactions, resulting in a monomeric NK1 molecule [35]. The resulting monomeric mutants functioned as MET receptor antagonists (Figure 2b, right), because they did not exhibit agonistic activity towards the MET receptor, and they blocked HGF-induced MET activation and subsequent cell migration and proliferation. This highlights a useful approach for transforming a receptor tyrosine kinase agonist into an antagonist and might be applicable to other ligands that function as homodimers, such as VEGFA, FGF and nerve growth factor (NGF).

Engineering soluble receptors to neutralize ligand activity

Soluble, secreted, extracellular domains of transmembrane receptors are used in nature to modulate ligand activity [37–39]. Leveraging this biological phenomenon, soluble receptor extracellular domains have been used in research and clinical applications to antagonize their activating ligands, as illustrated in Figure 2c. One example is etanercept, a biological therapeutic approved for the treatment of a range of autoimmune diseases. Etanercept consists of a fusion between the extracellular domain of p75 TNF receptor (TNFR-II) and the Fc domain of a human IgG1 [40]. The TNFRII extracellular domain functions as an antagonist by sequestering soluble TNF (also known as TNFα) and preventing it from binding to cell-surface receptors. By contrast, the Fc domain functions to increase the half-life of the drug in the bloodstream. It does this presumably both by increasing the size of the molecule to resist filtration by the kidneys and through its association with cells that express Fc receptors. Soluble receptor extracellular domains have also been used in preclinical cancer research to neutralize activating ligands against the MET receptor [41], VEGF receptor 1 (VEGFR1) [42,43] and FGF receptor 1 (FGFR1) [44]. The extracellular domains of a receptor are often multidomain structures; as such, truncated ligand binding domains have also been used to antagonize a target ligand. For example, the ligand binding domain of the macrophage-stimulating protein (MSP) receptor Ron [45], and a fusion of individual functional domains of VEGFR1 and VEGFR2 [46] were both shown to function as potent ligand antagonists against MSP and VEGF, respectively.
Engineering soluble receptors to inhibit cell-surface receptor activity

In some ligand–receptor systems, recombinant soluble receptors can be used to antagonize receptor activity. For example, a soluble form of the MET receptor has been shown to act as a decoy, antagonizing MET signaling by blocking both HGF ligand binding and cell-surface receptor–receptor contacts [41]. Receptors can also exist in an inactive complex before ligand binding. TNF mediates its biological effects by binding to TNF receptors TNFR-I (also known as p55) and TNFR-II (also known as p75). A pre-ligand assembly domain (PLAD), which mediates formation of inactive TNFR-I or TNFR-II multimers before ligand binding, was identified in the extracellular domain of TNFR (Figure 3, middle) [47]. Binding of TNF ligand to the receptor dissociates the PLAD interactions of the inactive receptor complex, inducing a conformational change in the receptor and resulting in an activated signaling complex (Figure 3, left). The addition of excess soluble PLAD, particularly TNFR-I PLAD, antagonizes TNF-induced receptor activation by competing for binding to the receptor (Figure 3, right) [48]. The formation of pre-existing inactive receptor complexes occurs in other systems, such as the Fas receptor (TNR6) [49], TRAIL
(TNF-related apoptosis-inducing ligand; also referred to as TNF10) receptors [50,51] and IL17 receptor A (IL17RA) [52,53], suggesting that this strategy could form a general approach to developing receptor-targeted antagonists.

**Factors to consider when playing with nature**

Despite the relative simplicity and successes of the examples presented above, several issues have risen in efforts to develop and use protein antagonists as therapeutic agents. First, the monomeric form of a dimeric ligand might show severely diminished binding affinity for its receptor, as was observed for monomeric VEGFA, which bound to the VEGFR2 receptor with an affinity >1000-fold weaker than that of natural dimeric VEGFA [54]. Second, ligand binding affinity can be significantly decreased when the receptor extracellular domain is removed from the constraints of the cell membrane. For example, the equilibrium binding constant of EGF binding for soluble EGFR extracellular domain is 2–3 orders of magnitude weaker than that of cell-surface EGF–EGFR interactions [55]. Third, receptors with clinical relevance are generally complex, multidomain mammalian proteins and can suffer from low levels of recombinant expression. Directed evolution can be helpful in overcoming each of these limitations. This powerful technology has been used to affinity-mature ligand–receptor interactions by more than 1000-fold [13] and to increase the levels of recombinant expression of complex soluble receptor extracellular domains to several milligrams per liter [26,56].

Kinetic and equilibrium binding parameters, receptor trafficking, protein stability, pharmacokinetics and immunogenicity can all impact on how effective a protein will be as a therapeutic. In one recent example, engineering a ligand to have a decreased receptor dissociation rate did not correlate with enhanced biological efficacy, because internalization of bound complexes within the cell occurred on a rapid time-scale that made the effects of the slower dissociation rate negligible [57]. Often, these undesired outcomes are difficult to predict or are not considered at the outset of a protein engineering project, and these are lessons learned the hard way. However, mathematical analyses are starting to play a more prominent role when choosing appropriate engineering parameters that will enhance therapeutic efficacy [58,59].

Mathematical modeling was used recently to explain the correlation between the binding affinity and biological activity of engineered hGH antagonists and agonists [15]. hGHR antagonists will bind to the receptor but will not activate it, maintaining the slow, basal internalization rate of these inactive receptors. Therefore, an affinity-enhanced hGH antagonist will possess persistent receptor binding, and hence increased antagonistic activity. However, a weaker-binding hGH antagonist (e.g. wild type hGH containing only the G—R mutation discussed above) will dissociate from the hGHR on a faster time scale than receptor internalization, limiting its antagonistic activity. By contrast, hGH agonists will bind to and activate the hGHR, thereby upregulating the internalization rate of these activated receptors. The hGH agonists described above, which possess 400-fold higher hGHR binding affinity than wild type hGH, did not exhibit improved activity, because both wild type hGH and the high-affinity hGH were internalized on a faster time scale than their dissociation from the hGHR. This elegant modeling study highlights an important distinction for engineering agonists versus antagonists. Improvements to the affinity of antagonists with moderate receptor binding affinity will often result in enhanced antagonism; however, the effects that affinity enhancements have on agonists are more complicated, largely owing to the ligand–receptor trafficking events discussed in Figure 1. The modeling study also suggests that faster association rates of receptor binding could yield improved agonistic activity [15]. Therefore, to generate improved agonists, it might be necessary to explicitly engineer for faster association rate, rather than simply stronger receptor binding affinity.

In cases in which the susceptibility to undergo proteolysis results in rapid degradation, a protein can be engineered by rational or combinatorial approaches to increase proteolytic stability [25,60,61]. Many therapeutic proteins also exhibit undesired pharmacokinetic properties, such as rapid clearance from the body. Researchers have adopted several approaches to improve the in vivo circulation half-life of protein therapeutics; for example, by increasing their molecular weight, so that these proteins resist filtration by the kidneys. Such approaches include conjugation to polyethylene glycol (PEGylation), fusion to the Fc domain of a human antibody, fusion to an albumin-binding protein, or...
altering glycosylation [62]. For instance, erythropoietin variants with two additional targeted glycosylation sites exhibit a three-fold increase in serum half-life [63], and PEGylation of interferon α-2b confers in vivo half-life improvements that enable dosing of once weekly, instead of three times per week [64]. Finally, the immunogenicity of modified or mutated proteins is also a crucial concern [65]. Generally, the less a protein deviates from its natural human form, the lower the probability that it will elicit an immune response. Engineered proteins that differ slightly from their native form have successfully passed clinical trials; however, it is difficult, if not impossible, to predict whether a particular protein will be immunogenic. In some cases, glycosylation [63] and PEGylation [66] have been shown to reduce immunogenicity by masking immunogenic epitopes.

Concluding remarks

Most successful attempts to engineer protein-based antagonists and agonists have used rational methods that rely on structural, computational or biochemical information about the ligand–receptor system of interest. However, as we have discussed here, rational and combinatorial engineering approaches are not mutually exclusive, and the combination of both is a powerful strategy for protein engineering, especially for protein-based antagonists for which the increased binding affinity often correlates with improved antagonism. Engineering enhanced protein agonists remains a challenge; novel methods that use high-throughput screening assays to identify protein variants with improved function, rather than improved binding affinity, will be important for the future development of more efficacious therapeutic agents.

Monoclonal antibodies have provided enormous clinical success, but they are not without their limitations. In certain cases, engineered ligands or receptors might have distinct advantages over antibodies. The large size of antibodies results in poor tissue perfusion, whereas smaller proteins could more fully interrogate a tumor mass [67]. Antibodies also possess a long in vivo half-life, which is advantageous for therapeutic applications; however, a long half-life results in high background signals for molecular imaging applications, where rapid systemic clearance is desirable. Engineered ligands or receptors present the flexibility of smaller size and established rational engineering approaches to modulate in vivo half-life when desired. Although smaller antibody fragments have been developed, they are often associated with weaker binding than the intact antibody, they can exhibit lower stability, and they might expose immunogenic epitopes that were previously masked [68]. Lastly, antibodies offer limited potential to engineer for agonistic activity. If simple receptor clustering results in receptor activation, the bivalent nature of antibodies could result in agonistic activity; however, antibodies usually exhibit antagonistic activity or no activity at all – known as ‘silent’ binding. By contrast, starting with a natural agonist, one could engineer ligands with enhanced biological activity. In addition, ligands and receptors naturally bind functionally important epitopes and provide a good starting point for engineering protein–protein interactions; conversely, antibody discovery typically requires screening of naïve libraries and testing of many clones to identify molecules that bind to desired epitopes.

As our understanding of biological systems continues to expand, direct engineering of ligand–receptor interactions will be increasingly used as a complement to, or in place of, antibody-based approaches. We envision that proteins generated from these efforts will help bring to bear the next generation of therapeutics, molecular imaging agents, targeted drug delivery agents, and selective tissue targeting probes.

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