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Engineering Selectivity and Discrimination into Ligand-Receptor Interfaces

Review

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Summary

The reengineering of protein-ligand (or enzyme-substrate) interfaces using a combination of chemical and genetic methods has become an increasingly common technique to create new tools to manipulate and study biological systems. Many applications of ligand receptor engineering require that the engineered ligand and receptor function independently of endogenous ligands and receptors. Engineered ligands must selectively interact with modified receptors, and modified receptors must effectively discriminate against endogenous ligands. A variety of chemical design strategies have been used to reengineer ligand-receptor interfaces. The advantages and limitations of various strategies, which involve the manipulation of hydrophobic, polar, and charged residues, are compared. New design strategies and potential applications of ligand-receptor engineering are also discussed.

Protein-ligand engineering is an increasingly important technique for creating new tools for the manipulation and study of biological systems. For almost two decades, chemical and genetic methods for manipulating the molecular structures of small molecule-protein interfaces have been used to alter enzyme substrate specificity or to generate new ligand-receptor pairs that can selectively regulate transcription, apoptosis, genetic recombination, signal transduction, or motor protein function (For reviews see [1–3]). Whereas many recent reviews have focused on the many applications of ligand-receptor engineering, this review aims to provide a general overview of the different design strategies that have been used in such studies, in terms of principles of molecular recognition and protein engineering. For simplicity, generic discussions will refer to the binding of ligand-receptor pairs. However, it should be understood that the same principles should apply to enzyme-substrate pairs, (i.e., substrate/transition state binding), as well. Throughout this review, examples will be cited that illustrate specific design principles, although the success of any particular design strategy may be dependent on a number of factors beyond the "design strategy." Thus, such examples should only be regarded as a qualitative guide.

Orthogonal and Functionally Orthogonal Ligand-Receptor Pairs

For most applications of ligand-receptor engineering, one generally seeks to design an "orthogonal ligand," a ligand that cannot bind to the natural receptor but can bind to an appropriately modified form of the receptor. For many applications it is also desirable to engineer "orthogonal receptors," which exclusively bind the synthetic ligand and not the natural ligand [1]. In practice, engineered ligand-receptor pairs need not be strictly orthogonal but need only be functionally orthogonal. Functionally orthogonal receptors can be defined as engineered receptors that are not activated by concentrations of the natural ligand found endogenously. In other words, a modified receptor can still have some measurable affinity for its natural ligand but would be unaffected by the natural ligand if the endogenous ligand concentration is well below its K_d (K_M or EC_{50}) (Figure 1A). The modified receptor's reduced affinity for the natural ligand can be thought of as the receptor's ability to discriminate against the endogenous ligand. Similarly, a ligand can be regarded as being functionally orthogonal if it has sufficiently higher affinity for the modified receptor than the endogenous receptor, such that there is a significant range of ligand concentrations wherein the modified receptor is exclusively occupied (Figure 1B). The difference in affinity for the modified receptor versus the wild-type can be regarded as a property of the ligand, the ligand's receptor selectivity. For many applications, it is necessary for both the designed ligand to selectively bind the engineered receptor, not the wild-type, and for the engineered receptor to not bind the natural ligand.

Steric Complementation

Bumps and Holes

One of the easiest ways to envision creating an orthogonal ligand-receptor pair is to modify the complementary shape of the ligand-receptor interface such that the engineered ligand is too large to fit within the binding site of the wild-type receptor but can bind to a modified form of the receptor whose binding site has been appropriately enlarged by mutagenesis (Figure 2A). The term "bumps and holes" was first used to describe an engineered cyclosporin/cyclophilin interface and the term has become almost synonymous with protein-ligand engineering in the common scientific vernacular [4]. However, not all bumps and holes are alike, and many other ligand design strategies have been employed to generate unique protein-ligand interfaces.

One common limitation to this strategy of ligand-receptor engineering is that whereas it is relatively straightforward to create a "bumped ligand" that does not fit in the endogenous receptor, the "hole-modified" receptor often retains significant affinity for the natural ligand because the generation of a hole does not actively discriminate against the natural ligand (Figure 2B). For example, bumped cyclosporin analogs can selectively bind to hole-modified cyclophilin mutants generated by a Phe→Ala substitution in combination with one or two other mutations (hCyP(Ser99→Thr/Phe113→Ala) and hCyP(Ser99→Thr/Phe113→Ala/Cys115→Met)) (Figure 3).

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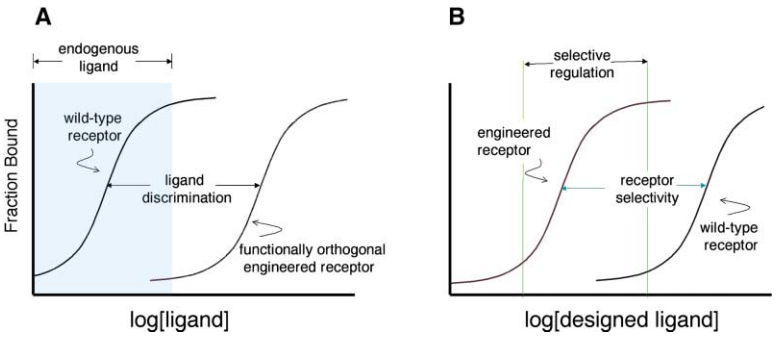

Figure 1. Definitions of Selectivity, Discrimination, and Functionally Orthogonal

(A) A functionally orthogonal receptor is not activated by endogenous concentrations of the natural ligand.

(B) A functionally orthogonal ligand is one capable of activating a modified receptor over a significant concentration range without significantly activating endogenous receptors.

However, these modified receptors bound the natural cyclosporin with almost equal affinity as the wild-type cyclophilin [4]. In another example, bumped ATP analogs have been created that selectively bind modified forms of myosin-Iβ modified by a Tyr61→Gly mutation within the ATP binding site. However, the modified receptor had nearly wild-type affinity for ATP [5]. Similarly, hole-modified forms of v-Src, by either Ile338→Ala or Ile338→Gly mutations, exhibited only a less than 7-fold reduction in ATP affinity [6]. These studies suggest that the bump and hole strategy is quite successful at generating ligands that selectively bind modified receptors, but often the modified receptors do not appreciably discriminate against their natural ligands.

It should be noted that not all applications of ligand-receptor engineering require that the receptor not be able to bind the endogenous ligand. Receptors with functionally silent mutations that can partially label specific downstream molecular targets or which can be selectively inhibited by unique inhibitors are powerful tools in chemical biology [1]. Nonetheless, it is often desirable and necessary to engineer systems such that both ligand and receptor are functionally orthogonal to the endogenous system. The challenge is to select mutations to the receptor that actively discriminate against binding the natural ligand and yet can be recognized by synthetic ligands with high affinity and selectivity.

Dual Bump-Hole/Hole-Bump Modifications

One obvious strategy to create mutually orthogonal ligand-receptor pairs has been to create dual bump and hole plus hole and bump combinations to provide mutually orthogonal ligand receptor pairs (Figure 4A). This strategy was recently demonstrated by Shokat et al., who designed orthogonal nucleotide triphosphate/kinase pairs [7]. In this case, substrate selectivity and receptor discrimination could be engineered into the new receptor. However, the new ligand-receptor pair had greatly reduced catalytic efficiency. While in principle it is possible to apply bump and hole strategies to make both a ligand and a receptor that are mutually orthogonal to the endogenous ligand-receptor pair, these results suggest that it may be difficult to maintain high affinity or catalytic efficiency when grossly modifying the protein-ligand interface by multiple opposing mutations. The use of random mutagenesis/selection techniques may be able to overcome these limitations [7].

Polar Ligand-Receptor Interactions Can Provide Specificity

Reversal of Hydrogen Bonding Patterns

Classic studies in molecular recognition and protein folding suggest that polar interactions, hydrogen bonds, and ion pairing are often associated with conferring specificity to molecular recognition processes [8, 9].

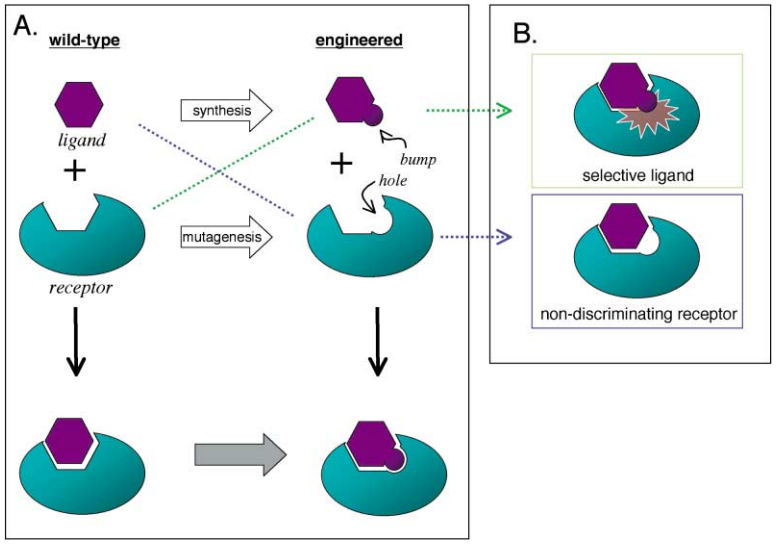

Figure 2. Altering Steric Complementarity by Bump and Hole Engineering

(A) Generation of an orthogonal ligand by bump and hole engineering.

(B) The ligand selectively binds only the engineered receptor, but the engineered receptor may still bind the endogenous ligand.

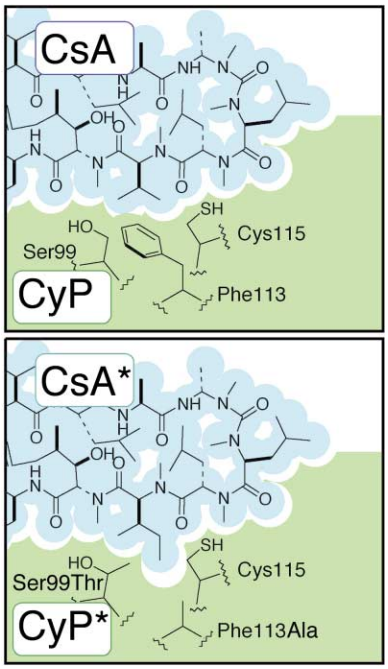

Figure 3. Key Cyclosporin/Cyclophilin Interactions

Bump and hole modifications alter the hydrophobic groups at the interface of Cyclosporin (CsA) and cyclophilin (CyP) [4].

Hwang and Miller had one of the early and most successful ligand-receptor design strategies for reengineering GTPases to selectively use xanthine-nucleoside triphosphates (XTP) as substrates by manipulating hydrogen bonding interactions [10]. The hydrogen bonding pair at the ligand-receptor interface of EF-Tu is reversed by exchanging a hydrogen bond acceptor for a hydrogen bond donor and vice versa (Figure 4B). In this case, the carboxylate side chain of Asp138, which contributes two hydrogen bond acceptors to the protein-ligand interface, is mutated to the amide of asparagine, which effectively exchanges one acceptor for a

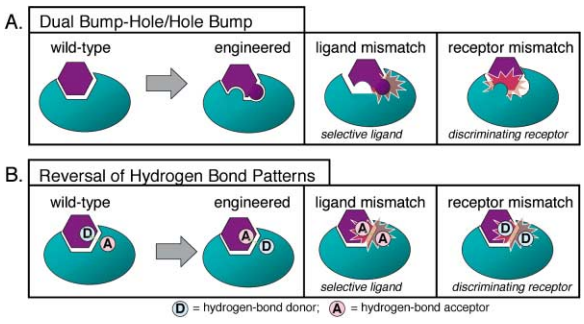

Figure 4. Selective Ligand Receptor Pairs May Be Created by Modification of Neutral Interactions

(A) Dual bump-hole/hole-bump modified receptors may provide improved substrate selectivity and receptor discrimination.

(B) Reversal of hydrogen bonding patterns can afford orthogonal ligand-receptor pairs without grossly affecting the shape of the ligand-receptor interface.

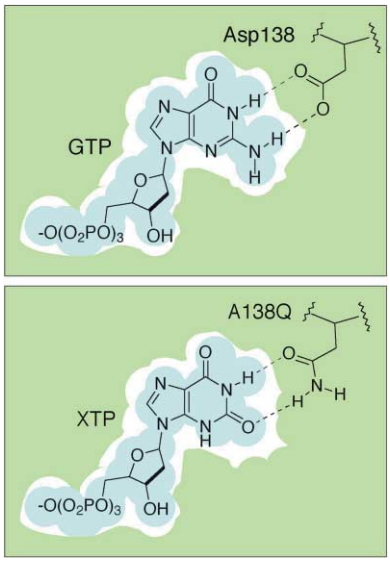

Figure 5. Key GTP/EF-Tu Interactions

Reversal of hydrogen bonding pairs was used to convert the GTPase EF-Tu (top) to a XTPase (bottom) [10].

hydrogen bond donor (Figure 5). The modified receptor shows substantially reduced affinity for the natural substrate GTP, which if bound would form a donor-to-donor mismatch. For analogous mutations in Ha-Ras p21, an Asp→Asn substitution reduces the GTP binding affinity by a factor of almost 10^3 but has a XTP affinity similar to the wild-type receptor's affinity for GTP [11]. Thus, altering hydrogen bonding patterns is one successful strategy to engineer selective ligands for mutant receptors that discriminate against their natural ligands or substrates. However, it is often the case that the amino acid side chains involved in hydrogen bonding are not amenable to reversing hydrogen bond patterns by simple substitution for another amino acid. In such cases, polar interactions may also be exchanged for nonpolar.

Manipulation of Charged Interactions

The manipulation of charged groups that are a part of or adjacent to the ligand-receptor interface can also have a significant effect on selectivity and discrimination. One of the principle advantages of this approach is that charged groups that do not directly contact the ligand can still influence ligand binding through space. Kampoor and Mitchson modified several conserved residues in the vicinity of N6 of the adenine of ATP in the ATP binding pocket of kinesin [12]. Within the obvious limitations of comparing different mutations at different positions within the protein, it is interesting to note that mutation of neutral residues Thr94→Ala, Pro17→Gly reduced the activity with ATP by only 60% of the wild-type, whereas modification of Arg14 (Arg14→Ala), which indirectly contacts the ligand through intervening water molecules, reduces the activity to below 1% of wild-type. In this case, the "hole" created by deleting the cationic arginine side chain could be complemented by a hydrophobic cyclopentyl group appended to N6 of adenosine of ATP. The process of exchanging polar ligand-receptor interactions with hydrophobic pairs can

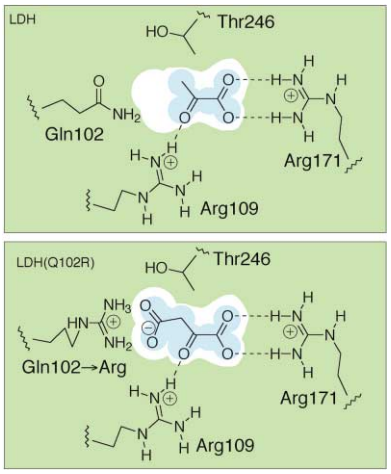

Figure 6. Key Lactate/Lactate Dehydrogenase Interactions

The introduction of a salt bridge converts a lactate dehydrogenase (top) to a malate dedehydrogenase (bottom)[13].

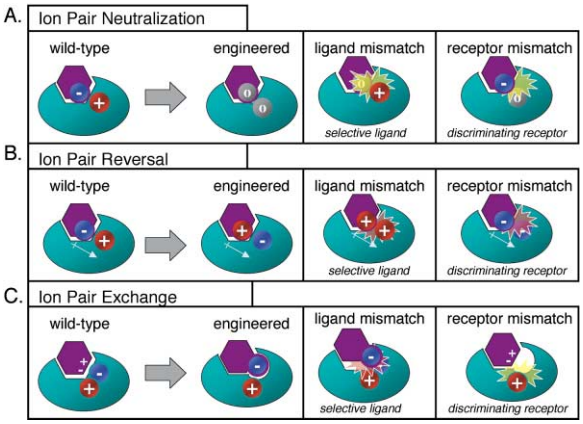

Figure 7. Selective Ligand-Receptor Pairs May Be Created by Modification of Ion-Pairs

(A) Charge to neutral substitutions are often effective at providing selective interactions because unpaired counter ions are ineffectively compensated for the cost of desolvation in the mismatched ligand-receptor pairs (yellow).

(B) Charge reversal may offer significant selectivity but is often associated with substantially lower affinity.

(C) An intramolecular salt bridge interaction can be converted to an intermolecular protein-ligand salt bridge.

indirectly provide selectivity and discrimination by leaving polar groups insufficiently compensated for the cost of desolvation in the mismatched complexes.

Introduction of New Ion Pairs

One of the early examples of reengineering enzyme substrate specificity reported by Holbrook involved converting a lactate dehydrogenase to a malate dehydrogenase by introducing a charged residue that can selectively ion pair with malate [13]. The Gln102→Arg mutant displayed a 30-fold higher K_M for pyruvate and a 25-fold decrease in K_M for oxaloacetate (Figure 6). This example illustrates that new polar interactions can be introduced to change substrate specificity. The arginine substitution does not dramatically reduce the enzymes affinity for pyruvate based on the measured K_Ms; however, this is a remarkably selective catalytic system when one compares K_{cat}/K_M. Similarly, Perham described a system wherein arginine substitutions within the cofactor binding site were used to convert pyruvate dehydrogenase from a NAD-dependent enzyme to an NADPH-dependent enzyme [14].

Ion Pair Neutralization

Whereas engineering high-affinity interactions by introducing ion pairs into the ligand-receptor interface can be difficult, the removal of existing ion pairing interactions between ligand and receptor can provide substantial discrimination against the natural substrate (Figure 7A). For example, manipulation of an intermolecular salt bridge between the carboxylate group of retinoic acid tRA and the guanidine group of Arg269 of RARβ has been used to alter the ligand binding specificity of a ligand-dependent transcriptional regulator [15]. In this case, a cationic to neutral/polar substitution, Arg269→Gln, caused a more than 700-fold reduction in activity for retinoic acid and increased its activity toward the structurally similar but neutral ligand retinol by over 110-fold. A similar charge neutralization strategy has been applied in conjunction with a bump and hole modification to the closely related receptor subtype RARγ. In this case, the double mutant RARγ(S289G, R278E) could be selectively activated by a neutral but longer ethyl amide of retinoic acid; the receptor's activity with the natural ligand tRA could not be determined due to significant endogenous concentrations of the wild-type receptor [16].

Charge Reversal

The reported successes of modifying receptors to discriminate against their natural ligands by "neutralizing" charged residues or reversing hydrogen bonding partners suggest that reversing the polarity of ion pairing interactions may also be a successful strategy to alter ligand binding or enzyme substrate specificity (Figure 7B). Kirsh successfully reengineered an aspartyl aminotransferase to preferentially accept L-arginine as a substrate by substituting the cationic residue Arg292, involved in ion pairing to substrate side chain, to an anionic aspartic acid [17]. In this case, the selectivity was modest, 16-fold based on Kcat/K_M ratios, and the modified enzyme was 43,000 times less active than the wild-type enzyme with its natural substrate.

Similarly, in the complex of retinoic acid (tRA) and the retinoic acid receptor (RAR), the carboxylate group of retinoic acid is bound via a salt bridge to Arg278. A cationic guanidine-functionalized retinol derivative could preferentially activate a engineered receptor RARγ(S289D), which presumably forms a salt bridge of opposite polarity of the wild-type complex [16]. However, this charge-reversed ligand receptor pair also shows dramatically reduced activity compared to the wild-type ligand-receptor pair. The general phenomenon, that reversal of salt bridge pairs does not afford structures (or complexes) of equal stability, has been well documented in the protein folding/stability literature [17–20]. Warshel has suggested that the partners involved within an intermolecular salt bridge are also stabilized by multiple interactions within the protein-ligand

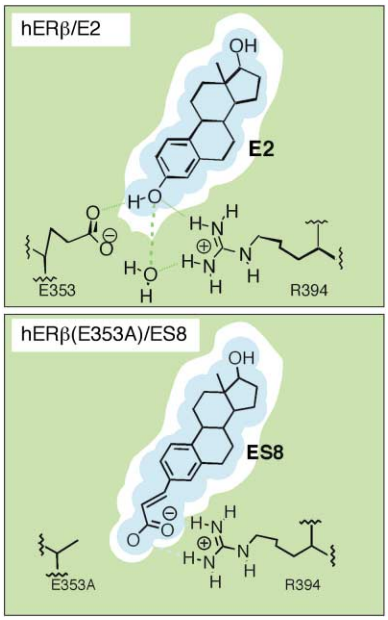

Figure 8. Key Estradiol/Estrogen Receptor Interactions

An intramolecular protein salt bridge which provides hydrogen binding interactions to bound estradiol (E2) in hERα (top) can be exchanged for an intermolecular salt bridge (bottom) [23].

complex, which are lost or may become destabilizing to the complex when the salt bridge polarity is reversed [20, 21]. This suggests that while charge reversal may in principle be capable of producing orthogonal ligand-receptor pairs, the general approach may in general be limited by a substantially reduced affinity of the modified complex.

Polar/Charged Group Exchange

Two recent reports by Tedesco et al. and Shi et al. have explored two different successful strategies to reengineer the interactions between estradiol (E2) and the estrogen receptor. Both involve the modification of Glu353, which forms an intramolecular protein salt bridge with Arg394 as well as forms hydrogen bonds to the phenol hydroxyl of E2 (Figure 8) [22, 23]. Together, these studies allow a more direct comparison of different strategies for complementing the same receptor mutations.

Tedesco et al. compared the relative potencies of estradiol for three mutants at positions 353. Substitution of Glu353 for Asp, Ser or Ala resulted in a 5-, 150-, or a 400-fold reduction in potency of the natural ligand E2, again suggesting that replacement of charge residues with neutral and nonpolar ones may be a general strategy for generating modified receptors which significantly discriminate against their natural ligands [22].

Tedesco et al. evaluated eight estradiol analogs that contain neutral, hydrophobic, or neutral hydrophilic pendant groups in place of the phenol hydroxyl effectively replacing the anionic partner of a salt bridge with a hydrophobic group. Their two best-matched hydrophobic extensions could activate ERα(E353A) mutant with 9 and 34 times higher potency than the wild-type ER [22]. Shi et al., using a strategy they termed "polar-group exchange," complemented the same mutation in an ERα

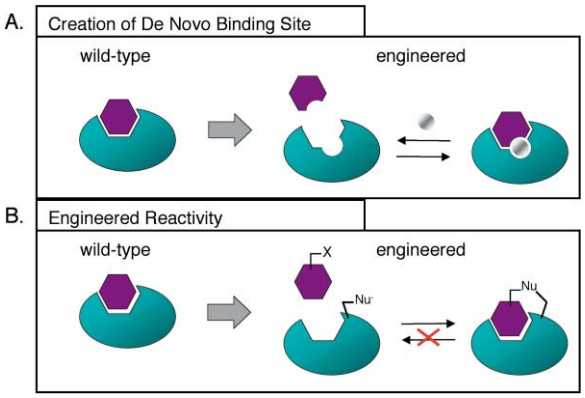

Figure 9. New Strategies in Protein-Molecule Engineering

(A) New binding sites can be engineered into both protein-ligand (enzyme-substrate) and protein-protein interfaces.

(B) The introduction of complementary reactive functionality may offer a new approach to increasing selectivity and high avidity.

variant by replacing the carboxylate group lost upon Glu→Ala substitution with one covalently attached to the ligand (Figure 8). They showed that carboxylate-functionalized analogs of estradiol could also effectively complement the Glu353→Ala mutant with a greater than 16-fold selectivity for the mutant over wild-type [23]. Shi et al. have recently confirmed their results using native ERα. Their best-matched carboxylate-functionalized ligand has a 95-fold preference for ERα(E353A) over the wild-type receptor (J.T.K. and Y. Shi, personal communication). The high affinity and increased selectivity achieved by exchanging an *intra*molecular protein salt bridge for an *inter*molecular protein ligand salt bridge is likely due to combining both steric and electrostatic interactions (Figure 7C).

The advantage of this "polar-group exchange" strategy for manipulating ion-paired charged residues compared to a "charge-reversal" strategy is that the new ligand receptor complex effectively differs from the wild-type complex only in the covalent connectivity of the same charged functional groups and therefore preserves the dipolar and other stabilizing interactions found in the native ligand-receptor complex. The generality of this approach remains to be demonstrated. Clearly, not all ligand binding sites are conveniently located adjacent to structural salt bridges, although in principle this strategy should also work for other neutral polar interactions. Furthermore, not all proteins will tolerate the loss of a structurally stabilizing salt bridge. In general, one should only expect to obtain high-affinity ligands when the polar groups in the engineered complex are precisely matched and when energetic cost of desolvating the polar partner on binding is not too large compared to the favorable pairing interactions gained upon binding.

Creating De Novo Binding Sites at Ligand-Receptor Interfaces

An alternative approach to engineering new complementary ligand-receptor interfaces is to engineer de novo ligand binding sites into the ligand-receptor or protein-protein interfaces. (Figure 9A). One of the first examples in this area was demonstrated by Craik et al., who engi-

neered a new metal binding site at the enzyme-substrate interface at the P2′ site of trypsin (Tn) by introducing metal-coordinating functional groups into both the enzyme and the substrate [24]. The Tn(Asn143→His/Glu151→His) mutant conditionally hydrolyzed peptide substrates that contain a histidine only in the presence of added nickel ions.

Schultz et al. recently demonstrated that a binding cavity for a neutral small molecule could be engineered into a protein-protein interface [25]. Binding and signal transduction could be conditionally activated in the presence of specific indole analogs that bound to a de novo binding site engineered into the dimerization interface between human growth hormone (hGH) and the human growth hormone receptor (hGHbp). Critical to the success of this strategy was the use of phage display methods to select forms of hGH(Thr175→Gly) capable of binding hGHbp(Trp104→Gly) only in the presence of added ligand. Importantly, this study demonstrated that applications of ligand-receptor engineering need not be limited to existing protein-ligand interfaces but may also, in principle, be applied to virtually any protein-protein interface, dramatically extending the range of potential applications of ligand-receptor engineering.

New Strategies and Applications

Ligand-receptor engineering continues to play an important role in developing new tools to manipulate and study biological systems. Recent studies suggest that the scope of ligand-receptor engineering may also be used to control the assembly of protein-proteins interfaces and that ligand-receptor engineering may offer new strategies for the potential treatment of certain human genetic diseases. Clearly, we are only beginning to scratch the surface of the possible applications that ligand-receptor engineering may hold. Regardless of whether rational molecular design, screening compound libraries, or genetic selections are used to develop new orthogonal ligand-receptor pairs, judicious choice of an appropriate ligand-receptor engineering strategy is often critical to its successful application.

Selectivity through Engineered Reactivity

It is often difficult to develop new ligands with very high avidity or that can act as the exclusive ligand (or substrate) for an engineered receptor in the presence of endogenous competitors. Such design strategies would be important for creating selective inhibitors that can act as conditional knockouts [26] or for limiting ligand diffusion in situations where ligands are only targeted to specific cells or regions of tissue [27]. New strategies are being developed to effectively increase the affinity between engineered ligand receptor pairs by introducing reactive functional groups, which could be used to selectively form covalent complexes (Figure 9B).

Engineered Ligands for Mutant Receptors Associated with Human Genetic Disease

The realization that many human genetic diseases are associated with mutations to receptors that impair ligand binding suggests that principles of ligand-receptor engineering may ultimately be applied to the designed of custom pharmaceuticals for certain genetic diseases. Ye et al. recently demonstrated that a thyroid hormone mimic could be designed to complement a mutationally impaired receptor associated with the human genetic disease resistance to thyroid hormone (RTH) [28]. Although applications of such strategies to the treatment of human disease are still many years away, this study emphasizes the need to understand and develop multiple strategies to complement receptor mutations, which discriminate against their natural ligands.

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