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Phage Display Evolution of a Peptide Substrate for Yeast Biotin Ligase and Application to Two-Color Quantum Dot Labeling of Cell Surface Proteins

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Abstract

Site-specific protein labeling with *Escherichia coli* biotin ligase (BirA) has been used to introduce fluorophores, quantum dots (QDs), and photocross-linkers onto recombinant proteins fused to a 15-amino acid acceptor peptide (AP) substrate for BirA and expressed on the surface of living mammalian cells. Here, we used phage display to engineer a new and orthogonal biotin ligase–AP pair for site-specific protein labeling. Yeast biotin ligase (yBL) does not recognize the AP, but we discovered a new 15-amino acid substrate for yBL called the yeast acceptor peptide (yAP), using two generations of phage display selection from 15-mer peptide libraries. The yAP is not recognized by BirA, and thus, we were able to specifically label AP and yAP fusion proteins coexpressed in the same cell with differently colored QDs. We fused the yAP to a variety of recombinant proteins and demonstrated biotinylation by yBL at the N-terminus, C-terminus, and within a flexible internal region. yBL is extremely sequence-specific, as endogenous proteins on the surface of yeast and HeLa cells are not biotinylated. This new methodology expands the scope of biotin ligase labeling to two-color imaging and yeast-based applications.

Introduction

Chemical probes, such as fluorophores, photoaffinity labels, and photoswitches, are extremely useful tools for the study of biomolecular structure, dynamics, activity, and interactions. However, the technical difficulty of conjugating these probes site-specifically to biomolecules of interest, particularly in living cells where thousands of competing biomolecules present the same range of functional groups, has limited their widespread use. In recent years, many new labeling methods have been developed to target chemical probes site-specifically to proteins in the cellular environment.[1,2] In most of these methods, the protein of interest is genetically fused to a recognition sequence that binds to or reacts with the chemical probe. Peptide recognition sequences[3–5] are more desirable than protein-based recognition sequences because they minimize perturbation to the target protein, but frequently their use is accompanied by lower labeling specificity.[6] Recently, we and others have made use of enzymes to mediate the covalent ligation between probes and peptide recognition sequences. By capitalizing upon the inherent sequence-specificity of enzymes such as biotin ligase,[7–9] transglutaminase,[10] and phosphopantetheinyl transferase,[11] highly specific labeling of recombinant cellular proteins bearing short peptide tags has been achieved.

Site-specific protein labeling using *Escherichia coli* biotin ligase (BirA) makes use of the extremely sequence-specific ATP-dependent transfer of biotin to the lysine side chain of a 15-

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amino acid recognition sequence (the acceptor peptide, or AP).[12] After site-specific biotinylation of AP fusion proteins, imaging can be performed using streptavidin conjugates to fluorophores or quantum dots (QDs).[8] QDs are semiconductor nanoparticles with complete resistance to photobleaching and extremely bright and narrow fluorescence emission, making them ideal probes for single molecule and multicolor imaging.[13] We used the biotin ligase technology to target QDs specifically to glutamate receptors, neuroligin, and epidermal growth factor receptors expressed on the surface of living cells, to image their single molecule trafficking behavior.[8,9] We also found that BirA can accept a ketone analogue in place of biotin.[7,14] Site-specific ligation of a ketone to AP fusion proteins allows derivatization with hydrazide- or hydroxylamine-functionalized biophysical probes, bypassing the need for bulky streptavidin conjugates.

To expand the usefulness of enzymatic protein biotinylation, we sought to discover a new biotin ligase–AP pair, which we call BL2–AP2. This new pair would have similar specificity and kinetics to the original BirA–AP pair, but it would represent an orthogonal system, in that BirA would not recognize the new AP2, and conversely, the new BL2 would not recognize the original AP (Figure 1A). If such specificity requirements were met, we envisioned using the BL2–AP2 pair in conjunction with BirA–AP to perform site-specific labeling of two different proteins on the surface of the same cell as shown in Figure 1B. An AP fusion and an AP2 fusion are coexpressed on the surface of the same cell. In the first labeling step, BirA is used to site-specifically biotinylate the AP, and then a streptavidin conjugate to a green fluorophore detects the introduced biotin. Provided that this streptavidin binding step completely saturates the introduced cell surface biotins, the second labeling step can then be performed using the BL2 enzyme to modify the AP2 fusions experiments that it is straightforward to saturate cell surface biotin sites with streptavidin conjugates.[8]

Two-color imaging of orthogonal carrier protein and O^6-alkylguanine-DNA alkyltransferase fusions has previously been demonstrated on yeast cell surfaces[15] and inside mammalian cells,[16] but the use of orthogonal peptide tags should be less structurally and functionally invasive due to their much smaller size. The development of an orthogonal BL2–AP2 pair should also enable duplexing of other applications for which site-specific biotinylation is used, such as protein purification and immobilization.[17]

We considered two possible approaches to developing the new BL2–AP2 pair. One possibility was to rationally design an AP2 sequence and then use in vitro evolution to engineer a matching BL2 enzyme. However, enzyme evolution is generally much more challenging than peptide evolution, so we preferred the reverse approach of rationally designing a BL2 enzyme and then using in vitro evolution to discover a matching AP2 substrate. We initially planned to design the BL2 by rationally mutating BirA to eliminate its recognition of the AP, but observations by our lab and others[18] led to a simpler approach. In our work with biotin ligase enzymes from different species, we discovered that *Saccharomyces cerevisiae* (yeast), *Bacillus subtilis*, and *Methanococcus jannaschii* biotin ligases (yBL, bsBL, and mjBL) do not recognize the AP.[19] This is particularly interesting in light of the fact that these three enzymes all efficiently biotinylate *E. coli* BCCP (biotin carboxyl carrier protein), the single natural protein substrate of BirA.[19–21] This suggests that the AP interacts with the BirA active site in a different manner than BCCP does. Like BirA, these other biotin ligases exhibit high sequence-specificity, only biotinylating between one and five endogenous protein substrates within their respective organisms.[22,23]

Starting from yBL, bsBL, and mjBL as our candidate BL2s, we used phage display to identify a new AP2 substrate. The original AP was discovered by in vivo panning from a peptide library fused to the lacI repressor,[12] but we decided against this method because it is not straightforward to replace the endogenous BirA activity with the activity of our candidate BL2s,

and in vivo selection permits less fine control over the specific selection conditions. In general, phage display has been used more often to discover new binding partners than catalytic substrates, but precedents exist for using phage display to discover new substrates for proteases,[24] kinases,[25] and trans-glutaminases.[26] In addition, phage display has already been used to identify the minimal BirA recognition elements for *Klebsiella pneumoniae* oxaloacetate decarboxylase[27] and yeast pyruvate carboxylase.[28]

To perform the selection, we used the scheme shown in Figure 2. An AP2 library of 15-mer peptides is displayed as a fusion to the pIII coat protein of the M13 bacteriophage. Because the phage are exposed to endogenous BirA during production in *E. coli*, the scheme permits facile incorporation of a negative selection step. A fraction of the phage that displays peptide substrates for BirA will emerge from the bacteria already pre-biotinylated, and these can be removed with streptavidin-coated beads. Thereafter, the remaining phage are treated with the BL2 enzyme, and the biotinylated phage are isolated using streptavidin-coated beads, before amplification and subjection to another round of selection.

Using this approach, we discovered a new 15-amino acid substrate for yeast biotin ligase, which we call the yeast acceptor peptide (yAP). We characterized the kinetics of yAP biotinylation, found that the yAP sequence is transposable and can be fused to many different proteins, and demonstrated labeling of the yAP with streptavidin–probe conjugates in vitro, on the surface of yeast cells, and on the surface of living mammalian cells. The yBL–yAP system is orthogonal to the BirA–AP pair, and thus we were able to label yAP and AP fusion proteins expressed in the same cell selectively with differently colored QDs. This new technology should be useful for multicolor labeling and imaging applications, and it establishes a general approach to the engineering of new biotin ligase–peptide substrate pairs.

Materials and Methods

First Generation Phage Display Selections

A total of (1–2) $\times 10^{11}$ plaque-forming units (PFU) of phage displaying the AP2 library were pre-blocked in 3% dBSA (dialyzed bovine serum albumin, to remove biotin) in tris-buffered saline (TBS) for 1 h at 30 °C. After precipitation with poly(ethylene glycol) (PEG), the phage were redissolved in 40 μL of 50 mM bicine pH 8.3 containing 5 mM magnesium acetate, 4 mM ATP, 50 μM biotin, and 100 μg/mL BSA. Biotinylation was initiated by the addition of the BL2 mix, consisting of 100 nM each of yeast biotin ligase, *B. subtilis* biotin ligase, and *M. jannaschii* biotin ligase. After incubation at 30 °C for 1 h, the reaction was quenched with 45 mM ethylenediaminetetraacetic acid (EDTA). The phage were precipitated with PEG 3 times to remove excess biotin, then redissolved in 40 μL of 3% dBSA in TBS.

A total of 10 μL of streptavidin-coated magnetic beads (Roche) was blocked in 100 μL of 3% dBSA/TBS at 37 °C for 30 min. The beads were pelleted with a magnet, resuspended in 40 μL of 3% dBSA/TBS, added to the phage solution, and incubated at room temperature with the phage for 30 min. The beads were washed once with 100 μM biotin to remove streptavidin binding peptides, then 5 times with TBS + 0.1% Tween, 3 times with TBS, and once with water before resuspending in 22 μL of water. The captured phage were eluted from the beads by heating at 80 °C for 5 min[29] and then mixing with 2.5 μL of 10× TBS and 0.5 μL of 100 μM biotin. For the first two rounds of selection, the phage were re-amplified by adding 24 μL of the bead suspension to 20 mL of BM4092F′ *E. coli* (1:100 dilution of a saturated culture) in Luria–Bertani broth (LB) supplemented with 100 μg/mL avidin (Rockland). For the third and fourth rounds, 13 μL of the bead suspension was used to infect 20 mL of ER2738 *E. coli* (New England Biolabs) in LB. Negative selections were performed with phage produced from ER2738, by removing the pre-biotinylated phage with streptavidin-coated polystyrene beads as described in the Supporting Information.

Second Generation Phage Display Selections

Selections performed with the AP2.2 libraries were identical to the first generation with the following modifications: (1) in the generation 2 selections, 10 nM yBL was used for each round, and (2) in the generation 2′ selections, the yBL concentration was decreased successively over rounds, from 100 nM in the first round, to 10 nM in the second and third rounds, to 1 nM in the fourth and fifth rounds, and to 0.1 nM in the sixth round. As before, phage were prepared from the BM4092F′ strain for the first two rounds, then from the ER2738 strain for all subsequent rounds. Negative selections were performed when ER2738 was used for phage amplification.

Labeling of yAP Fusion Proteins with QDs on the Surface of Living HeLa Cells

HeLa were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37 °C under 5% CO_2. HeLa were transfected with the yAP-YFP-TM plasmid using Lipofectamine 2000 (Invitrogen). After 36–48 h, the cells were rinsed once with Dulbecco's phosphate-buffered saline pH 7.4 (DPBS), then biotinylated for 1 h at 32 °C with 10 μM biotin, 1 mM ATP, 4.7 μM yBL, and 5 mM magnesium chloride in 0.75× DPBS containing 10% FBS. The reaction also contained 0.2 mM EDTA, 20 μM dithiothreitol, and 1% glycerol from the yBL storage buffer. The cells were rinsed with DPBS containing 6% bovine serum (DPBS + BS), then labeled with the 10 nM streptavidin–QD655 conjugate (Invitrogen) in DPBS containing 1% dBSA for 5 min at room temperature. The cells were washed once with DPBS + BS and twice with DPBS before imaging on a Zeiss Axiovert 200M inverted epifluorescence microscope using a 40× oil-immersion lens. YFP (495DF10 excitation, 515DRLP dichroic, and 530DF30 emission), QD655 (405DF20 excitation, 585DRLP dichroic, and 655DF20 emission), and DIC (630DF10 emission) images were collected and analyzed using Slidebook software (Intelligent Imaging Innovations). Acquisition times ranged from 100 to 400 ms.

Two-Color Labeling and Imaging of yAP and AP Fusions on the Surface of Living HeLa Cells

HeLa transfected with the yAP-YFP-TM plasmid were plated together on the same dish with HeLa stably expressing AP-CFP-TM[7,8] or HeLa stably expressing AP-CFP-TM that had also been transiently transfected with yAP-YFP-TM. After 40–48 h of protein expression, the cells were washed with DPBS, then biotinylated with yBL, and labeled with streptavidin–QD655 as stated previously. Then, the AP tag was labeled by incubating the cells with 0.5 μM BirA, 10 μM biotin, 1 mM ATP, and 5 mM magnesium chloride in DPBS for 4 min at room temperature. After washing with DPBS + BS, the cells were labeled with 10 nM streptavidin–QD565 conjugate (Invitrogen) in DPBS + 1% dBSA, incubated for 4 min at room temperature, then washed 3 times with DPBS. Imaging was performed as stated previously, with CFP (420DF20 excitation, 450DRLP dichroic, and 475DF40 emission) and QD565 (405DF20 excitation, 565DRLP dichroic, and 565DF20 emission) filter sets.

Results and Discussion

Model Selections Give 2000-Fold Enrichment of AP-Displaying Phage in a Single Round of Selection

Before beginning selections with peptide libraries, we wished to determine the enrichment factor that could be obtained using our selection protocol. We prepared two phage constructs: an AP phage, displaying the 15-amino acid AP substrate for BirA,[30] and an Ala phage, displaying an AP mutant where the lysine biotinylation site is mutated to alanine. To perform the model selection, we combined the AP phage and Ala phage in a 1:100 ratio and then implemented steps 2 and 3 of the selection scheme shown in Figure 2, using 0.65 nM BirA enzyme. To compare the composition of the phage pools before and after the selection, we

isolated and sequenced the DNA. Figure S1 shows that in the initial mixture, the Ala phage dominates and the AP phage is undetectable. After selection, however, the AP phage dominates the mixture by about 20:1. Thus, we estimate an enrichment factor of ~2000 in a single round of selection.

Design of the AP2 Library for First Generation Phage Display Selections

All characterized biotin ligase enzymes biotinylate between one and five endogenous protein substrates within their respective organisms.[31] These biotin acceptor proteins exhibit some degree of sequence overlap, especially in the residues that immediately surround the lysine biotinylation site.[32] Structures of three of these acceptor proteins suggest a conserved tertiary structure, in which the lysine biotinylation site is presented at the tip of a tight hairpin loop.[33–35] It is difficult to recapitulate such a three-dimensional structure in a peptide sequence, and it is probably for this reason that the AP mostly likely interacts with the BirA active site in a different manner than BCCP does.

To design an AP2 library to be used in selections with our three candidate BL2s, we aligned the sequences of their natural biotin acceptor proteins (Figure 3A). Aside from the lysine biotinylation site, five residues flanking the lysine appear to be highly conserved. We noticed that these residues are well-conserved in the biotin ligase substrate proteins of other organisms as well. Thus, in a manner analogous to Schatz,[12] we fixed these five positions and the lysine biotinylation site and randomized the remaining positions of the 15-mer by saturation mutagenesis to create an AP2 library of diversity 4.2×10^7 (Figure 3A). Because our library also resembles the sequence of BirA's BCCP substrate, we recognized the importance of performing negative selections, to remove any sequences still recognized by BirA.

First Generation Phage Display Selections Produce a Conserved M(T/E)F Motif

In our first attempts at selection, we suffered an initial setback due to inadvertent amplification of the AP phage. Although the AP was only a minor contaminant (<0.01%) in the construction of the AP2 library, incomplete removal during the negative selection step, coupled with low levels of biotinylation of desired AP2 sequences under the selection conditions used (0.65 nM of BL2 enzymes), may have led to the complete dominance by the AP phage in the third round of selection. In our next attempt, we made two key changes in the protocol to avoid unintentional enrichment of the AP phage. First, the BL2 enzyme concentration was increased to 100 nM, to increase the biotinylation extent of desired AP2 substrates. Second, for the first two rounds of selection, the phages were produced in the *E. coli* strain BM4092F′, in biotin-depleted media. This strain lacks the ability to biosynthesize biotin, and it expresses a mutant of BirA with a higher K_m value for biotin (see Supporting Information).[36] Thus, even phage that display the AP will not become pre-biotinylated when produced in BM4092F′. For the third and fourth rounds, we returned to the original *E. coli* ER2738 strain and performed negative selections to remove substrates of BirA.

With these two modifications in the selection protocol, we observed strikingly different results when we re-selected from the same library using a mixture of yBL, bsBL, and mjBL enzymes (each 100 nM). Using a phage ELISA (enzyme-linked immunosorbent assay), we assayed the recovered phage pools from each round of selection for biotinylation by both the BL2 mixture and BirA. We observed a steady increase in the extent of biotinylation by the BL2 mixture but no increase in the biotinylation extent by BirA (Figure S2A). Deconvolution of the mixture revealed that yBL was solely responsible for the biotinylation activity (Figure S2B). It is interesting that we did not observe any enrichment of mjBL substrates, even though the fixed amino acids in the AP2 library exactly match those of mjBL's natural protein substrate.

Evaluating individual clones from the third and fourth rounds by sequencing and phage ELISA revealed that all of the peptides that were biotinylated by yBL contained an M(T/E)F motif immediately following the lysine (Figure S2C). The enrichment of this motif is remarkable because the two amino acids following the methionine were completely randomized in the design of the initial library, suggesting that these two amino acids are crucial for peptide recognition by yBL. It was also striking that the isolated yBL-active peptides bore little resemblance to the natural acceptor domains in yeast, or to the AP, suggesting that these substrates all bind to yBL in a different manner. Unfortunately, no crystal structure of yBL is yet available to provide insight into the mechanism of peptide or protein substrate recognition. By phage ELISA, the best peptide isolated from the first generation selections was biotinylated by yBL roughly 90-fold worse than AP was biotinylated by BirA, under identical conditions. Thus, we sought to improve the kinetics of this peptide by constructing a new library and performing a second generation set of selections.

Second Generation Phage Display Selections with Improved AP2.2 Library

We designed a new library for the second generation selections that was biased to include the MTF motif that emerged from the first generation selections. We completely randomized seven positions by saturation mutagenesis. Instead of rigidly fixing the residues at the remaining positions, we decided to mutate each of them (besides the lysine biotinylation site, which remained completely fixed) so that 34% of the sequences would contain the designed amino acid, while the remaining 66% would contain any of the other 19 amino acids or stop codons (Figure 3A). This design enabled us to explore sequence space previously unavailable to us in the first generation selections, and yet the designed amino acid was represented in >10-fold excess over any other amino acid at the indicated positions.

We constructed two libraries for the second generation selection: a smaller library of diversity 1.2×10^8 and a larger library of diversity 7.1×10^8. In one set of selections (generation 2), we performed four rounds of selection with the smaller library using 10-fold less yBL (10 nM) to isolate peptides with improved kinetics. In another set of selections (generation 2′), we performed six rounds of selection with the larger library, progressively decreasing the yBL concentration from 100 to 0.1 nM over the rounds of selection. In the first two rounds, it was necessary again to produce the phage in the BM4092F′ strain to minimize pre-biotinylation by BirA and prevent enrichment of peptides that were better substrates for BirA than yBL. As in the first generation selections, we observed a steady increase in the biotinylation extent of recovered phage after yBL treatment but not after treatment with BirA (results for generation 2′ are shown in Figure 3B). The activity with yBL did not increase beyond the fourth round, as streptavidin binding peptides (identifiable by the HPQ tripeptide motif[37]) began to dominate the selection. We also observed that the extent of biotinylation by yBL was higher for the phage pools isolated from the second generation selections than from the first (Figure 3B), suggesting that we had selected for more kinetically competent peptides by lowering the yBL concentration during selection.

Sequences of Best Clones and Identification of yAP

Figure 3C shows the sequences of the best peptides isolated from both of the second generation selections (ranked in order of activity toward yBL). The best peptide from the first generation selections is also shown for comparison. Despite randomization in the design of the second generation library, the AMKMTF motif re-emerged largely intact, although some of the best peptides possessed M → F mutations in the position preceding the lysine. Again, KMTF emerged as an immutable sequence shared by all of the substrates. By contrast, there was less preference for the original VL motif, particularly in the best peptides. We did not notice any pattern to the amino acid preferences at either end of the 15-mer, other than the presence of a large number of polar residues. By phage ELISA, the best peptide we isolated was biotinylated

by yBL 9-fold worse than the AP was biotinylated by BirA, under identical conditions. This represents a 10-fold improvement over the best peptide isolated from the first generation selection. We named this sequence the yAP, and all subsequent characterizations were performed with this peptide.

Characterization of yAP Kinetics and Transposability

We fused the yAP to the N- and C-terminal ends of two different proteins, cyan fluorescent protein (CFP) and heterochromatin protein 1 (HP1), and tested if they could be biotinylated by yBL. Figure S3A shows the ATP-dependent biotinylation of both CFP and HP1 fusions, detected by streptavidin blot. Figure S3B compares the biotinylation efficiency of all four fusion proteins by a [^{3}H]-biotin incorporation assay, showing that the yAP is preferred at the C-terminus of CFP, while it is preferred at the N-terminus of HP1. Thus, yAP biotinylation by yBL does exhibit some context-dependence, but yAP is recognized by yBL at both ends of the fusion proteins.

We measured the biotinylation kinetics of one of the fusion proteins, yAP–HP1, using a [^{3}H]-biotin incorporation assay (Figure 4). We determined a k_{cat} value of 0.10 ±0.01 min^{-1}and a K_m value of 130 ± 25 μM, which gives a catalytic efficiency about 780-fold lower than that of the BirA–AP pair.[38] The discrepancy between yAP and AP biotinylation is larger than predicted by phage ELISA, but this may reflect the different kinetic behaviors observed under single turnover and multiple turnover conditions. The inferiority of the current yAP is largely due to a poor k_{cat}, which reflects the limitations of our phage display system for selecting for multiple turnover substrates.

Orthogonality of the yBL–yAP Pair and Application to Site-Specific Labeling with QDs on Live Cells

We tested the orthogonality of the new yBL–yAP pair using three assays. First, we used phage ELISAs to demonstrate that the yAP is not biotinylated by BirA and conversely that the AP is not biotinylated by yBL (Figure 5A). Next, we observed the same result when yAP and AP fusions to the Aga2p mating protein[39] of yeast were biotinylated, stained with streptavidin–phycoerthyrin, and analyzed by flow cytometry (Figure S4). Both fusion proteins were displayed at the same levels on yeast cell surfaces, indicating that the observed orthogonality was not due to diminished availability of either peptide. We also observed that biotinylation was site-specific for the lysine of the yAP (Figure S4C).

We then showed that the new orthogonal pair enables two-color imaging of two distinct mammalian cell surface proteins when used in conjunction with BirA–AP. First, we created a yAP-YFP-TM construct where we fused the yAP and the yellow fluorescent protein (YFP) marker to the extracellular side of the transmembrane helix (TM) of the platelet-derived growth factor receptor (Figure 5B). We then biotinylated human HeLa fibroblasts transfected with the yAP-YFP-TM construct with yBL and observed that the labeling with a streptavidin–QD conjugate was specific for the transfected cells (Figure S5). Negative controls showed that the biotinylation was ATP-dependent and site-specific for the lysine of the yAP (Figure S5). Thus, yBL exhibits the same high degree of specificity for the yAP that BirA exhibits for the AP.

We next performed two-color labeling on HeLa cells. HeLa cells transfected with the yAP-YFP-TM plasmid were plated in the same dish together with HeLa cells stably expressing the AP-CFP-TM construct.[7,8] The cells were biotinylated with yBL for 1 h at 32 °C, then labeled with streptavidin–QD655. Next, the cells were biotinylated with BirA for 4 min at room temperature and labeled with streptavidin–QD565. Figure 5B shows that yAP-displaying cells were labeled only with QD655 and that the AP cells were selectively labeled with QD565. This

demonstrates that the orthogonality of the yBL–yAP pair is also preserved on the mammalian cell surface.

AP and yAP fusion proteins could also be selectively labeled when coexpressed in the same cell. In the same culture dish, we plated together HeLa expressing only the yAP, HeLa expressing only the AP, and HeLa expressing both yAP and AP. We used yBL biotinylation to selectively label the yAP fusion proteins with QD655. Then, BirA biotinylation was used to target QD565 to the AP fusion proteins. Figure 5C shows that cells coexpressing AP and yAP (white arrows) exhibit both QD655 and QD565 staining. Neighboring cells expressing only AP or only yAP exhibit single color QD staining.

Conclusion

We have evolved a new peptide substrate for yeast biotin ligase, called the yAP. Our first set of phage display selections identified an M(T/E)F motif that appeared to be crucial for yBL recognition. In the second set of phage display selections using a more focused library, we improved the kinetics of yAP biotinylation approximately 10-fold via successive dilution of yBL. Our yAP peptide is only the second peptide substrate ever reported for a biotin ligase, and our phage display approach is novel and more general than the lacI repressor panning method previously used to find the AP.[12]

We showed that the yAP could be recognized and biotinylated by yBL when fused to the N-terminus, C-terminus, or an internal position of a variety of recombinant proteins. yBL is extremely specific for the yAP, as endogenous proteins on the surface of yeast and HeLa mammalian cells are not biotinylated. We measured the k_{cat} and K_m values for a yAP–HP1 fusion protein and determined a catalytic efficiency of 13 M^{-1} s^{-1}, approximately 780-fold lower than that of the BirA–AP pair.

As expected based on our choice of a BL2 enzyme, and on the implementation of negative selections, the yAP–yBL pair was orthogonal to the BirA–AP pair. We demonstrated the lack of cross-reactivity between yBL and AP, and BirA and yAP, using phage ELISA and a yeast cell surface display assay. The orthogonality of these two pairs allowed us to selectively target QDs to HeLa cells coexpressing yAP and AP fusion proteins in the same cell.

In addition to two-color imaging applications, yAP technology should also be useful for protein detection, labeling, and purification, especially in *S. cerevisiae* cells, where it may be possible to rely upon endogenous yBL activity (we are currently testing this). In many cases, expression of recombinant proteins in yeast is superior to expression in *E. coli*, due to the opportunities for glycosylation, disulfide bond formation, and folding/assembly of multidomain complexes.

The relatively poor k_{cat} value of our yAP reflects the limitations of phage display, where it is impossible to discriminate between high-turnover peptide substrates and low-turnover substrates. Yeast cell surface display,[39] in which thousands of copies of each peptide are displayed on each cell, will allow for highly biotinylated yeast to be separated from moderately biotinylated yeast by fluorescence-activated cell sorting. This evolution platform should provide access to yAP sequences with improved kinetics. We plan to pursue this in future work, having already shown that the yAP and AP peptides can be displayed on yeast and recognized by yBL and BirA (Figure S4).

It may also be possible to improve the properties of the yAP through the design of more sophisticated libraries. The AP2 and AP2.2 libraries used here were arbitrarily designed to resemble the AP. It may be beneficial to move the lysine biotinylation site to different positions along the 15-mer. In addition, because truncation of the AP by more than one residue at either

end leads to a greatly reduced k_{cat} value,[38] slightly longer peptide libraries may result in improved yAP biotinylation rates.

Previous efforts to truncate the natural biotin acceptor proteins have led to minimal domains 70–80 amino acids in length[40] because biotin ligases recognize a stably folded structure. The three-dimensional structure of this fold is likely to be highly evolutionarily conserved, given the ability of biotin ligases from different species to recognize acceptor domains from other organisms.[40] The fact that the AP and yAP are substrates only for their respective biotin ligases suggests that these peptides bind to their enzymes in a different mode than the natural acceptor protein substrates.

QDs are extremely useful probes for single molecule imaging in living cells. The yAP–yBL technology, in conjunction with BirA–AP, can be used to image and study the trafficking and localization of two different cellular proteins at once. This can be used to investigate, for example, how different isoforms of a particular protein (e.g., neuroligin) traffic to excitatory versus inhibitory synapses during development or how two different receptors diverge in the endosomal pathway after internalization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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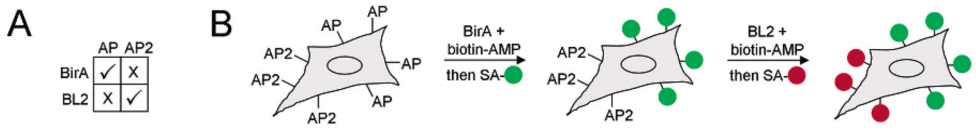

Figure 1.

(A) Specificity requirements for two orthogonal biotin ligase–AP pairs. (B) Site-specific labeling of two different cell surface proteins (an AP fusion and an AP2 fusion), using two orthogonal biotin ligase enzymes (BirA and BL2). Streptavidin (SA)– fluorophore conjugates bind to the site-specifically introduced biotins. Biotin-AMP is the activated adenylate ester of biotin, which can be used in place of biotin + ATP for the enzymatic biotinylation.

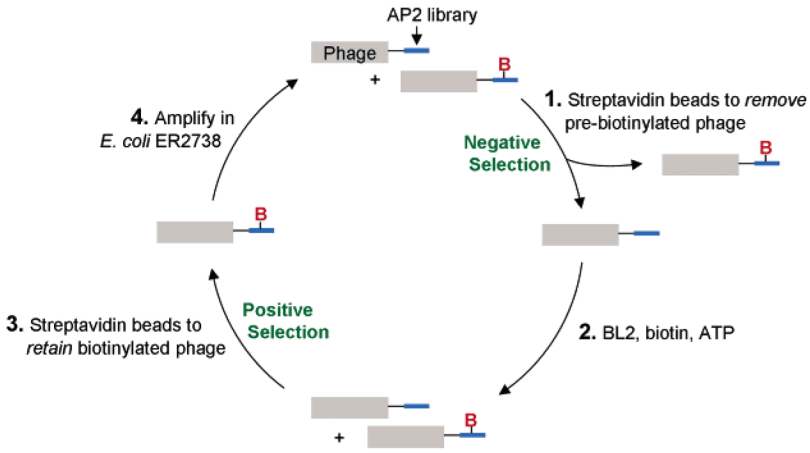

Figure 2.

Phage display selection scheme. The AP2 library (blue) is fused to the pIII coat protein. B represents biotin. After phage production in *E. coli* strain ER2738, a fraction of the phage pool is pre-biotinylated by endogenous BirA. In step 1 of the selection, streptavidin-coated beads are used to remove these pre-biotinylated phages (negative selection). In step 2, the remaining phage are treated with the BL2 enzyme. In step 3, streptavidin-coated beads are used to isolate the biotinylated phage from this mixture (positive selection). Finally (step 4), the recovered biotinylated phage are amplified in ER2738 for another round of selection.

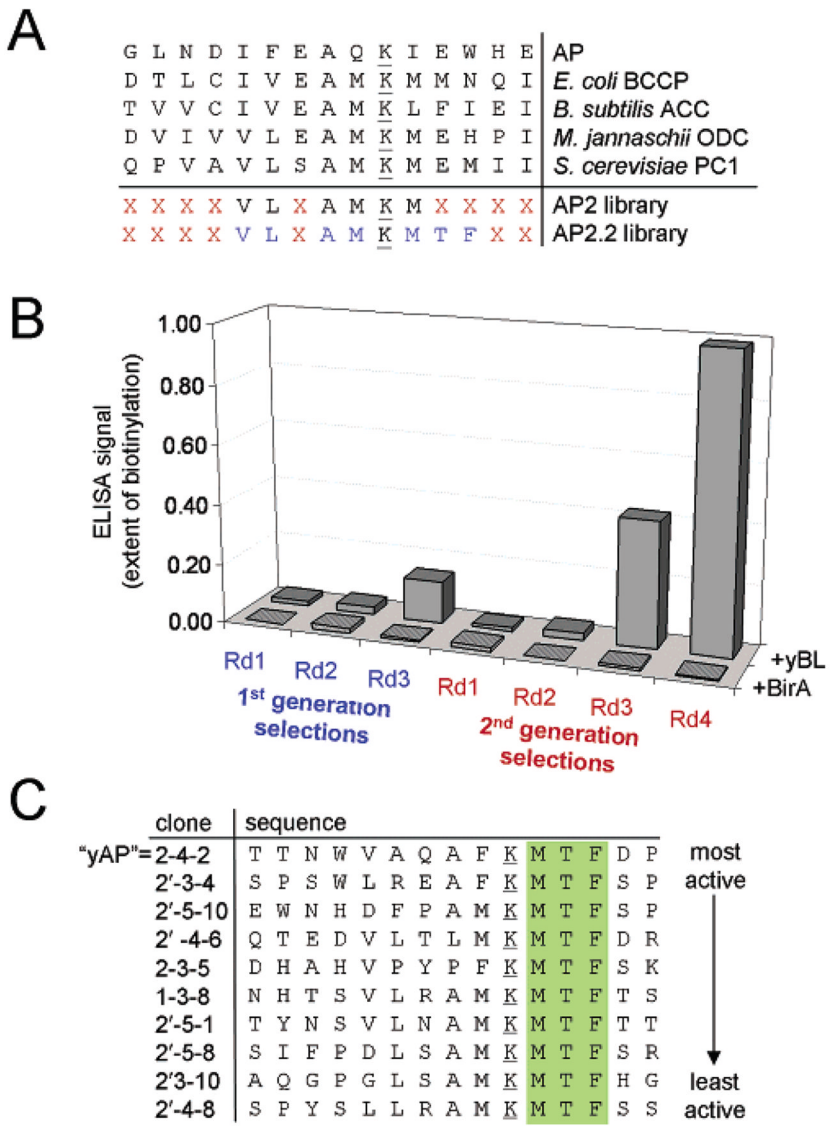

Figure 3.

Library design and selection results. (A) Sequences of AP and biotin ligase substrate proteins from four different species. The lysine biotinylation sites are underlined. BCCP is the biotin carboxyl carrier protein; ACC is acetyl-CoA carboxylase; ODC is oxaloacetate decarboxylase; and PC1 is pyruvate carboxylase 1. Sequences of the first generation library (AP2 library) and second generation library (AP2.2 library) are shown. Red positions are completely randomized, while blue positions are partially randomized (~34% the indicated amino acid). (B) ELISAs showing the extent of biotinylation by BirA and yBL of recovered phage from different rounds of selection. For each reaction, 1×10^{11} PFU of phage were incubated with 10 nM enzyme at 30 °C for 1 h. (C) Ten of the best clones isolated from different rounds of selection (clone nomenclature: generation-round-clone). The MTF motif observed in all clones is highlighted. The clones span a 20-fold window of activity toward yBL, by phage ELISA. The top clone was named yAP and characterized further.

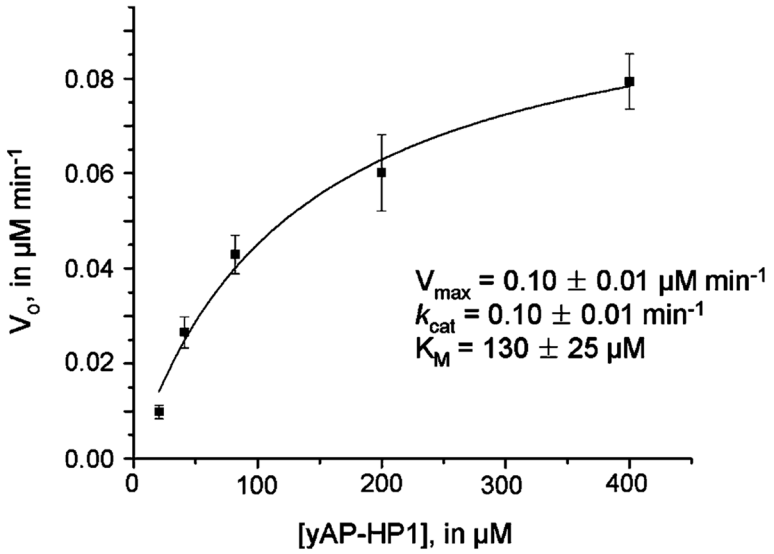

Figure 4.

Kinetics of yAP biotinylation. The Michaelis–Menten curve shows the initial rates of biotinylation of a yAP–HP1 fusion protein by yBL, as a function of yAP–HP1 concentration. Measurements of initial rates at each concentration were performed in triplicate (error bars, SD).

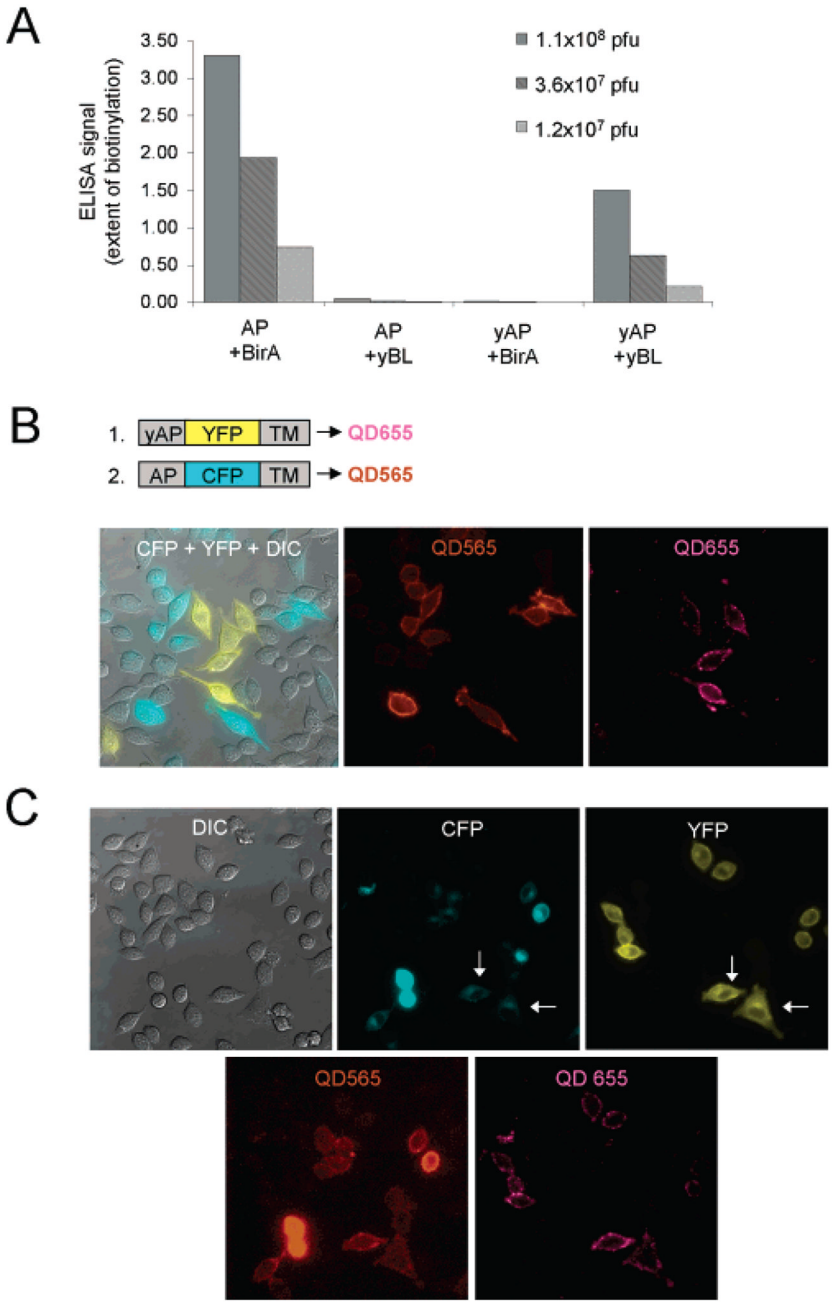

Figure 5.

Orthogonal labeling with the yBL–yAP pair. (A) Phage ELISA demonstrating the orthogonality of the yBL–yAP pair as compared to the BirA–AP pair. 1.8×10^{10} PFU of yAP phage or AP phage were incubated with 10 nM enzyme (BirA or yBL) at 30 °C for 1 h. Measurements for three phage dilutions are shown. (B) Selective labeling of live HeLa cells expressing yAP or AP fusion proteins (domain structures shown at top) with QDs. First, yAP expressing cells (indicated by YFP fluorescence) were selectively labeled with streptavidin–QD655 conjugate, using yBL biotinylation. Then, AP expressing cells (indicated by CFP fluorescence) in the same dish were labeled with streptavidin–QD565 conjugate, using BirA biotinylation. CFP and YFP images were merged and overlaid with the DIC image. (C)

Selective labeling of live HeLa cells coexpressing yAP and AP fusion proteins. HeLa expressing the yAP-YFP-TM construct alone, the AP-CFP-TM construct alone, or both constructs simultaneously (white arrows) were plated together and labeled as in panel B.