Published in final edited form as:

Mol Biotechnol. 2013 July ; 54(3): 829–841. doi:10.1007/s12033-012-9631-7.

Directed Evolution of a Fluorogen-Activating Single Chain Antibody for Function and Enhanced Brightness in the Cytoplasm

Bradley P. Yates, Michelle A. Peck, and Peter B. Berget

Department of Biological Sciences & Molecular Biosensor and Imaging Center, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, PA 15213, USA

Peter B. Berget: p.berget@uscience.edu

Abstract

Directed evolution is an exceptionally powerful tool that uses random mutant library generation and screening techniques to engineer or optimize functions of proteins. One class of proteins for which this process is particularly effective is antibodies, where properties such as antigen specificity and affinity can be selected to yield molecules with improved efficacy as molecular labels or in potential therapeutics. Typical antibody structure includes disulfide bonds that are required for stability and proper folding of the domains. However, these bonds are unable to form in the reducing environment of the cytoplasm, stymieing the effectiveness of optimized antibodies in many research applications. We have removed disulfide-forming cysteine residues in a single chain antibody fluorogen-activating protein (FAP), HL4, and employed directed evolution to select a derivative that is capable of activity in the cytoplasm. A subsequent round of directed evolution was targeted at increasing the overall brightness of the fluoromodule (FAP–fluorogen complex). Ultimately, this approach produced a novel FAP that exhibits strong activation of its cognate fluorogen in the reducing environment of the cytoplasm, significantly expanding the range of applications for which fluoromodule technology can be utilized.

Keywords

Directed evolution; Fluorogen activating protein; Fluoromodule; Intrabody; scFv; Fluorogenic dyes

Introduction

Advances in fluorescence-based probes, reagents, and techniques have provided methods and customizable, highly sensitive assays that can be used to address current biological questions. Fluorescent proteins and their variants, as well as other genetically encoded reporters, have been invaluable in aiding our collective understanding of in vivo biological processes. One such class of reporters is the recently developed fluoromodule technology, comprising cognate pairings of fluorogenic dyes and their respective fluorogen-activating proteins (FAPs) [1]. FAPs are derived from single chain antibody variable fragments (scFvs) that have been selected from a yeast surface-display library [2] for their ability to bind to an

organic dye, such as thiazole orange (TO), malachite green (MG), or dimethylindole red (DIR) [1, 3]. Upon binding, the scFv constrains the dye such that absorbed energy normally released during intramolecular rotation is emitted as fluorescence, referred to as dye activation [4]. Binding of the FAP to the dye, also referred to as a fluorogen, results in dramatic increases in detectable fluorescence over background levels, which have been harnessed in a variety of uses including surface labeling and monitoring receptor internalization and trafficking [5–8].

For the most part, however, applications of fluoromodules have been restricted to instances where the FAP is presented on the cell surface or in an otherwise non-reducing environment, e.g., vacuoles and compartments in the secretory pathway. The underlying cause of this limitation is that the IgG variable heavy (V_H) and variable light (V_L) domains comprising FAPs rely on the formation of conserved intradomain disulfide bonds for proper folding, stability, and function [9, 10]. In reducing environments, such as the cytoplasm of cells, these bonds fail to form properly, and under such conditions many antibody fragments have shown marked decreases in stability and function [9, 11]. Thus, most scFv-based FAPs would be predicted to be incapable of properly folding and activating fluorogens in the intracellular milieu. Just as intrabodies—antibody fragments that are active inside of cells—hold great promise for use in therapeutics [12], FAPs exhibiting activity in similar environments will serve to expand the research utility of fluoromodules. To establish a family of FAPs that have no dependence on disulfide bond formation, we took directed evolution and activity-based selection approaches.

Directed evolution attempts to mimic and focus the power of natural selection for the alteration of a specific chosen characteristic. Commonly this entails creating, in a host system, a library of genes expressing high genetic variation centered on the trait. The desired trait is then screened for and the carrier hosts selected. Any underlying genetic variation that led to that trait can then be identified by DNA sequencing. Directed evolution has been successfully carried out in several systems—including phage display [13, 14], ribosome display [15, 16], and yeast display [17, 18]—and for a vast array of purposes such as increased promoter function [19], enzymatic activity [20], and binding affinity [17, 21].

The research presented here applies directed evolution methods to a malachite green-binding FAP, HL4-MG, in order to adapt it for intracellular activity and to further enhance the brightness of the fluoromodule as a step toward optimizing its utility for in vivo applications. Through use of directed evolution techniques we demonstrate, first, the development and selection of a fluoromodule with intracellular activity and, second, the enhancement of that fluoromodule's brightness characteristics while maintaining disulfide bond independence.

Materials and Methods

Targeted Elimination of Cysteine Residues in HL4-MG by Overlap PCR

One cysteine residue in each variable domain of HL4-MG was targeted for site-specific mutagenesis to be changed to alanine. The residues were H92 in the V_H domain and L88 in the V_L domain. Numbering of the residues is according to the Kabat numbering system [22]. Single mutants were made using hi-fidelity (Phusion enzyme, Thermo Scientific) overlap PCR, and nucleotide substitutions were chosen for facile restriction enzyme screening. Template scFv was in pPNL6 vector (Pacific Northwest National Laboratory). Cysteine H92 was mutated from TGT to GCC, which eliminated a native BsrGI restriction site; cysteine L88 was changed from TGT to GCG, creating a unique FspI restriction site. Primer pairs for creation of the Cys^{H92}Ala mutant were: (P3) Forward—5'-GCTGTGTATTACGCCACAAGGGCCAG with (P6) Reverse—5'-

GTACGAGCTAAAAGTACAGTG, and (P1) Forward—5'-TAGATACCCATACGACGTTC with (P2) Reverse—5'-CTGGCCCTTGTGCGTAATACACAGC. Primer pairs for creation of the Cys^{L88}Ala mutant were: (P5) Forward—5'-GCAACTTACTATGCGCAAGAGGGTAGC with (P6) Reverse, and (P1) Forward with (P4) Reverse—5'-GCTACCCTCTTGCGCATAGTAAGTTGC. Nucleotides that have been altered from wild type are underlined. Primer annealing locations can be seen in Fig. 1. For each targeted mutation, the two purified overlapping PCR product fragments underwent five cycles of self-templated PCR to create a new, mutant template. The new templates underwent standard PCR for 30 cycles using the two outermost primers (P1 and P6) to amplify the entire two-domain antibody fragment. Following sub-cloning of H92 and L88 single mutants into pPNL6 yeast surface-display vector, the H92-L88 double mutant was created by BamHI/NotI restriction digest and ligation of the mutant L88 *V_L* into the construct already containing the mutant H92 *V_H* domain. All primers were custom ordered from Integrated DNA Technologies, and enzymes from New England Biolabs.

Assessment of FAP Activity of HL4-MG Cysteine Mutants

pPNL6 vectors containing wild type HL4-MG, the H92 and L88 Cys-Ala single mutants, and the H92-L88 Cys-Ala double mutant were transformed into EBY100 strain yeast (Life Technologies) using the EZ Yeast transformation kit (MPBiomedicals). Transformed yeast were grown in liquid culture, induced and fluorescent antibody-labeled according to the Yeast Display scFv Antibody Library User's Manual from Pacific Northwest National Laboratory (Richland, WA 99352 <http://www.sysbio.org/dataresources/usermanual031113.pdf>) and as previously described [1, 2]. For each sample, 10⁶ yeast cells expressing surface-displayed scFvs were labeled with mouse monoclonal primary antibody against the c-myc epitope, washed with 1 × PBS/2 mM EDTA/0.1 % Pluronic F-127, and then incubated with Alexa-fluor488 goat anti-mouse secondary antibody. Samples were mixed with malachite green dye MG-11P at a final concentration of 100 nM, incubated on ice for 30 min, and then assayed by two-color FACS on a Becton Dickinson FACS-Vantage SE with a FACSDiva option. Dye activation was measured by excitation with a 633 nm laser and emission read at 685 nm. Levels of FAP yeast cell surface expression were assessed by fluorescence of the Alexa-fluor488 secondary antibody, excited with a 488 nm laser, with emission read at 535 nm.

Creation of Mutant scFv Library by Error-prone PCR

The Cys^{H92}Ala, Cys^{L88}Ala double mutant scFv gene was used as the parent gene for random mutagenesis. The library was created using error-prone PCR according to a previously established protocol [23]. Reaction mixes were typical for standard Taq polymerase, but included 2 nM final concentrations of both 8-oxy-dGTP and dPTP. Two reactions were run for this template, one for 10 cycles of denaturation, annealing and extension, and one for 20 cycles. Primers used for the PCR reactions were Forward—5'-GACAATAGCTCGACGATTGAAGGTAGATACCCATACGACGTTCCAGACTACGCTCTGCAG and Reverse—5'-GATCTCGATGCGGCCGCGAGCTATTACAAGTCTTCTTCAGAAATAAGCTTTTGT TCTAGAAT. These primers ensure sufficient regions of homology for the subsequent gap repair step, and cover the 5' HA epitope and 3' c-myc epitope to protect against mutagenesis of either tag. Mutagenic PCR products were run on a 1.0 % agarose gel in the absence of ethidium bromide, for 10 h at 50 V. The gel was stained for 30 min in ethidium bromide, destained for 30 min in water, visualized with low-dose UV light, and the bands excised. DNA was extracted from agarose slabs according to the GeneJET Gel Extraction Kit protocol (Fermentas). Extracted DNA product was then amplified by standard PCR,

using the primers indicated above. Amplification products were then prepared for gap repair using the GeneJET PCR Purification Kit (Fermentas).

Gap Repair and Transformation of scFv Mutants into *S. cerevisiae*

Mutagenized PCR products were integrated into pPNL6 vector and simultaneously transformed into EBY100 yeast by gap repair [24]. The pPNL6 vector was prepared by restriction digests (first with *NheI* and *BamHI*, then subsequently with *SalI*), and concentrated by ammonium acetate precipitation. An overnight starter culture of EBY100 yeast in YEPD was used to inoculate 200 mL of YEPD for overnight growth in a shaking incubator at 30 °C. The next day, when the cultures had reached an OD₆₀₀ of 0.87, they were centrifuged for 5 min at 3,000 rpm in a Beckman GPKR centrifuge. Cell pellets were washed with water, centrifuged again as above, and finally resuspended in 1 mL of water. Resuspended cells were separated into 100 µL aliquots and combined with 326 µL of transformation mix (PEG3400, LiAc, and boiled salmon sperm DNA). Cell/transformation mixes contained 1 µg tri-cut vector and 9 µg of purified PCR product as insert (one mix for the 10 mutagenic cycle PCR sample and one mix for the 20 cycle sample). Transformation mixes were heat shocked for 40 min at 42 °C, pelleted in a 5804 tabletop centrifuge (Eppendorf International), and resuspended in SD + CAA + dextrose yeast growth medium. At this time dilutions of the resuspended cells were made to determine the total number of TRP prototrophs generated by the gap repair process. This number gives the diversity of the mutated library.

Enrichment of Population for Recovery of FAP Activity

After a diversity of at least 10⁶ was obtained for each PCR population, the 10-cycle and 20-cycle mutagenic PCR populations were combined in equal amounts. The new, combined mutant library was grown and induced as described above for yeast surface display of the mutant scFvs. Fluorescence-activated cell sorting (FACS) enrichments were carried out in the presence of 100 nM MG-11P, a cell-impermeant derivative of malachite green. Use of 1 µM propidium iodide as a vital dye allowed subtractive gating of fluorescent dead cells. Of the remaining live cells, the brightest ca. 1 % of the mutant library in terms of malachite green fluorescence was sorted into a flask containing 25 mL of SD + CAA + dextrose growth medium, pH = 4.5. This enriched population was grown, induced and sorted twice more, again taking the brightest 1 % each time. Aliquots of cells were removed and frozen for long term storage after each round of enrichment.

Induction-Dye Plates and Selection of Candidate HL4-MG Mutant Clones

During the final round of enrichment, the brightest 1 % of the population was sorted onto an induction medium agar plate [3] containing 200 nM of either MG-11p or MG-Ester. The plate was shielded from light, and incubated at room temperature for 2 days, then shifted to 20 °C for 3 days of induction, and imaged. Images were taken with a cooled CCD camera using a 635 nm LED spotlight for excitation and a 740/140 nm filter for emission. Alternatively, plates were imaged in a custom DIGE gel imager (courtesy of Jon Minden, Carnegie Mellon University), using Cy5 channel emission filters. Brightfield images of the plates were also taken for analysis of colony size. Using ImageJ software [25], brightness intensity of each of the colonies was measured and normalized to colony area (from brightfield image). All colonies were ranked according to their brightness/area ratio. Colonies were selected from both extremes to be analyzed further.

Sequence Confirmation and Characterization of Selected HL4-MG Mutant Clones

5 mL of YEPD medium was inoculated with an individual selected colony and grown overnight with shaking at 30 °C. The following day, cultures were separated into five

Eppendorf tubes, spun for 2 min at 1,500 rpm in an Eppendorf 5415D centrifuge and each pellet was resuspended in 250 μ L of resuspension buffer from the Fermentas GeneJET Plasmid Miniprep Kit. Roughly 50–100 μ L of 600 nm glass beads were added to each tube, which was then vortexed at maximum speed for 5 min. Then 250 μ L Lysis Buffer was added to each tube and mixed by inversion, followed by 350 μ L Neutralization Buffer. After mixing, the tube was centrifuged at maximum speed for 10 min, as per Fermentas miniprep kit instructions. Supernatant from the 10 min spin was applied to a kit-included column and centrifuged for one minute at maximum speed. The remaining supernatant fractions from the same clone were applied to the same column and spun as above. Washing and elution of the DNA in 30 μ L of water were as described in the miniprep protocol. Yeast minipreps obtained by the above protocol were used to transform *E. coli* Mach cells. DNA from a single bacterial colony was sent for DNA sequencing (Retrogen, Inc.). pPNL6 vectors containing novel mutant gene clones were retransformed into EBY100 yeast. scFv genes were further subcloned into the CytEx vector by NheI and NotI restriction digest, then also transformed into EBY100. Both pPNL6- and CytEx-transformed yeast were grown and induced as described above.

Flow Cytometry: Surface Display and Cytoplasmic Activity

Following induction, 10^6 pPNL6 construct-containing yeast were washed twice with $1 \times$ PBS/2 mM EDTA/0.1 % pluronic F-127 and resuspended to a final volume of 500 μ L in that same buffer in preparation for flow cytometry analysis. Cells were labeled with c-myc primary antibody and Alexa-fluor488 secondary antibody as described above. Each sample was analyzed on the cytometer in the presence of 100 nM MG-2P. Mean fluorescence from fluorogen activation (685 nm) was normalized to scFv expression as measured by c-myc Alexa-fluor488 signal (530 nm). For cytoplasmic assay, 10^6 CytEx construct-containing yeast were prepared in the same manner as above, except that MG-Ester was used as the dye and no labeling with primary or secondary antibodies was carried out. Only the fluorogen activation (at two wavelengths: 685 and 780 nm) was measured for these samples.

Fluorimetry: Surface-Displayed K_d Determination

Following induction, 5×10^5 pPNL6-expressing yeast cells were assayed in triplicate in a 96-well plate. Cells were transferred to black 96-well plates in 200 μ L final volume of $1 \times$ PBS/2 mM EDTA/0.1 % pluronic F-127 with MG-2P concentrations ranging from 0.1 to 100 nM. Plates were incubated at 4 $^{\circ}$ C overnight before reading. Fluorescence was read in a TECAN Safire² fluorimeter with excitation at 635 and 685 nm emission.

Fluorimetry: Peak Surface-Display and Cytoplasmic Activity

Following induction, 10^6 pPNL6-expressing yeast cells were assayed in triplicate in a 96-well plate. Cells were transferred to black 96-well plates in 100 μ L final well volume of $1 \times$ PBS/2 mM EDTA/0.1 % F-127 with a final concentration of 500 nM MG-Ester in each well. Following an overnight incubation at 4 $^{\circ}$ C, endpoint peak fluorescence was measured in the TECAN Safire² fluorimeter with excitation at 625 nm and emission of 650 nm. Cytoplasmic assay was carried out as described above, but using CytEx-expressing yeast cells.

Directed Evolution of p13 in Cytoplasmic Expression (CytEx) System

A new primer was designed for the error-prone PCR step to adapt the method to the CytEx vector, (forward) 5'-CTTCATACATTTTCAATTAAGATGAGATACCCATACGACGTTCCAGACTACGCTCTGCAG. The reverse primer paired with this was the same as for the creation of the mutant scFv library of HL4-MG (Cys^{H92}Ala, Cys^{L88}Ala) described above. CytEx vector was triple restriction enzyme-digested in the same manner as pPNL6, described above, and all

subsequent steps followed the same methods. Template DNA was p13, and the gap repair vector used was CytEx. Flow cytometry enrichments were carried out in the presence of 1 μ M MG-Ester fluorogen and 1 μ M propidium iodide as a vital dye. Since c-myc could not be utilized to assess expression levels during this process, fluorogen activation was the only parameter used to gate for the top 0.5 % of the population. In total, four enrichments were undertaken.

Subcloning Mutants into CytExGreen for Expression Normalization

CytExGreen was made by digesting the existing CytEx vector with NheI, then cloning in an in-frame eGFP gene fragment that had a 5' AvrII site and a 3' NheI site created by anchored PCR. Subcloning in this manner destroys the site where AvrII ligates into NheI, while keeping the original NheI site intact for standard domain subcloning. Synthetic DNA coding for two (Gly₄Ser) repeat units was included as a linker 3' of eGFP. Single chain antibodies, their individual domains, or their mutant variants were then subcloned into CytExGreen as a NheI and NotI restriction fragment. The final result is cytoplasmic expression of a fusion gene-of-interest with an N-terminal eGFP tag in yeast. N-terminal HA and C-terminal c-myc epitope tags remain intact.

Visualization of FAPs by Confocal Microscopy

Induced cells used for microscopy were prepared in the same way as for fluorimetry and flow cytometry. Glass-bottom 35 mm dishes (MatTek) were treated with concanavalin A and overlaid with 200 μ L of prepared yeast cells (2×10^7 cells/mL). The cells were allowed to settle and adhere for 2 min, washed with 1 mL of PBS, and then overlaid with 2 mL of SD + CAA + dextrose medium supplemented with 100 nM MG-Ester. Following incubation at 30 °C for 2.5 h, the dishes were then imaged on a Carl Zeiss LSM-510 Meta DuoScan Inverted Confocal Microscope (Zeiss). Samples were observed through a Plan-Apochromat 100 $\times$ /1.40 Oil DIC M27 objective. Green fluorescent protein signal was excited with a 488 nm Argon laser and captured using a BP 505–550 filter. Malachite green fluorescence was detected by excitation with a 633 nm helium–neon laser and collected via the LP 650 emission filter. Capture and processing of images was achieved using Zen 2009 software (Zeiss).

Results

Directed Evolution of HL4 for Intracellular Activity

HL4-MG was selected as a candidate scFv to undergo directed evolution for activity in the cytoplasm. Cysteine residues H92 and L88 (Kabat numbering system [22]) were individually targeted for site-directed mutagenesis, both being replaced with alanine (Fig. 1). The Cys^{H92}Ala-Cys^{L88}Ala double mutant was also created. These mutations were expected to eliminate the intradomain disulfide bonds of HL4, carrying the added consequence of possibly lessening the inherent stability and functionality of the original scFv. To assess whether the site-directed mutants exhibited loss of FAP activity and to what extent, mutant proteins were expressed on the surface of yeast and evaluated for malachite green activation by flow cytometry (Table 1). Compared to wild type HL4, the Cys^{H92}Ala mutant exhibited 48 % fluorogen activation while Cys^{L88}Ala retained only 6 % activity. The double mutant showed the same FAP activity as the Cys^{L88}Ala mutant.

Since the Cys^{H92}Ala-Cys^{L88}Ala gene had both intra-domain disulfide bonds disrupted yet maintained some ability to activate malachite green, it was selected as the parent for directed evolution to regain the activity lost to the site-directed mutations. Using error-prone PCR, a library of random mutants was constructed with a diversity of ca. 1.6×10^6 , expressed as proteins on the surface of yeast as a population, and interrogated for fluorogen activation by

flow cytometry. The brightest ca. 1 % of the population was collected, grown, induced, and subjected to another flow cytometry enrichment. This process was repeated for a total of three enrichments of increasing stringency for both malachite green activation and expression level (as determined by c-myc staining). Enrichment of the populations is depicted in the flow analysis output as shown in Fig. 2.

Analysis during the flow cytometry enrichment indicated a successful trend in isolating a FAP clone with increased activity. By comparing the output of the three enrichment windows (Fig. 2d–f) it is apparent that the cell population in the lower right quadrant of each output window moves in an upward, rightward direction indicating a population average increase in fluorogenic dye activation and an increase in FAP expression, respectively. These populations represent the enrichment of desired mutants created by the mutagenic PCR used to create the library. Following the third enrichment, the brightest 0.75 % of the population was sorted onto induction-dye plates. After growth, colonies on the plates were imaged, and clones were ranked by the ratio of colony fluorescence intensity to colony size. Eleven individual clones in total were selected from both extremes of the rank-list and sequenced. Sequencing data revealed two unique, novel sequences, which were designated p13 and o4. Compared to HL4-MG, p13 contained a single point mutation, Gln^{L89}Leu as well as the two designed cysteine mutations (Cys^{H92}Ala and Cys^{L88}Ala). Clone o4 differed from the parent construct at four residues: Thr^{H28}Ala, Ser^{L22}Cys, and Phe^{L71}Leu were novel mutations, while the designed Cys^{L88}Ala mutation had reverted back to cysteine (Fig. 3). Since p13 was less complicated (in that it only had a single point mutation compared to its parent) and retained both designed cysteine mutations, it was selected for further characterization.

Comparison of p13 to HL4 and Intracellular Activity Assay

The pPNL6 yeast surface-display system [2] was used to express the derived scFvs on the surface of yeast in order to measure malachite green activation by either fluorimetry or flow cytometry. Fluorimetry assays revealed that fluorogen activation for p13 was 44 % that of wild type HL4 levels (Fig. 4a). To gain an understanding of the importance of the new point mutation discovered in p13, a mutant version of HL4 was created (HL4Gln^{L89}Leu), which contained only that single residue change (all cysteine residues were left intact). Interestingly, on the surface of yeast, this pointmutant showed higher levels of fluorogen activation than either p13 or HL4, at 141 % HL4 activity. Figure 4b shows flow cytometry analyses, which were in concordance with the fluorimetry assays.

While these assays confirmed that the p13 clone was capable of activating malachite green, it was still unknown whether this activity would be preserved in the reducing environment of the cytoplasm. To investigate whether p13 was able to function in the cytoplasm, a modified version of pPNL6, pCytEx, (gift from Chris Szent-Gyorgyi, Carnegie Mellon University) was used. This vector differed from pPNL6 in only one respect—the fusion to AGA-2 was deleted, leaving protein-of-interest to be expressed in an untargeted manner in the cytoplasm of yeast. Cytoplasmically expressed scFvs were measured for fluorogen-activating ability by both fluorimetry and flow cytometry, basically as above except using MG-Ester, a cell-permeable version of malachite green. As anticipated, HL4 was unable to activate malachite green to any appreciable degree in the cytoplasm. As shown in Fig. 4c, d, the double cysteine mutant was capable of activating malachite green > threefold that of HL4 whether measured by fluorimetry or flow cytometry. Clone p13 showed a dramatically improved ability to activate the fluorogen compared to HL4 (>20-fold by fluorimetry, >30-fold by flow cytometry). In separate experiments, the affinity of p13 for malachite green was measured in titrations using yeast cell surface expressed protein. The FAP-fluorogen K_d was determined to be 9.6 nM (data not shown). In identical experiments, the HL4 parent had a K_d of 3.9 nM, in close agreement with the previously published results of 3.2 nM [1]. Thus,

compared to the HL4 parent, the p13 clone lost some affinity for malachite green, but gained the ability to function in the cytoplasm. Surprisingly, the point mutation Gln^{L89}Leu, alone, was sufficient to enable HL4 to activate malachite green in the cytoplasm. Although there was some variation between methods of measurement, HL4 Gln^{L89}Leu exhibited robust activation of fluorogen in the cytoplasm (>23-fold by fluorimetry, >15-fold by flow cytometry) (Fig. 4c, d, respectively).

To evaluate how well this improvement could be visualized in living cells, yeast expressing scFvs in the cytoplasm were imaged by confocal microscopy. In the presence of 100 nM MG-Ester, both p13 and HL4 Gln^{L89}Leu demonstrate clear and robust activation of the fluorogen while HL4 and the parent Cys^{H92}Ala-Cys^{L88}Ala construct essentially showed background levels of activity (Fig. 5).

Directed Evolution for Enhanced Brightness of p13

The directed evolution experiments above produced an HL4 derivative that is capable of activity in the cytoplasm. However, we noted that p13 was significantly less bright than HL4 in cell surface activity measurements, reaching only slightly more than 40 % the brightness of HL4 (Fig. 4b). In the initial characterization of malachite green-binding FAPs, HL4 was shown to have a modest quantum yield of 0.16 [1]. Thus, to ensure that p13 would provide adequate utility in applications as an intracellular FAP label, we sought to increase the inherent brightness of its malachite green-binding activity, again by directed evolution.

While the overall methodology of this process was similar to that previously described, a few aspects were altered to address the specific goal of enhanced brightness while maintaining intracellular activity. To ensure that all clones isolated would be functional in the cytoplasm without an additional re-screening step, the directed evolution was carried out using the pCytEx (cytoplasmic expression) vector. Also, as enhanced brightness (as opposed to affinity, for example) was the ultimate goal, each enrichment step was performed at a higher fluorogen concentration (1 μ M MG-Ester). Since the enrichment and sorting were assessed based on fluorescence signal from the cytoplasm, the standard practice of using c-myc antibody signal to measure expression levels was not feasible. Instead, sorting was strictly based on fluorescence brightness. The parent gene used for this directed evolution was p13. Mutagenesis and mutant library construction by gap repair into pCytEx were the same as described before.

During each round of enrichment of the new mutant library, the brightest 0.5 % of the population was sorted and re-grown for the subsequent round. A total of four rounds of enrichment were performed. During these enrichments, 246 yeast cells were autocloned from the brightest 0.01 % of the population after the second round, and 400 more after the fourth round (200 from the brightest 0.2 % of the population, and another 200 from the brightest 0.05 % of the population). Induction-dye plates of these autocloned colonies were imaged and analyzed as described before (data not shown). Colonies were ranked according to their absolute brightness and brightness-per-area, with 10 colonies being chosen from the second enrichment and 22 colonies being chosen from the fourth enrichment.

Assessment of Brightness of Selected p13 Maturants

Both qualitative and quantitative measurements indicated that every isolated clone from this directed evolution experiment was in fact brighter than p13, to varying degrees (data not shown). However, these measurements could not take into account any differences in the level of FAP gene expression in the cytoplasm. In order to accurately compare the brightness enhancement of these p13 maturants a method of normalizing the fluorogen activation was needed. To accomplish this, a modified version of the pCytEx vector system,

pCytExGreen, was constructed that expresses the FAP protein as a C-terminal fusion to eGFP. In this way we could measure the activity of the FAP as dye activation brightness per unit of green fluorescence from eGFP. Of the 32 clones, the brightest and most consistent 11 were chosen to be subcloned into pCytExGreen.

Flow cytometry analysis of induced cultures of these clones indicated that there was, in fact, variation in gene expression level as measured by eGFP signal. The data summarized in Table 2 show that the FAP expression ranged from 51 to 135 % of p13 parent expression levels, with five of the clones exhibiting higher expression than that of p13, and six showing lower levels of expression relative to p13. Using the ratio of MG dye fluorescence per unit of eGFP signal, only the clone named p13-MW had a lower ratio than p13 (at 95%). The ratio for other clones, ranged from 124 to 201 % of p13—the brightest was the clone named p13-FW at 206 %. The brightest clones with the best level of protein expression (p13-CW, p13-RW1, and p13-FW) were selected for further analysis to determine whether these FAPs showed significant brightness enhancement in a fluorescence microscopy assay setting.

Observation of Enhanced Brightness by Microscopy

To examine how well quantitative increases in brightness would translate into observable differences in fluorescence during an assay scenario, induced cultures of the maturants p13-CW, p13-FW, and p13-RW1 were compared to p13 by confocal microscopy. Yeast cells expressing each FAP in the cytoplasm from pCytExGreen were imaged in the presence of 100 nM MG-Ester. Qualitatively, all three maturants showed easily discernible enhancements in fluorescence detection by microscopy compared to p13 (Fig. 6).

Discussion

Here we describe two sequential directed evolution experiments performed on a single chain antibody, each selecting for a unique property. Both experiments produced populations of candidates comprising multiple distinct clones exhibiting the desired trait (activity in the absence of intra-domain disulfide bonds or enhanced brightness). We have demonstrated the ability to carry out directed evolution in an intracellular context in yeast under conditions when critical disulfide bonds are unable to form. Previous research has shown that scFvs selected by display methodologies often fail to retain their antigen-binding activity when expressed in the cytoplasm. Visintin et al. [26] reported successful design and application of a two-hybrid system for in vivo identification of antibodies that are active in the cytoplasm. Here we present a similar method that exploits the fluorescent output of fluoromodules to directly detect and select variants capable of intracellular activity. In this way, our method ensures cytoplasmic function of isolated clones, eliminating the need for an extra subcloning and/or re-screening step after the final enrichment.

Proteins containing an Ig-fold often rely on highly conserved intradomain disulfide bonds for stability and proper functioning [27]. In the first directed evolution, we demonstrated that the ability of HL4 to bind and activate the fluorogen malachite green was indeed dependent upon the presence of both intradomain disulfide bonds. Initial flow cytometry analysis following cysteine mutagenesis further suggests that the stability provided from the disulfide bridge in the V_L is more critical to the function of HL4 than that from the V_H (Table 1). This may indicate that the V_H of HL4 is more inherently stable even in the absence of its disulfide bond than its V_L . Such differential stability of V_H and V_L domains is a characteristic that has been noted in other scFvs [28, 29].

Using the approaches described in this paper, we were able to isolate a FAP containing a single compensatory mutation that not only restored some of the activity lost to the disulfide bond-free mutant, but also conferred FAP activity in the intracellular milieu. Our results

indicate that this glutamine-to-leucine mutation was both necessary and sufficient for intracellular fluorogen activation by HL4. In experiments not presented here, we isolated and combined the various mutations that arose from the first directed evolution to try to establish a brighter intracellular FAP by “semi-rational” design. From 35 different mutants tested, only those containing Gln^{L89}Leu mutation were functional in the cytoplasm, further emphasizing the importance of this particular mutation. Attempts to crystallize either the HL4 or p13 FAP in order to determine their crystal structures thus far have been unsuccessful. So the mechanism by which this single point mutation functions remains elusive. The residue in question at position 89 in the V_L chain is the first residue of the complementarity-determining loop 3 (CDR3) of HL4’s variable light chain. Amino acids at this position in structures of other antibodies rarely make antigen contacts [30]. In cases where the most frequent glutamine is replaced by less common amino acids, L89 residues have been postulated to be important contributors to catalytic or enzymatic roles [31, 32]. However, here L89 is contextually situated in a non-catalytic FAP and additionally the glutamine had changed to leucine—the third most common amino acid at that position according to the Abysis amino acid distribution database [33]—so the role played by leucine in this case will likely be distinct from those instances. Furthermore, it has been noted that when glutamine is in position L89, it can make hydrogen-bonding interactions with framework residue tyrosine L36 [34]. In the Gln^{L89}Leu mutation this potential for stabilization is lost. Thus, until a structural understanding of the environment surrounding position L89 is obtained, determination of the precise role of the mutant leucine at that location will remain a challenge.

Similarly perplexing was the result that Cys^{H92}Ala-Cys^{L88}Ala double mutant was better at activating malachite green than HL4 when expressed in the cytoplasm (Fig. 4c, d). In general, antibody fragments that have had one or both of their intradomain disulfide bonds removed lose a substantial source of stability. Such proteins are often seen to have problems folding properly and functioning [9, 11]. Because of this, and under the assumption that the double cysteine mutant would be analogous to HL4 in a reducing environment, it was expected that both HL4 and the Cys^{H92}Ala-Cys^{L88}Ala double mutant would behave similarly in the cytoplasmic activity assays. One possible explanation could be that the alanine residues that replaced the critical cysteine residues are able to allow more favorable folding conditions for the FAP. This could result in two scenarios: if improperly folded FAP is quickly degraded by cellular machinery, slightly increased folding could result in a larger pool of active FAP, relative to the fraction that is degraded. Alternatively, if folding is improved, the Cys^{H92}Ala-Cys^{L88}Ala FAP may present a more stable or conformationally favorable binding pocket for the fluorogen. Another interpretation could be that the mutations themselves have a direct effect on FAP-fluorogen interactions. Replacement of the H92 and L88 cysteine residues with alanine could provide a more hydrophobic environment near the dye binding pocket that would make it more favorable for the hydrophobic fluorogen to bind. Even if not directly in the binding pocket, such mutations could potentially create an aberrant hydrophobic patch elsewhere on the FAP that would be of sufficient size to nonspecifically bind some fluorogen. Finally, the two unaltered cysteine residues could be factors in the increased activity of Cys^{H92}Ala-Cys^{L88}Ala. It has been shown in ABPC48, an scFv naturally lacking one of the conserved cysteine residues, that the remaining reduced cysteine becomes accessible to the solvent and can interact with various reagents [10]. Either the residue itself or the conformational change that allows the cysteine to become solvent-exposed could potentially lead, in part, to the unpredicted activity of the double cysteine mutant of HL4.

Ultimately these data show, to our knowledge, a novel variation of established directed evolution methods for the detection, isolation, and selection of scFvs exhibiting antigen-binding activity in the cytoplasm. By also including fusions to eGFP in future directed

evolution endeavors, this approach demonstrates great potential for ensuring the intracellular activity of a particular FAP, even while selecting for a distinct parameter (e.g., affinity or brightness). As a direct result of our intracellular directed evolution protocol, the FAP clone named p13-CW was isolated, and has proven to be the relatively brightest malachite green-binding FAP to date in vitro (data not shown). As p13-CW is also active in the cytoplasm, this FAP is a strong candidate to be a platform upon which future FAP-based biosensors can be constructed.

Acknowledgments

The authors gratefully acknowledge: Yehuda Creeger for skillful assistance in flow cytometry and induction plate imaging; Nina Senutovitch for assistance in imaging induction plates with the DIGE imaging system; Chris Szent-Gyorgyi for providing the CytEx vector before publication; and Joseph Franke for the eGFP clone used to construct the CytExGreen vector. This research was supported by Grant U54 RR022241 to Alan S. Waggoner (PI) from the National Institutes of Health. M.A.P was supported by an NSF REU Grant, #0452908 to Carnegie Mellon University (David Hackney, PI and Brooke McCartney, co-PI).

References

1. Szent-Gyorgyi C, et al. Fluorogen-activating single-chain antibodies for imaging cell surface proteins. *Nature Biotechnology*. 2008; 26:235–240.
2. Feldhaus MJ, et al. Flow-cytometric isolation of human antibodies from a nonimmune *Saccharomyces cerevisiae* surface display library. *Nature Biotechnology*. 2003; 21:163–170.
3. Ozhalici-Unal H, et al. A rainbow of fluoromodules: A promiscuous scFv protein binds to and activates a diverse set of fluorogenic cyanine dyes. *Journal of the American Chemical Society*. 2008; 130:12620–12621. [PubMed: 18761447]
4. Silva G, et al. Experimental and computational investigation of unsymmetrical cyanine dyes: understanding torsionally responsive fluorogenic dyes. *Journal of the American Chemical Society*. 2007; 129:5710–5718. [PubMed: 17411048]
5. Fitzpatrick J, et al. STED nanoscopy in living cells using fluorogen activating proteins. *Bioconjugate Chemistry*. 2009; 20:1843–1847. [PubMed: 20976031]
6. Fisher GW, et al. Detection and quantification of beta2AR internalization in living cells using FAP-based biosensor technology. *Journal of Biomolecular Screening*. 2010; 15:703–709. [PubMed: 20488980]
7. Glover M, et al. Pharmacological rescue of mutant CFTR detected using a novel fluorescence platform. *Molecular Medicine*. 2012; 18:685–696. [PubMed: 22396015]
8. Holleran J, et al. Fluorogen-activating proteins as biosensors of cell surface proteins in living cells. *Cytometry Part A*. 2010; 77:776–782.
9. Glockshuber R, Schmidt T, Pluckthun A. The disulfide bonds in antibody variable domains: effects on stability, folding in vitro, and functional expression in *Escherichia coli*. *Biochemistry*. 1992; 31:1270–1279. [PubMed: 1736986]
10. Proba K, Honegger A, Pluckthun A. A natural antibody missing a cysteine in VH: consequences for thermodynamic stability and folding. *Journal of Molecular Biology*. 1997; 265:161–172. [PubMed: 9020980]
11. Biocca S, et al. Redox state of single chain Fv fragments targeted to the endoplasmic reticulum, cytosol and mitochondria. *Biotechnology (N.Y.)*. 1995; 13:1110–1115. [PubMed: 9636285]
12. Zhou C, Przedborski S. Intrabody and Parkinson's disease. *BBA*. 2009; 1792(7):634–642. [PubMed: 18834937]
13. Dunn S, et al. Directed evolution of human T cell receptor CDR2 residues by phage display dramatically enhances affinity for cognate peptide MHC without increasing apparent. *Protein Science*. 2006; 15:710–721. [PubMed: 16600963]
14. Persson H, et al. In vitro evolution of an antibody fragment population to find high-affinity hapten binders. *Protein Engineering, Design & Selection*. 2008; 21:485–493.

15. Hanes J, Plückthun A. In vitro selection and evolution of functional proteins by using ribosome display. *Proceedings of the National Academy of Sciences of the United States of America*. 1997; 94:4937–4942. [PubMed: 9144168]
16. Zahnd C, et al. Directed in vitro evolution and crystallographic analysis of a peptide-binding single chain antibody fragment (scFv) with low picomolar affinity. *The Journal of Biological Chemistry*. 2004; 279:18870–18877. [PubMed: 14754898]
17. Richman S, Kranz D, Stone J. Biosensor detection systems: Engineering stable, high-affinity bioreceptors by yeast surface display. *Methods in Molecular Biology*. 2009; 504:323–350. [PubMed: 19159105]
18. Colby DW, et al. Development of a human light chain variable domain (VL) intracellular antibody specific for the amino terminus of huntingtin via yeast surface display. *Journal of Molecular Biology*. 2004; 342:901–912. [PubMed: 15342245]
19. Alper H, et al. Tuning genetic control through promoter engineering. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:12678–12683. [PubMed: 16123130]
20. Song L, Siguier B, Dumon C, Bozonnet S, O'Donohue MJ. Engineering better biomass-degrading ability into a GH11 xylanase using a directed evolution strategy. *Biotechnology for Biofuels*. 2012; 5:3–18. [PubMed: 22244361]
21. Colby DW, et al. Potent inhibition of huntingtin aggregation and cytotoxicity by a disulfide bond-free singledomain intracellular antibody. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:17616–17621. [PubMed: 15598740]
22. Kabat, EA.; Wu, TT.; Perry, HM.; Gottesman, KSA.; Foeller, C. Sequences of proteins of immunological interest. Bethesda, MD: U.S. Dept. of Health and Human Services, Public Health Service, National Institutes of Health; 1991.
23. Chao G, et al. Isolating and engineering human antibodies using yeast surface display. *Nature Protocols*. 2006; 1:755–768.
24. Orr-Weaver TL, Szostak JW. Yeast recombination: The association between double-strand gap repair and crossing-over. *Proceedings of the National Academy of Sciences of the United States of America*. 1983; 80:4417–4421. [PubMed: 6308623]
25. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*. 2012; 9:671–675. [PubMed: 22930834]
26. Visintin M, et al. Selection of antibodies for intracellular function using a two-hybrid in vivo system. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96:11723–11728. [PubMed: 10518517]
27. Wörn A, Plückthun A. Stability engineering of antibody single-chain Fv fragments. *Journal of Molecular Biology*. 2001; 305:989–1010. [PubMed: 11162109]
28. Wörn A, Plückthun A. Different equilibrium stability behavior of ScFv fragments: Identification, classification, and improvement by protein engineering. *Biochemistry*. 1999; 38:8739–8750. [PubMed: 10393549]
29. Wang T, Duan Y. Probing the stability-limiting regions of an antibody single-chain variable fragment: A molecular dynamics simulation study. *Protein Engineering, Design & Selection*. 2011; 24:649–657.
30. MacCallum RM, Martin AC, Thornton JM. Antibody-antigen interactions: Contact analysis and binding site topography. *Journal of Molecular Biology*. 1996; 262:732–745. [PubMed: 8876650]
31. Zhu X, et al. The origin of enantioselectivity in aldolase antibodies: Crystal structure, site-directed mutagenesis, and computational analysis. *Journal of Molecular Biology*. 2004; 343:1269–1280. [PubMed: 15491612]
32. Golinelli-Pimpaneau B. Novel reactions catalysed by antibodies. *Current Opinion in Structural Biology*. 2000; 10:697–708. [PubMed: 11114507]
33. Martin, ACR. Abysis amino acid distributions. Division of Biosciences, University College London; 2012. Retrieved August 27, 2012 from <http://www.bioinf.org.uk/abysis/sequences/distribution.html>

34. Xiang J, et al. Light-chain framework region residue Tyr71 of chimeric B72.3 antibody plays an important role in influencing the TAG72 antigen binding. *Protein Engineering*. 1999; 12:417–421. [PubMed: 10360982]

Fig. 1.

Schematic of MG-binding FAP HL4. Layout of domains and critical cysteine residues of HL4-MG. This schematic depicts the primary sequence of HL4, oriented with the N-terminal residue on the *left*. The V_H and V_L domains are shaded in *dark* and *light gray*, respectively, and the flexible $(Gly_4Ser)_3$ linker connecting the two is *left unshaded*. Two disulfide-forming cysteine residues are present in each variable domain, labeled according to Kabat numbering and connected to its bonding partner by a *solid line*. *Symbols* above the scFv represent primers used to create overlapping fragments for the site-directed mutagenesis of targeted cysteine residues (detailed in “Materials and Methods” section). *Filled arrowheads* indicate directionality (5′–3′). Primers P1 and P6 were non-mutagenic and annealed to regions outside the coding sequence of the scFv. Primers P2–P5 contained designed regions of nucleotide mismatch to introduce site-specific mutations (shown as *peaks* in *arrow tails*)

Fig. 2.

FACS enrichments of mutant library for recovered fluorogen activation. Flow cytometry scatter plots showing expression level (c-myc, as Alexafluor488) and fluorogen activation (MG-11P) for populations of 20,000 surface-displaying yeast cells. The *x*-axis for each plot is fluorescence at 530 nm (c-myc) and the *y*-axis is fluorescence at 685 nm (MG). Note that both axes have a logarithmic scale. **a** Uninduced HL4 (wild type) cells, **b** Cys^{H92}Ala-Cys^{L88}Ala (parent), **c** HL4 (wild type), **d** mutant library (1st enrichment), **e** mutant library (2nd enrichment), **f** mutant library (3rd enrichment). All analyses and sorts were carried out in the presence of 100 nM MG-11P fluorogen. The P2 gate as shown in each plot was designed to select subpopulations of the brightest 1 % for sorting and enrichment

V_H		A	ABC	•	ABCDEF
HL4	QVQLVESEGGVLVQPGGSLRLSCAASGFTFSYEMNWVRQAPGKLEWVSRIDGGSSTNYADSVKGRFTISRDNASTLYLQMNSLRAEDTAVYYCTRARVFGSVSPYGMVDVWGQGT	TVTS			
Cys ^{H92} Ala-Cys ^{L88} Ala	QVQLVESEGGVLVQPGGSLRLSCAASGFTFSYEMNWVRQAPGKLEWVSRIDGGSSTNYADSVKGRFTISRDNASTLYLQMNSLRAEDTAVYYA	TRARVFGSVSPYGMVDVWGQGT			
o4	QVQLVESEGGVLVQPGGSLRLSCAASGFTFSYEMNWVRQAPGKLEWVSRIDGGSSTNYADSVKGRFTISRDNASTLYLQMNSLRAEDTAVYYA	TRARVFGSVSPYGMVDVWGQGT			
p13	QVQLVESEGGVLVQPGGSLRLSCAASGFTFSYEMNWVRQAPGKLEWVSRIDGGSSTNYADSVKGRFTISRDNASTLYLQMNSLRAEDTAVYYA	TRARVFGSVSPYGMVDVWGQGT			
p13-CW	QVQLVESEGGVLVQPGGSLRLSCAASGFTFSYEMNWVRQAPGKLEWVSRIDGGSSTNYADSVKGRFTISRDNASTLYLQMNSLRAEDTAVYYA	TRARVFGSVSPYGMVDVWGQGT			
p13-FW	QVQLVESEGGVLVQPGGSLRLSCAASGFTFSYEMNWIRQAPGKLEWVSRIDGGSSTNYTDSVKGRFTISRDNASTLYLQMNSLRAEDTAVYYA	TRARVFGSVSPYGMVDVWGQGT			
p13-RW1	QVQLVESEGGVLVQPGGSLRLSCAASGFTFSYEMNWVRQAPGKLEWVSRIDGGSSTNYVDSVKGRFTISRDNASTLYLQMNSLRAEDTAVYYA	TRARVFGSVSPYGMVDVWGQGT			
Linker					
HL4	SGILSGGGSGGGSGGGSGGGG				
Cys ^{H92} Ala-Cys ^{L88} Ala	SGILSGGGSGGGSGGGSGGGG				
o4	SGILSGGGSGGGSGGGSGGGG				
p13	SGILSGGGSGGGSGGGSGGGG				
p13-CW	SGILSGGGSGGGSGGGSGGGG				
p13-FW	SGTLGGGGSGGGSGGGSGGGG				
p13-RW1	SGILSGGGSGGGSGGGSGGGG				
V_L				•†	
HL4	DIRVTQSPSSVSASVGDRTISCRASQGIATWLGWYQQKPGKPPQLLIYSASTLQTVPSRFGSGSGTDFTLTISLQPEDVATYYAQEGSTFPLTFGGG	KVDIK			
Cys ^{H92} Ala-Cys ^{L88} Ala	DIRVTQSPSSVSASVGDRTISCRASQGIATWLGWYQQKPGKPPQLLIYSASTLQTVPSRFGSGSGTDFTLTISLQPEDVATYYAQEGSTFPLTFGGG	KVDIK			
o4	DIRVTQSPSSVSASVGDRTISCRASQGIATWLGWYQQKPGKPPQLLIYSASTLQTVPSRFGSGSGTDFTLTISLQPEDVATYYAQEGSTFPLTFGGG	KVDIK			
p13	DIRVTQSPSSVSASVGDRTISCRASQGIATWLGWYQQKPGKPPQLLIYSASTLQTVPSRFGSGSGTDFTLTISLQPEDVATYYALEGSTFPLTFGGG	KVDIK			
p13-CW	DIRVTQSPSSVSASVGDRTISCRASQGIATWLGWYQQKPGKPPQLLIYSASTLQTVPSRFGSGSGTDFTLTISLQPEDVATYYALEGSTFPLTFGGG	KVDIK			
p13-FW	DIRVTQSPSSVSASVGDRTISCRASQGIATWLGWYQQKPGKPPQLLIYSASTLQTVPSRFGSGSGTDFTLTISLQPEDVATYYALEGSTFPLTFGGG	KVDIK			
p13-RW1	DIRVTQSPSSVSASVGDRTISCRASQGIATWLGWYQQKPGKPPQLLIYSASTLQTVPSRFGSGSGTDFTLTISLQPEDVATYYALEGSTFPLTFGGG	KVDIK			

Fig. 3.

Primary sequence alignment of HL4 family. Amino acid sequences for HL4 and its derivatives are shown. The V_H domain, glycine/serine linker, and the V_L domain are shown separately. Complementarity-determining regions (CDRs), defined in accordance with Kabat convention, are *underlined*. Positions H92 and L88, disulfide-forming cysteines in HL4, are marked with a large *dot symbol*. Light chain residue L89, site of the suppressor GlnL89Leu, is denoted by a *dagger*. Small *dot markers* are placed every ten residues. Mutations relative to HL4 are *bolded*. Residue numbering in the V_H ranges from H1 to H112, and includes the following modifications in agreement with Kabat: H52A after H52; H82A, H82B, and H82C after H82; H100A, H100B, H100C, H100D, H100E, and H100F after H100; there are no residues at Kabat positions H113 and H114. Numbering in the V_L (L1–L107) does not include any additional sites; however, no residues are present at Kabat positions L108, L109, L110, or L111

Fig. 4.

Fluorescence analysis of surface-displayed and cytoplasmically expressed FAPs. FAPs were expressed in EBY100 strain yeast cells for surface display from the pPNL6 plasmid (**a, b**) or for cytoplasmic expression from pCytEx (**c, d**). Fluorogen activation of clonal yeast populations was measured by fluorimetry (**a, c**) and flow cytometry (**b, d**). **a** Fluorimetry data collected for 10^6 yeast cells displaying the indicated FAP on the cell surface in the presence of 500 nM (cell-permeant) MG-Ester. Samples were tested in triplicate with the resulting fluorescent signal values averaged, corrected for background (uninduced p13-expressing yeast) and normalized to HL4 (HL4 = 1.0). **b** Flow cytometry analysis of FAPs displayed on the surface of yeast cells. Fluorogen activation and c-myc signal were measured at 685 and 530 nm, respectively, for 20,000 individual yeast cells from clonal

populations in the presence of 100 nM MG-Ester. A ratio was calculated for the population average fluorogen activation divided by population average c-myc signal to generate values of fluorescence per expressed FAP. Ratios were then normalized to HL4 (HL4 = 1.0). **c** Fluorimetry measurements of 10^6 cytoplasmically expressing yeast cells in the presence of 500 nM MG-Ester. Triplicate trials were run for each sample, and the data was collected, corrected, and normalized as in **a**. **d** Flow cytometry of yeast expressing FAPs in the cytoplasm in the presence of 100 nM MG-Ester. Interrogation of 20,000 individual yeast cells for each sample produced population average values. Intracellular expression precluded calculation of ratios to c-myc signal. Consequently, only fluorogen activation population averages (measured at 685 nm) were analyzed. Population averages were again normalized to HL4 levels (HL4 = 1.0)

Fig. 5.

Fluorescence microscopy of cytoplasmically expressed FAPs. EBY100 yeast expressing the indicated FAP in the cytoplasm from pCytEx was imaged for malachite green fluorescence in the presence of 100 nM MG-Ester. *Top* differential interference contrast (DIC) images of the yeast. *Bottom* malachite green activation as evaluated by excitation with a 633 nm laser and detection with a long pass 650 nm filter. Yeast containing pCytEx with p13 maintained in non-inducing growth medium (lacking galactose) are shown at the *far right* to illustrate background signal

Fig. 6. Microscopy of p13 maturants in the cytoplasm of yeast. Yeast cells cytoplasmically expressing eGFP-FAP fusions from pCytEx-Green were grown, induced, and imaged as described. For each of the different fusions expressed, images were taken in the eGFP channel (eGFP), for fluorogen activation (MG) and using differential interference contrast microscopy (DIC). The overlay of all three images can be seen in the *bottom row* (Merge)

Table 1

Population averages of fluorogen activation from designed cysteine mutants

FAP gene	c-myc	MG-2P	MG/c-myc ratio	Normalized ratio
HL4	9938.36	2615.77	0.267	=1.00
Cys ^{H92} Ala	10906.17	1343.56	0.127	0.48
Cys ^{L88} Ala	12049.90	182.04	0.017	0.06
Cys ^{H92} Ala, Cys ^{L88} Ala	6931.06	91.52	0.017	0.06

Population averages of fluorescence intensities were measured from induced yeast cells by flow cytometry. Protein expression was evaluated by Alexafluor 488 signal (c-myc) and fluorogen activation was assessed by measured malachite green fluorescence (MG-2P)

The ratio of population averages of MG-2P fluorescence to c-myc expression for each of the cysteine mutants were normalized to that of the parent, HL4. Each population consisted of 20,000 individually interrogated yeast cells

Table 2

Flow cytometry analysis of isolated p13 maturants expressed in the cytoplasm of yeast

p13 Mutant	eGFP	MG-Ester	MG/eGFP
p13-FW	0.78	1.59	2.06
p13-RW1	0.87	1.75	2.01
p13-GW2	0.51	0.91	1.81
p13-CW	1.18	2.12	1.79
p13-HW1	0.74	1.27	1.71
p13-AW	0.99	1.38	1.40
p13-HW2	1.01	1.41	1.39
p13-RW2	0.89	1.14	1.28
p13-BW	1.04	1.30	1.25
p13-GW1	1.21	1.49	1.24
p13	=1.00	=1.00	1.00
p13-MW	1.35	1.28	0.95

FAPs were expressed in the cytoplasm of yeast as C-terminal fusions to eGFP and induced as before. Cells were analyzed by flow cytometry for both malachite green and eGFP fluorescence in the presence of 1 μ M MG-Ester. Clonal population averages were obtained from analysis of 20,000 individual cells. To assess unit brightness, the population mean malachite green signal was divided by the population mean eGFP signal. For each data series, the values were normalized to p13 (p13 = 1.00, bolded)