Published in final edited form as:

Chembiochem. 2013 March 4; 14(4): . doi:10.1002/cbic.201200718.

Engineering New Protein-Protein Interactions on the β -Propeller Fold by Yeast Cell Surface Display

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Keywords

enzymes; genetic engineering; yeast cell surface display; Kelch-repeat; β -propeller fold

The β -propeller fold is a widespread structural module responsible for specific protein-protein interactions in the cell. ^[1] Typically β -propellers consist of four to eight blades that are made of antiparallel β -sheets twisting around a central tunnel (Figure 1 and Figure S1 in the Supporting information). The flexible loops connecting the β -strands in the blades put together docking sites for diverse cellular targets. Often the blades of β -propellers are tandem repeats of Kelch or WD motifs of 40–50 residues so β -propellers are also known as the Kelch- or WD-repeats (Figure S2 in the Supporting Information). ^[2–3] They have many important functions in the cell including recruiting substrate proteins to Cullin-RING ligases for protein ubiquitination, ^[4–7] mediating signal transduction through receptor-G protein coupling, ^[8] organizing caspases to initiate cell apoptosis, ^[9] regulating microtubule dynamics, ^[10] and detecting DNA damage. ^[11] The β -pinwheel domain of DNA topoisomerases also adopts a fold similar to the β -propeller to wrap around double-stranded DNA and induce DNA supercoiling. ^[12–13] Here we engineered the binding specificity of the Kelch-repeat (KR) domain of the Keap1 protein ^[14] by yeast cell surface display. Our results showed that the β -propeller fold can be redesigned to generate new protein-protein interactions.

Keap1 is the substrate receptor of Cullin 3-RING ubiquitin (UB) ligase that binds to Nrf2 for UB modification. Nrf2 is a transcription factor in the cell activating many antioxidant genes. ^[15] Keap1 functions as a dimer with the two C-terminal KR domains recognizing distinct sequence motifs (degrons) in the Neh2 domain of Nrf2. ^[16] One degreon known as the DLG motif covers residues ²³LWRQDIDLG³¹ of Nrf2 and binds to KR with a K_d of 500 nM. The other degreon known as ETGE spans the sequence of ⁷⁶LDEETGE⁸² of Nrf2 and binds to KR with a K_d of 8.1 nM. ^[16] Cancer related mutations are found in both degrons of Nrf2 to interfere with Keap1 recognition and render the cancer cells to be drug resistant. ^[17–18] For an example, the Glu79Lys mutation in the ETGE degreon of Nrf2 is associated with lung cancer, adenocarcinoma, and large cell neuro-endocrine carcinoma. ^[18]

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We expressed residues 31–98 of the Neh2 domain of Nrf2 excluding the DLG degron to measure the binding of the ETGE degron with the KR domain of Keap1. We refer to the truncated Neh2 as Neh2[ETGE] to denote that it only has the ETGE degron. Neh2[ETGE] was fused with a N-terminal peptidyl carrier protein (PCP) that can be labeled with biotin in the presence of Sfp phosphopantetheinyl transferase and a biotin-coenzyme A (CoA) conjugate.^[19] We immobilized the biotin-labeled Neh2[ETGE] on a Biacore sensor chip and measured its affinity with the KR of Keap1 by surface plasmon resonance (SPR). We found that Neh2[ETGE] binds to KR with a K_d of 5.2 nM, matching the value in the literature (Table 1 and Figure S3A in the Supporting Information).^[16] In contrast the Glu79Lys mutant of Neh[ETGE] (Neh2[ETGE]-E79K) did not show a SPR response to KR binding at a concentration as high as 20 μ M (Figure S3B). We thus decided to engineer KR to restore its recognition with the mutant Neh2[ETGE].

We tested to see if we can use yeast cell surface display to engineer the binding specificity of KR. We cloned the KR of Keap1 into the yeast vector pCTCON2 to express KR as a fusion to agglutinin protein Aga2p that is attached to the yeast cell wall through disulfide bonds to Aga1p (Figure 2A).^[20–21] To measure the display level of KR on the cell surface, we incubated the cells with a mouse anti-HA antibody and a chicken anti-Myc antibody that would bind to the cognate peptide tags fused to the N- and C-termini of KR, respectively. We then incubated the cells with a mixture of a goat anti-mouse antibody conjugated with Alexa Fluor 647 and a goat anti-chicken antibody conjugated with Alexa Fluor 488 to label the tags with distinct fluorophores (Figure 2A). Flow cytometry analysis of the yeast cells showed that 37% of the cells were doubly labeled with the fluorophores, suggesting the efficient display of KR on the surface of the yeast cells (Figure 2B).

Next we assayed if KR displayed on the cell surface was functional in binding to Neh2[ETGE]. We used biotin-labeled Neh2[ETGE] and the chicken anti-Myc antibody as the primary reagents, and streptavidin-phycoerythrin (SA-PE) and goat anti-chicken antibody-Alexa Fluor 488 conjugates as the secondary reagents to detect the binding of Neh2[ETGE] to KR on the surface of the yeast cells (Figure 2A). The flow cytometry data showed that 40% of the cells can bind to both Neh2[ETGE] and the anti-Myc antibody (Figure 2C). By comparison, only 0.8% of the cells were doubly labeled when biotin-labeled Neh2[ETGE]-E79K was used for KR binding (Figure 2D). These results confirmed the activity of KR anchored on the cell surface for Neh2 binding. They also suggested that the Glu79 residue in the ETGE degron of Neh2 plays a key role in KR recognition. We could thus display a KR library on the surface of the yeast cells to select for KR variants that would bind to the Glu79Lys mutant of Neh2 with high affinity.

We relied on previously solved crystal structure of the KR domain of Keap1 in complex with the ETGE degron to guide the design of KR library (Figure 1 and S1).^[22–23] The crystal structure shows that KR folds into a γ -propeller with six blades. The ETGE degron peptide binds to the top face of the propeller with the Glu79 residue forming multiple salt bridges and hydrogen bonds with the Arg415, Arg483 and Ser508 residues of KR. Glu79 is also buried in a hydrophobic pocket comprised of KR residues Ile461, Phe478, and Tyr525 and the back bonds of Gly462 and Gly509. The KR residues interacting with Glu79 of the degron are spread out in five loops extending from three blades of the propeller (Figure S1A and S2). With the Glu79Lys mutation in Neh2, the positive charged Lys side chain in the ETGE degron may be repelled by Arg415 and Arg483 of KR to disrupt the binding between Neh2 and KR (Figure S1B).

We first constructed a KR library with randomized residues at Arg415, Phe478, Arg483 and Ser508 and displayed the library on the surface of yeast cells (Figure S5A in the Supporting Information). We then carried out iterative rounds of selection of the yeast library with

biotin labeled Neh2[ETGE]-E79K based on the protocol for the selection of antibody libraries (Figure 3).^[20–21] In the first round of selection we incubated the yeast library with 0.1 μ M Neh2[ETGE]-E79K followed by binding the yeast cells to magnetic beads coated with streptavidin. The use of magnetic-activated cell sorting (MACS) enabled a large quantity of yeast cells ($\sim 1 \times 10^{10}$) to be selected based on the affinity of KR with the Neh2 mutant. In the second round of selection, the yeast cells were bound to a mixture of the anti-HA and anti-Myc antibodies to enrich cells displaying full length KR on the cell surface. For the subsequent rounds of selection, library cells were bound to biotin-labeled Neh2[ETGE]-E79K and a chicken anti-Myc antibody. Stringency of the selection was raised from rounds 3 to 6 by decreasing the concentration of Neh2[ETGE]-E79K from 100 nM to 10 nM for library binding, and by narrowing the sorting gate from 1.0% to 0.1% to collect the brightest cells with strong display of KR and tight binding with the Neh2 mutant (Figure 3). Through iterative rounds of selection, we observed a steady increase in the yeast cell population at the diagonals of the flow cytometry plots. This suggests that cells displaying KR mutants with high affinities with Neh2[ETGE]-E79K were enriched.

After five rounds of selection, 20 KR clones were sequenced and their alignments showed that mutants KR1-3 were the dominant clones in the library (Table 2). These clones have a negatively charged Glu residue to replace Phe478, and aromatic residues Phe, Tyr and Trp to replace Arg483 and Ser508. Residue Arg415 randomized in the KR library was unanimously changed to a homologous Lys side chain. The Phe478Glu mutation in the selected mutants may compliment the Glu79Lys mutation in Neh2 to restore the salt bridge interactions between KR and Neh2. The Arg483Trp/ Phe mutations in KR1-3 may transform the repellent electrostatic interactions between Arg483 of the wild type (wt) KR and Lys79 of the mutant Neh2 to cation- stacking interactions that strengthen the binding of the mutant KR-Neh2 pair. Aromatic Tyr or Trp residues replacing Ser508 may also engage Lys79 of Neh2 with cation- interactions and hydrophobic interactions. Mutants KR2 and KR4 also have extra mutations Asn469Asp and Cys434Arg, respectively, at the sites that were not randomized in the library (Table 2). These mutations are not in the binding pocket of the ETGE degon and they were generated because of single base changes in the codons (Figure S1A). Most likely these mutations were introduced by errors of the polymerase chain reactions (PCR) when the KR gene was amplified for library construction. After the sixth round of selection, the KR library was converged to KR1 with 17 appearances among the 20 sequenced clones (Table 2).

To confirm the binding between KR1 and KR2 with the Neh2 mutant, we measured the dissociation constants of the KR mutants with Neh2[ETGE]-E79K (Table 1 and Figure S3). KR1 binds to Neh2[ETGE]-E79K with a K_d of 61 nM, more than 330 fold stronger than the binding of wt KR with the Neh2 mutant with a K_d larger than 20 μ M. KR2 has less affinity than KR1 for binding to the Neh2 mutant but still it binds to Neh2[ETGE]-E79K with a K_d of 240 nM, corresponding to a 83-fold improvement from the wt KR. Interestingly both KR1 and KR2 can bind to Neh2[ETGE] with the wt degon, though the K_d 's of KR1 and KR2 with Neh2[ETGE] (51 nM and 140 nM, respectively) are 10–27 fold higher than the K_d of wt KR (5.2 nM). The affinities of KR mutants with wt Neh2[ETGE] may rise from the common interactions of the wt and mutant KRs with the native sequence flanking the Glu79Lys mutation in the Neh2 degon. Mutated residues in KR1 and KR2 may have also been selected for better binding with the wt residues in the degon. To acquire KR mutants specific with Neh2[ETGE]-E79K, the KR library may first be counter-selected with wt Neh2[ETGE] to remove clones that bind to the wt degon followed by selection with Neh2[ETGE]-E79K to enrich KR mutants that specifically bind to the mutant degon.

To see if other residues may be mutated for better interactions with the Neh2 mutant, we constructed a second library based on KR1 with randomized residues at Ile461, Gly462,

Gly509 and Tyr525 (Figure 1 and S1). These residues are shown by the crystal structure of KR to be in close vicinity to Glu79 of the ETGE degron.^[23] Five rounds of selection were carried out with the yeast library and 20 clones from round 5 of the selection were sequenced (Figure S4 in the Supporting Information). Alignments of the sequenced clones showed that half of the clones in the selected yeast pool were the KR1 mutant with native residues at positions 461, 462, 509 and 525. Besides the original KR1, selected KR mutants may have conserved mutations with Leu or Met to replace Ile461, and Phe to replace Tyr525 (Table 2). Gly462 and Gly509 show quite different tolerance with mutations. The two Gly residues belong to two consecutive Kelch motifs and can be aligned with each other in a loop just before the 1 strand of the Kelch motif (Figure S2). While Gly462 is absolutely required for binding to Neh2[ETGE]-E79K, Gly509 can be substituted by Gln, Asn, Ser or Met with polar side chains. Affinity measurements show that mutants KR10, KR12 and KR16 from the second library have K_d 's ranging from 100 to 260 nM with the wt or the Glu79Lys mutant of Neh2[ETGE] (Table 1). Their affinities with the Neh2 mutant are not as high as the original KR1 with a K_d of 61 nM, but are certainly much stronger than the wt KR. The sorting of two KR libraries identified mutants with different active site residues but similar affinities with the same target protein. This suggests that the β -propeller fold can elicit diverse interactions to achieve high affinity binding with a target protein.

In this study we used yeast cell surface display to restore the binding of the KR domain of Keap1 with a mutated Nrf2 degron found in cancer patients. It would be of interest to incorporate the engineered KR into Keap1 to test if Keap1 with altered binding specificity can mediate the ubiquitination of the Nrf2 mutant in the cancer cell. Previously chimeric receptors or synthetic ligands have been used to induce protein ubiquitination in the cell.^[24–28]

The β -propeller fold has been engineered in a number of ways including introducing point mutations to increase the thermo stability of the β -propeller,^[29–30] and creating artificial repeats of the propeller blades to assemble new β -propeller proteins.^[31–33] Library of galactose oxidase with a β -propeller fold has also been screened to identify mutant enzymes that can oxidize glucose.^[34] The yeast display method reported here enables the engineering of β -propeller proteins by high throughput FACS sorting. Our results show that the sequences of the flexible loops extending from the β -propeller fold can be redesigned to bind to new targets just as the loops of the complementarity-determining regions (CDR) of an immunoglobulin fold are engineered to optimize antibody binding with an antigen. Our work has thus revealed the great potential of engineering the β -propeller fold as a new platform for target protein recognition.

Experimental Section

Please see supporting information for experimental procedures on cloning, protein expression, KR library construction, FACS sorting of the library, and characterization of the KR mutants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a lab startup grant from the University of Chicago, and a National Science Foundation CAREER award (1057092) to JY; the Howard Hughes Medical Institute (N.Z.) and National Institutes of Health (R01-CA107134 to N.Z.). This work was also funded in part by the Chicago Biomedical Consortium with support

from the Searle Funds at The Chicago Community Trust (J. Y.). We thank Professor K. Dane Wittrup (MIT) for providing the pCTCON2 plasmid and yeast strain EBY100, and helpful discussions.

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Figure 1.

Crystal structure of the Kelch-repeat (KR) domain of Keap1 in complex with the ETGE degron peptide of Nrf2 (PDB ID 1X2R).^[23] Key residues of KR contacting Glu79 of Nrf2 degron are shown with residues randomized in the first library in grey and residues randomized in the second library in orange.

Figure 2. Selection of the KR library by yeast cell surface display. (A) Detecting KR expression on the cell surface by antibody binding to the HA and Myc tags fused to the N- and C-termini of KR, and selection of KR by binding to biotin-labeled Neh2[ETGE]-E79K. (B) Flow cytometry analysis of wt KR display with anti-HA and anti-myc antibodies. (C) Flow cytometry analysis of wt KR binding to biotin-labeled Neh2[ETGE]. (D) Flow cytometry analysis of wt KR binding to Neh2[ETGE]-E79K. Percentages of cells that were labeled with both fluorophores were shown. *F*: fluorescence intensity.

Figure 3.

Sorting of the first KR library displayed on the yeast cell surface. (A) Sorting with magnetic beads coated with streptavidin. Binding of cells to Neh2[ETGE]-E79K and an anti-Myc antibody was analyzed by flow cytometry. (B) FACS sorting by antibody binding to the HA and Myc tags flanking KR on the cell surface. (C) – (F), FACS sorting by binding to Neh2 mutant and an anti-Myc antibody. Percentages of doubly labeled cells were shown in the plots. Red frames are the sorting gates used for the collection of yeast cells. Percentages of cells in the sorting gate are in red. *F*, fluorescence intensity.

Table 1

Characterization of the binding interactions between KR mutants and the Neh2 domain of Nrf2.

	Neh2[ETGE]-E79K					
	k_{on} ($M^{-1}s^{-1}$) ($\times 10^3$)	k_{off} (s^{-1}) ($\times 10^{-4}$)	K_d (nM)	k_{on} ($M^{-1}s^{-1}$) ($\times 10^3$)	k_{off} (s^{-1}) ($\times 10^{-4}$)	K_d (nM)
wtKR	126 $\pm$ 5	6.5 $\pm$ 0.1	5.2	- [a]	- [a]	>2.0 $\times 10^4$
KR1	3.9 $\pm$ 0.5	2.0 $\pm$ 0.1	51	4.1 $\pm$ 0.7	2.5 $\pm$ 0.1	61
KR2	3.9 $\pm$ 0.4	5.5 $\pm$ 0.4	140	2.5 $\pm$ 0.5	6.1 $\pm$ 0.4	240
KR10	1.1 $\pm$ 0.3	2.9 $\pm$ 0.4	260	0.8 $\pm$ 0.2	1.2 $\pm$ 0.2	150
KR12	2.4 $\pm$ 0.3	2.7 $\pm$ 0.4	110	0.7 $\pm$ 0.2	1.4 $\pm$ 0.3	210
KR16	3.9 $\pm$ 0.3	5.5 $\pm$ 0.5	140	2.8 $\pm$ 0.5	2.8 $\pm$ 0.3	100

[a] Not determined because of low binding affinity.

Table 2

KR mutations selected from the libraries.

Selection of the 1 st library						No. of times selected	
wtKR	R415	F478	R483	S508	Extra mutations	Round 5	Round 6
KR1	K	E	W	Y		4	17
KR2	K	E	F	Y	N469D	2	2
KR3	K	E	F	Y		2	0
KR4	R	K	R	S	C434R	1	0
KR5	K	E	W	F		1	0
KR6	R	R	R	S		1	0
KR7	K	C	D	Y		1	0
KR8	K	D	F	F		1	0

Selection of the 2 nd library based on KR1						Round 5	
KR1	I461	G462	G509	Y525		10	
KR9	I	G	Q	Y		1	
KR10	M	G	Q	Y		2	
KR11	L	G	N	Y		1	
KR12	M	G	N	Y		2	
KR13	I	G	S	F		1	
KR14	I	G	M	F		1	
KR15	M	G	M	F		1	
KR16	M	G	S	Y		2	