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Selective Inhibition of Engineered Receptors via Proximity-Accelerated Alkylation

Konstantin Levitsky,[†] Christopher J. Ciolli,[†] and Peter J. Belshaw*

Departments of Chemistry and Biochemistry, University of Wisconsin−Madison, Madison, Wisconsin 53706

belshaw@chem.wisc.edu

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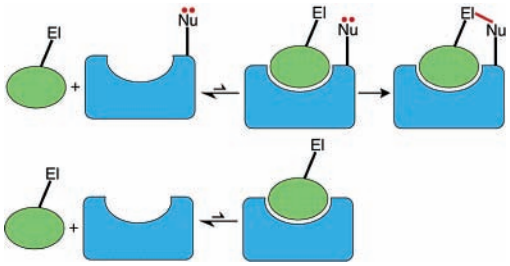
ABSTRACT



A new approach for creating allele-specific inhibitors is demonstrated. In this approach, a receptor and ligand are engineered to contain complementary reactive groups that form a covalent bond via a proximity-accelerated reaction upon formation of the receptor–ligand complex, irreversibly modulating the biological function of the receptor. This approach is demonstrated in the cyclophilin–cyclosporin receptor–ligand system by introducing thiol and acrylamide functional groups in the receptor and ligand, respectively.

In recent years, engineered receptor−ligand pairs have been used extensively for the investigation of biological systems. A common strategy involves reshaping the receptor−ligand interface through the addition of "bumps" on the ligands and the engineering of compensatory "holes" in the receptors.[1] This approach has been used to generate isoform-selective inhibitors[2,3] and activators[4−10] of engineered receptors, providing experimental approaches to investigate the cellular functions of individual receptor isoforms. A significant challenge in the development of allele-specific ligands is the identification of compensatory mutations in the receptor that are functionally silent. The cellular activity of the modified receptors are often altered when mutations are introduced into the ligand binding pocket, complicating interpretation of function.[3,11] We have taken an alternative approach wherein the engineered ligand can covalently modify the engineered receptor through the introduction of suitably reactive functional groups on both the ligand and the receptor allowing for the selective formation of covalent complexes via a proximity-accelerated reaction. In particular, we were interested in creating engineered receptor−ligand pairs that would form covalent complexes via affinity labeling by an

* To whom correspondence should be addressed.
[†] These authors contributed equally to this work.

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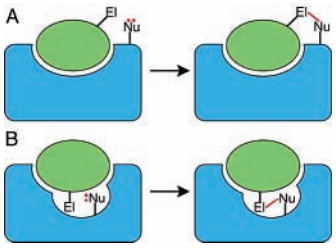


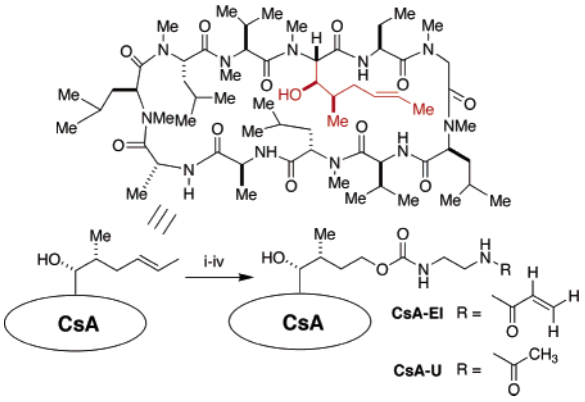
Figure 1. Illustrations of the exo (A) and endo (B) mechanisms for proximity-dependent modification of receptors. In the exo mechanism, the reactive group is located outside the active site on the surface of the receptor. In the endo mechanism, the reactive group is located within the active site of the receptor. Nucleophile (Nu), electrophile (El).

exo mechanism rather than an endo mechanism[12] (Figure 1). We anticipated that exo mechanism inhibitors would facilitate the creation of engineered receptors with wild-type biological activity, as mutations could be introduced on the surface of the receptor at residues outside the ligand-binding site.

Recently, several groups have utilized receptor−ligand interactions to induce proximity-accelerated reactions for several distinct purposes. For example, Chmura and Meares engineered reactive antibody−chelate ligand pairs that form covalent complexes for applications in the targeted delivery of imaging reagents or therapeutic agents to cells and tissues.[13] The Sharpless group demonstrated "click chemistry in situ" where two ligands simultaneously bound to a target protein generate a high-affinity bivalent ligand through proximity-accelerated formation of a covalent link.[14] Wells and co-workers at Sunesis developed "tethering", an approach using proximity-accelerated dynamic disulfide exchange to identify low-affinity lead inhibitors with both wild-type and engineered receptors.[15] These leads can be optimized to give high-affinity noncovalent inhibitors of wild-type protein targets. Gartner and Liu have demonstrated a variety of proximity-accelerated reactions templated by sequence-specific formation of DNA duplexes containing covalently tethered reactive groups.[16] In this paper, we demonstrate that the proximity-accelerated formation of a covalent bond in engineered receptor−ligand pairs can provide a method for obtaining selective inhibitors of the engineered receptor over the wild type.

We selected the cyclophilin A−cyclosporin A receptor−ligand system for our experiments.[17] Cyclosporin A (CsA) is a reversible inhibitor (Ki ≈ 5 nM) of the peptidyl-prolyl cis−trans isomerase (PPIase) activity of cyclophilin A (CypA). Examination of the X-ray crystal structure of the CypA−CsA complex[18] indicated that residues 103−106 of CypA were in close proximity to the MeBmt-1 side chain of CsA yet not in direct contact with CsA. As the MeBmt-1 side chain can be substantially modified while retaining binding to cyclophilin,[19] we chose to introduce reactive groups into the MeBmt-1 residue of CsA and residues 103−106 of CypA. In the selection of reactive groups for the formation of covalent receptor−ligand complexes, the Michael addition of a thiolate with an acrylamide was chosen, as these groups have been shown to be effective for proximity-accelerated additions both in vitro[13] and in vivo.[20] The acrylamide is sufficiently unreactive toward common cellular nucleophiles (e.g., glutathione) to be useful in vivo. We installed this Michael acceptor into CsA via reductive ozonolysis of the MeBmt-1 side chain and elaboration of the resulting alcohol to CsA-El (Scheme 1). We also prepared

Scheme 1. Synthesis of CsA-El and CsA-U[a]



[a] Reagents: (i) O_3, DCM. (ii) $NaBH_4$, MeOH (81%). (iii) (a) $CO(Im)_2$, DIEA, THF; (b) $NH_2-CH_2-CH_2-NH_2$, DIEA, THF (67%). (iv) Acryloyl chloride, TEA, DCM (CsA-El, 81%) or acetic anhydride, TEA, THF (CsA-U, 70%).

an unreactive derivative CsA-U with an acetyl group replacing the acrylamide. CsA-El and CsA-U retain the ability to bind to wild-type CypA with moderate affinity (Kds ≈ 200 nM) as determined in fluorescence binding assays.[1]

We introduced cysteines into CypA by site-directed mutagenesis creating A103C, G104C, P105C, and N106C CypA-Nu mutants. Figure 2A shows the selective modification of the A103C, G104C, and P105C mutants by CsA-El as revealed by a shift in apparent molecular weight, whereas

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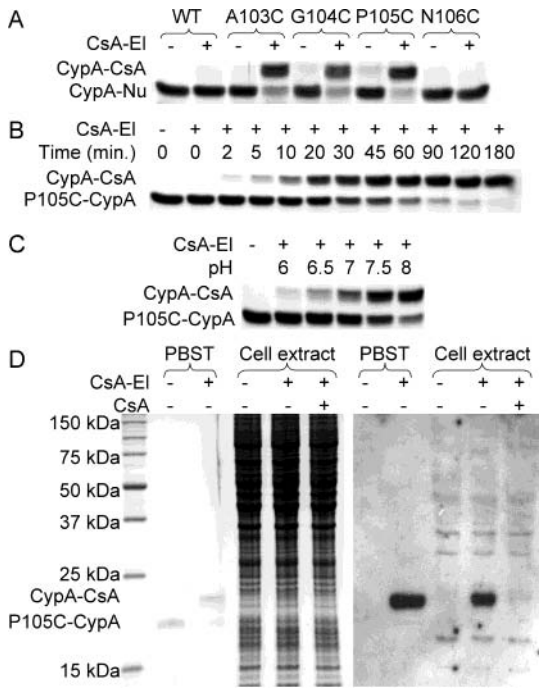

Figure 2. Alkylation of CypA and CypA-Nu mutants by CsA-El. (A) alkylation at pH 7.5, 16 h. (B) Time course for alkylation at pH 7.5. (C) pH profile for alkylation at 30 min. (A−C) CypA-Nu (4 μM) was incubated in phosphate-buffered saline, 0.05% Tween-20 (PBST), and glutathione (1 mM) with or without CsA-El (8 μM) at 37 °C. Samples were resolved on 12% SDS-PAGE gels and visualized with coomassie blue. (D) Specificity of alkylation. P105C−CypA (1 μM) was incubated with CsA-El (10 μM) in PBST or a Jurkat T-cell cytosolic extract (6 mg/mL) for 1 h, with or without an excess of CsA (60 μM). Duplicate gels were analyzed by SDS-PAGE and stained with coomassie blue (left panel) or western blot (right panel) probed with anti-CsA 1° antibody (Research Diagnostics) and goat anti-mouse alkaline phosphatase 2° antibody (Sigma).

wild-type and N106C−CypA were unaffected. A model of the N106C−CypA−CsA-El complex suggests that the C106 thiol is too distant to react with CsA-El. We selected P105C−CypA for further investigations, including a time course profile (Figure 2B) and pH dependence (Figure 2C) for reaction with CsA-El. These experiments show that the covalent complex can form under biologically relevant conditions. To investigate the specificity of labeling in a biological context, we tested the ability of CsA-El to specifically alkylate P105C−CypA in a Jurkat T-cell cytosolic extract. P105C−CypA was spiked into the extract at 1 μM (0.33% of total cytosolic protein), a concentration similar to the levels of endogenous CypA in cytosolic extracts (0.1−0.4%).[21] Within the limits of detection, we observed the exclusive modification of P105C−CypA by CsA-El in the extract (Figure 2D). The alkylation was dependent on the noncovalent association of CsA-El with the P105C−CypA, as an excess of CsA completely abrogated affinity labeling.

Table 1. Peptidyl-prolyl Cis−Trans Isomerase Activity of CypA and CypA-Nu Mutants and Inhibition by CsA-El and CsA-U

CypA mutant	wt	A103C	G104C	P105C	N106C
% relative kcat/Km[a]	100	111	97	129	105
% activity (+ CsA-U)[a,b]	83	71	77	82	78
% activity (+ CsA-El)[a,b]	76	42	44	**9**	77

[a] Average of three independent determinations with succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as the substrate (SD ≤ 14%). [b] % PPIase activity (relative first-order rates) following incubation of protein (5 nM) with CsA-U or CsA-El (40 nM) for 16 h at 37 °C prior to measurement of activity.

This level of specificity is striking and typically observed only with highly optimized ligands or mechanism-based inhibitors. These results show that exo mechanism proximity-accelerated alkylation with a weak electrophile can achieve highly selective targeting.

To investigate the utility of proximity-accelerated alkylation for selective inhibition of enzyme isoforms, the enzymatic activities of the CypA-Nu mutants were determined in PPIase assays[22] (Table 1) in the presence and absence of CsA-U and CsA-El. Notably, all of the CypA-Nu mutants retained wild-type levels of PPIase activity relative to CypA (11 $\mu M^{-1}s^{-1}$), showing that the mutations on the surface of the protein, outside the active site, did not significantly alter the enzymatic activity. In our inhibition studies, CsA-El, but not CsA-U, mediated the selective inhibition of the P105C−CypA mutant via proximity-accelerated alkylation. The level of inhibition is concentration dependent and approaches 100% over time. The activity of the P105C−CypA mutant was reduced to 9% in the presence of CsA-El, whereas the wild-type CypA retained 76% activity, the apparent level of noncovalent inhibition under the experimental conditions.

We have demonstrated the use of exo mechanism proximity-accelerated alkylation as an approach to develop allele-specific inhibitors of protein function. This approach should simplify the production of engineered receptor−ligand pairs wherein the modified receptor retains wild-type cellular function, as mutations can be introduced on the surface of the receptor at some distance from the ligand-binding site. We anticipate that this approach will complement and extend the use of engineered receptor−ligand pairs as tools for the investigation of biological systems. Indeed, this approach may be particularly effective when used in conjunction with a partially selective "bump and hole" engineered system to provide ligands with near absolute specificity without having to make drastic mutations in the active site. In cases where a covalent receptor−ligand complex manifests a gain in function, specific activation should be possible through limited dosing of the electrophilic ligand.

We anticipate that our approach could be useful for investigating the functions of the 14 predicted human cyclophilin isoforms in diverse cellular phenomena such as

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protein folding, apoptosis, RNA splicing, transcription, and oxidative stress.[23]

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Supporting Information Available: Materials, methods, experimental procedures, and structural characterization for CsA-El, CsA-U, and synthetic intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

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