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Transforming a $(\beta/\alpha)_8$-Barrel Enzyme into a Split-Protein Sensor through Directed Evolution

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Summary

Split-protein sensors have become an important tool for the analysis of protein-protein interactions in living cells. We present here a combinatorial method for the generation of new split-protein sensors and demonstrate its application toward the $(\beta/\alpha)_8$-barrel enzyme N-(5′-phosphoribosyl)-anthranilate isomerase Trp1p from *Saccharomyces cerevisiae*. The generated split-Trp protein sensors allow for the detection of protein-protein interactions in the cytosol as well as the membrane by enabling *trp1* cells to grow on medium lacking tryptophan. This powerful selection complements the repertoire of the currently used split-protein sensors and provides a new tool for high-throughput interaction screening.

Introduction

Protein-protein interactions are at the center of almost all biological processes, and the development of methods for the identification and characterization of such interactions continues to be one of the main challenges in functional genomics and proteomics. The introduction of the yeast-two hybrid system by Fields and Song in 1989 was a milestone for the analysis of protein-protein interactions in living cells [1]. The success of this approach was highlighted by its application for the screening for protein-protein interactions on a genome-wide scale [2]. However, a major limitation of the two-hybrid system lies in its restriction to the detection of those protein-protein interactions that can be reproduced within the nucleus of a yeast cell. To overcome this restriction, an alternative to the two-hybrid method was introduced in 1994 by Johnsson and Varshavsky [3]. Here, the two interacting proteins are expressed as fusion proteins with an N- and a C-terminal fragment of ubiquitin. Upon interaction of the two proteins, a quasi-native ubiquitin is formed and subsequently recognized by ubiquitin-specific proteases, resulting in the cleavage of a reporter protein from the C-terminal fragment of ubiquitin. The split-ubiquitin system allows for the detection of interactions between cytoplasmic as well as membrane proteins [3–5]. Since the introduction of split-ubiquitin, a variety of other split-protein sensors have been developed, including pairs of fragments of dihydrofolate reductase (DHFR), β-galactosidase, β-lactamase, inteins, and green fluorescent protein (GFP) [6–12]. Among these systems, only split-ubiquitin was successfully applied to screen for binding partners [13–15]. Other sensors were used to monitor the interactions between selected pairs of proteins rather than to find new partners by a random library approach. Robust systems that can be used for identifying interaction partners at any location inside the cell and in different hosts are therefore still needed. Ideally, the interaction-induced reassociation of such a split-protein sensor would provide the cell with a growth advantage, thus allowing a selection for interacting proteins. However, generating new split-protein sensors is technically demanding, as it depends critically on identifying suitable fragments that can reconstitute a native-like and active protein. The chosen fragmentation site has to fulfill the following criteria: (1) it must yield two fragments that efficiently fold into quasi-native protein only when fused to two interacting proteins; (2) it must not significantly impair the activity of the reconstituted protein; (3) it must yield soluble protein fragments that are not readily degraded in vivo. In previous studies, the challenge of finding such sites has been mostly tackled by trial and error.

Here, we introduce a combinatorial approach to generate split-protein sensors and apply it to the $(\beta/\alpha)_8$-barrel enzyme N-(5′-phosphoribosyl)-anthranilate isomerase Trp1p from *Saccharomyces cerevisiae* to yield a split-Trp sensor [16, 17]. One of the selected split-Trp pairs was successfully applied to monitor protein-protein interactions both at the membrane as well as in the cytosol of yeast. Its selected fragmentation site would not have been easily predicted by theoretical considerations, thus justifying our evolutionary approach.

Results and Discussion

For the development of a new split-protein sensor, we chose the $(\beta/\alpha)_8$-barrel enzyme N-(5′-phosphoribosyl)-anthranilate isomerase Trp1p from *Saccharomyces cerevisiae* [16, 17]. Trp1p is a relatively small (25 kDa) monomeric protein that catalyzes the isomerization of N-(5′-phosphoribosyl)-anthranilate in the biosynthesis of tryptophan [17]. Creating a pair of Trp1p fragments (split-Trp) that only reconstitute the enzymatic activity when linked to interacting proteins would allow monitoring of this protein interaction through a simple growth assay; otherwise *trp1* yeast strains expressing such a split-Trp fusion pair would be able to grow on medium lacking tryptophan. As many different *trp1* strains exist, the interaction assay could be applied immediately in different genetic backgrounds, adding a further attractive feature to a split-Trp sensor. Trp1p is a well-studied member of the prominent class of proteins that fold into

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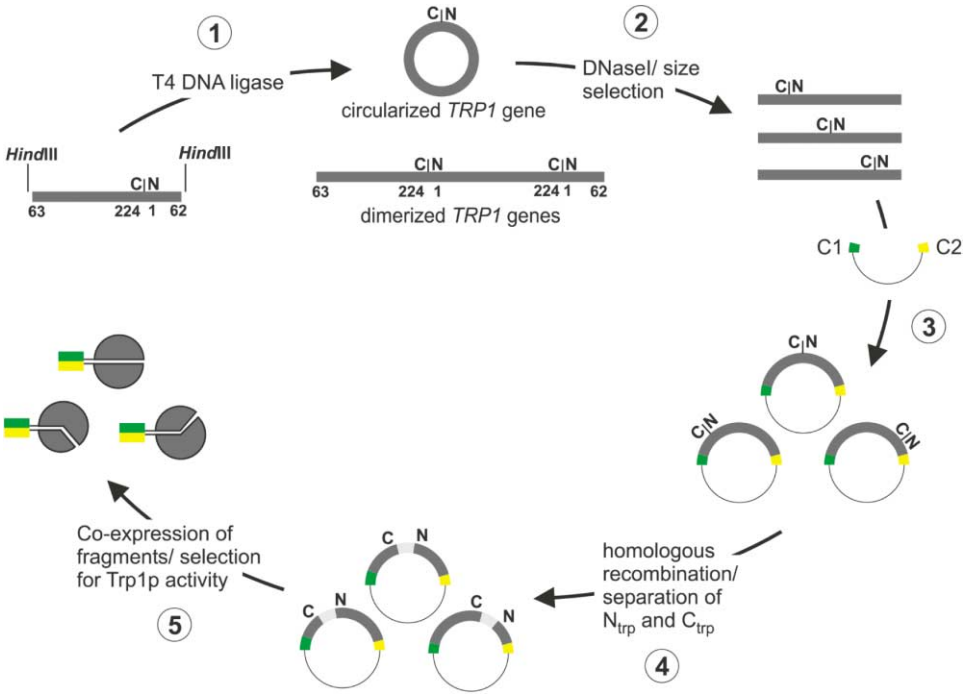


Figure 1. Combinatorial Approach toward the Generation of Split-Trp Proteins

As a starting point, a rearranged copy of the *TRP1* gene (gray) was used in which the original N and C termini of Trp1p were connected by a short linker containing a unique AvrII site. (1) The linear fragment was incubated with T4 DNA ligase to circularize the gene. At the same time, dimers and higher oligomers were formed. (2) Treatment of the ligation mix with DNaseI resulted in randomly cut linear molecules and fragments corresponding to the size of *TRP1* were isolated. (3) Isolated fragments were cloned into a yeast expression vector containing two polypeptides that associate into an antiparallel coiled coil (green [C1] and yellow [C2] boxes). It should be noted that due to the blunt-end cloning step, the majority of the clones will carry *TRP1* fragments that are out of frame with one or both of the polypeptides that form the coiled coil or will be inserted in a wrong orientation into the plasmid. (4) Homologous recombination in yeast cells was used to insert a terminator sequence and the P_{GAL1} promoter (light gray box, see text for details) between the original N and C termini of Trp1p. (5) Coexpression of the two fragments and selection for complementation of tryptophan auxotrophy of yeast cells allowed the isolation of functional split-Trp pairs.

a $(\beta/\alpha)_8$-barrel, which is the most commonly occurring fold among enzymes. It has been previously subjected to circular permutation and has been expressed as two separate fragments that spontaneously associate into a functional enzyme [18, 19]. Furthermore, it has been proposed that the $(\beta/\alpha)_8$-barrel evolved by tandem duplication from a $(\beta/\alpha)_4$ domain [20]. It would therefore add to our understanding of this protein fold to determine whether and, if yes, where the $(\beta/\alpha)_8$-barrel can be split into two fragments that reconstitute quasi-native Trp1p only when fused to interacting proteins.

Generation of New Split-Protein Sensors through Directed Evolution

To generate split-protein sensors based on Trp1p (split-Trp), we adapted an approach originally developed by Graf and Schachmann for creating random circular permutations of proteins [21]. In short, circularized or oligomerized genes of *TRP1* were randomly cut with DNaseI, creating new N and C termini (Figure 1). The collection of the randomly cut gene(s) was then ligated into a vector so that the new termini are fused to DNA that codes for a pair of two polypeptides (C1 and C2) that associate with each other into an antiparallel coiled coil [22, 23]. The antiparallel coiled coil formed by C1 and C2 represents the interacting proteins whose association brings the two halves of the split-Trp into spatial proximity. Using homologous recombination, a DNA fragment containing a terminator sequence and the P_{GAL1}-promoter was then inserted between the original N and C termini of Trp1p [24]. In the final construct, the C-terminal fragment fused to C1 (C1-C_{trp}) is under the control of the inducible but leaky P_{CUP1}-promoter, and the N-terminal fragment fused to C2 (N_{trp}-C2) is under the stringent control of the P_{GAL1}-promoter. Out of a library of approximately 1600 clones generated in this way, 11 potential split-Trp pairs were selected in the yeast strain EGY48 by complementation of its tryptophan auxotrophy and analyzed by DNA sequencing (Figure 2). Five of the eleven analyzed clones led to the expression of Trp1p fragments in which both fragments were fused in frame to the polypeptides C1 and C2. In the other six clones, C2 was not in frame to N_{trp} but extended by an unrelated peptide. We assume that N_{trp} and C_{trp} of these clones associate spontaneously without the help of interacting proteins. This assumption is supported by the work of Eder and Kirschner, who generated two Trp1p fragments that spontaneously assemble into a functional enzyme [19].

We then analyzed those five clones in detail that express both Trp1p fragments in frame with C1 and C2

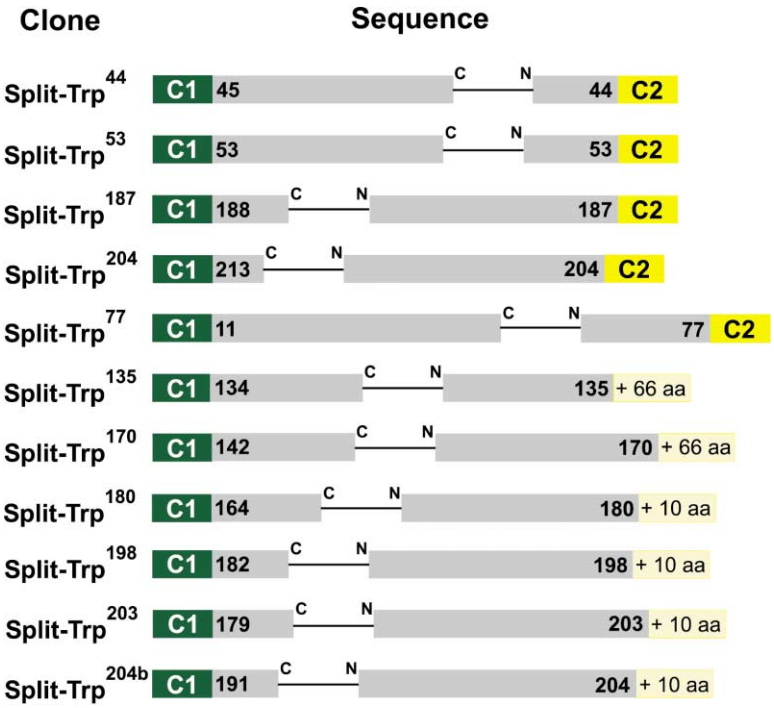


Figure 2. Selected Split-Trp Protein Pairs Capable of Complementing Tryptophan Auxotrophy in Yeast

The clones are named after the last residue of each N-terminal fragment. The gray rectangles represent the corresponding N- and C-terminal fragments of Trp1p. C1 and C2 are the two polypeptides that associate into the antiparallel coiled coil [22]. Due to a shift in the reading frame, in six of the eleven clones C2 is replaced by peptides of 10 or 66 amino acids (see Experimental Procedures). Six of the eleven isolated clones possess an overlapping *TRP1* sequence. This requires that they were generated by 2-fold digestion of oligomerized *TRP1* genes.

(Figure 3). To test whether the *trp1* complementation depends on the presence of both Trp1p fragments, we repeated the growth assays on plates lacking tryptophan and galactose but containing glucose and copper, thereby repressing the expression of N_{trp}-C2. Of the five clones tested, only split-Trp^{77} conferred tryptophan auxotrophy to the *trp1* yeast in the presence of glucose, indicating that its large C-terminal fragment spanning residues 11–224 already possesses enzymatic activity. On galactose, split-Trp^{44}, split-Trp^{187}, and split-Trp^{77} complemented tryptophan auxotrophy at 30°C and 23°C, whereas split-Trp^{53} and split-Trp^{204} complemented tryptophan auxotrophy only at 23°C (Figure 3). To test the contribution of the interaction between the two coils to the reassociation of the Trp1p fragments, we deleted C2 from N_{trp}-C2 in split-Trp^{44}, split-Trp^{53}, split-Trp^{187}, and split-Trp^{204}. The deletion of C2 abolished the capacity of these four clones to complement tryptophan auxotrophy (Figure 3). This finding demonstrates that the formation of a functional Trp1p from these fragments indeed depends on the fusion to a pair of interacting polypeptides.

Structural Interpretation of the Fragmentation Sites

Since the structure of Trp1p from *S. cerevisiae* has not yet been solved, we aligned its sequence with the sequences of the N-(5′-phosphoribosyl)-anthranilate isomerases from *E. coli* (ePRAI) and *Thermotoga maritima* (tPRAI) and identified the fragmentation sites in the known crystal structures of the homologous enzymes (Figure 4) [19, 25]. The fragmentation site of split-Trp^{44} lies in one of the active site loops between β2 and α2, two residues away from an arginine residue that interacts with the carboxyl group of the substrate N-(5′-

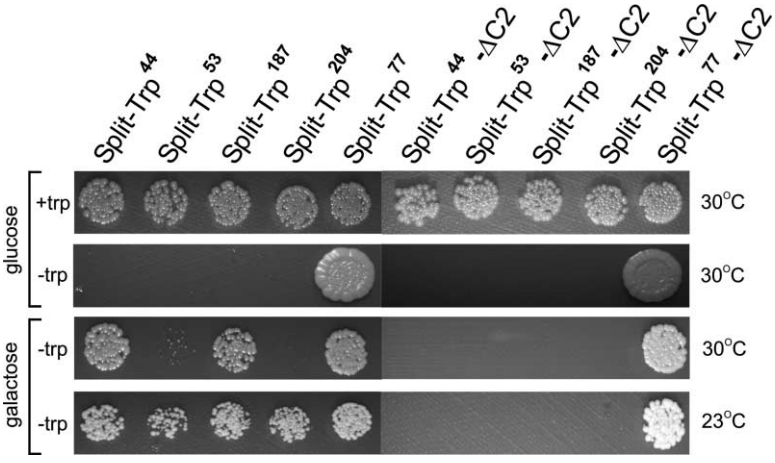
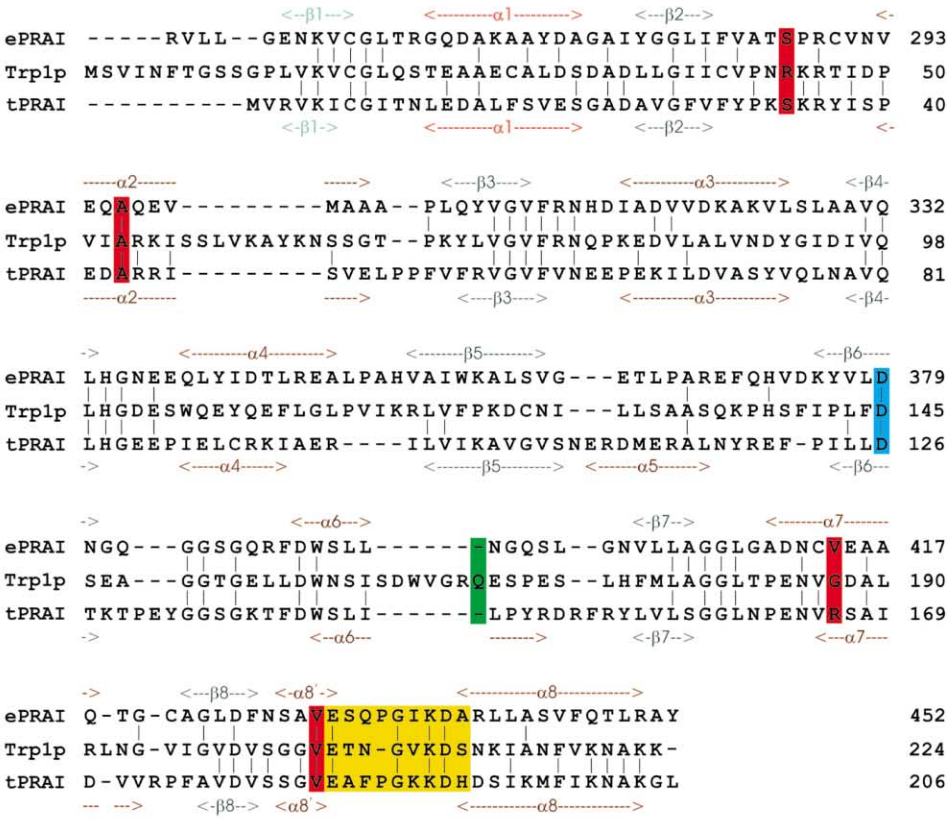


Figure 3. Growth Assays of Yeast Strains Expressing split-Trp^{44}, split-Trp^{53}, split-Trp^{187}, split-Trp^{204}, or split-Trp^{77} on Different Selective Plates

In these experiments, the split-Trp protein fragments are fused to the interacting polypeptides C1 and C2 (see Figure 2) [22]. Yeast strains expressing the same split-Trp proteins but lacking the sequence encoding C2 (split-Trp-ΔC2) were used for control experiments. C1-C_{trp} is under control of the leaky P_{CUP1}-promoter, and N_{trp}-C2 is under the control of the P_{GAL1}-promoter. A colony of yeast cells EGY48 expressing different split-Trp protein pairs was suspended in 1 ml water, and 5 μl was spotted on medium with or without tryptophan (trp), galactose, and glucose, but always containing copper and was grown at two different temperatures (23°C and 30°C). Images were taken after 8 days.

A



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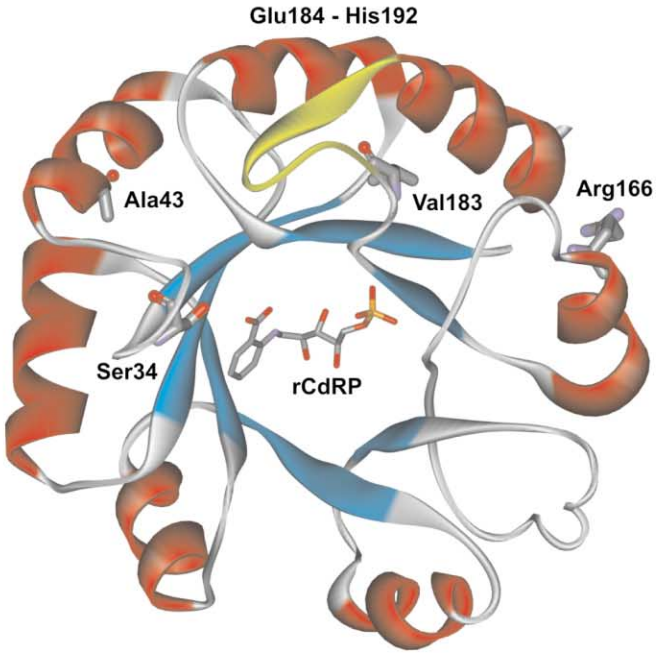

Figure 4. Structural Interpretation of the Fragmentation Sites

(A) Sequence alignment of N-(5′-phosphoribosyl)-anthranilate isomerases from *E. coli* (ePRAI), *S. cerevisiae* (Trp1p), and *T. maritima* (tPRAI) [19, 25]. Note that ePRAI is the second protein of a bifunctional enzyme. The amino acid numbering, therefore, starts with the corresponding residue of the original sequence (Arg251). The position and the numbering of secondary elements (α helices and β strands) for ePRAI and tPRAI are shown as arrows above and below the sequences of ePRAI and tPRAI, respectively. Gaps in the sequence are indicated by dashes. Identical residues are connected by vertical bars. Red boxes indicate the positions of fragmentation sites in split-Trp^{44}, split-Trp^{53}, split-Trp^{187}, and split-Trp^{204}. Residues deleted in the mutant split-Trp^{204} are marked in yellow. Circular permuted variants of Trp1p constructed by Luger et al. [18] are boxed in turquoise and green, and the fragmentation site used by Eder et al. [19] is labeled in green.

phosphoribosyl)-anthranilate. Although combinatorial mutagenesis experiments have indicated that turn sequences in general are highly mutable in $(\beta/\alpha)_8$-barrels, the proximity of this position to an active site residue would not have made it an obvious candidate for a fragmentation site [26]. In split-Trp^{187} and split-Trp^{53}, the fragmentation sites are located in α helices α7 and α2 of the $(\beta/\alpha)_8$-barrel, respectively. This is in agreement with the mutability of α-helical residues in combinatorial mutagenesis experiments on $(\beta/\alpha)_8$-barrels and with earlier random circular permutation experiments of other folds in which new termini were introduced into α helices [21, 26]. Furthermore, α helix α2 is extended by nine amino acids in Trp1p compared to ePRAI and tPRAI, making it plausible that the introduction of a fragmentation site could be tolerated without significantly affecting the activity or the folding of the $(\beta/\alpha)_8$-barrel. Particularly interesting is split-Trp^{204}, in which a stretch of eight amino acids (205–212), including four highly conserved residues, is deleted from Trp1p. This results in a very short C_{trp} of only twelve residues that is fused to C1, corresponding to α helix α8 in the structure of tPRAI and ePRAI. The eight deleted amino acids form a loop in the vicinity of the active site, directly after the short α helix α8′. Helix α8′ is believed to participate in the binding of the phosphate group of the substrate and is not present in the regular structures of other $(\beta/\alpha)_8$-barrels [19, 25]. While split-Trp^{204} complements tryptophan auxotrophy only at 23°C, indicating a decreased stability of the split enzyme, this finding nevertheless questions the significance of this loop with its four completely conserved residues in the function of N-(5′-phosphoribosyl)-anthranilate isomerases. However, it is unknown how much residual Trp1p activity is sufficient to complement tryptophan auxotrophy in yeast, and a more detailed interpretation of this finding will therefore require the kinetic characterization of Split-Trp^{204} in in vitro assays.

Eder and Kirschner have shown that the N-terminal fragment 1-167 folds in the absence of its C-terminal partner [19]. Furthermore, it has been proposed that this N-terminal subdomain is an intermediate in the folding of Trp1p [26–30]. In agreement with these studies, all of the selected split-Trp pairs that spontaneously assemble into a functional protein possess relatively large N-terminal fragments, incorporating at least the first five (β/α)-motives. This observation suggests that a spontaneous assembly of Trp1p fragments depends on the presence of a folded N-terminal domain and that the location of the fragmentation site reflects the folding pathway of the natural protein. Shorter N-terminal fragments, such as $^{44}N_{trp}$ and $^{53}N_{trp}$, might not fold independently, and the chances to spontaneously reconstitute active protein from unfolded fragments without induced proximity would be greatly diminished. It should also be noted that most of the isolated split-Trp pairs that reassemble spontaneously consist of Trp1p fragments that overlap for at least 16 residues. This overlap prevents us from exactly localizing the fragmentation site from the sequence data (Figure 2). An exception is split-Trp^{135}, where, according to the structure of tPRAI, the fragmentation site is located in a loop at the N-terminal side of the $(\beta/\alpha)_8$-barrel [25].

Detection of Membrane Protein Interactions Using Split-Trp Sensors

An important application for new split-protein sensors will lie in the detection and characterization of protein-protein interactions occurring at the membranes of intracellular organelles. To test whether the split-Trp system operates at the membrane, we attached the interaction-dependent split-Trp pairs to the membrane proteins Sec62p and Sec63p [31–33]. Sec62p and Sec63p directly bind to each other and are part of the heptameric Sec complex that is responsible for translocating proteins posttranslationally across the membrane of the endoplasmic reticulum (ER) (Figure 5A) [31–33]. *SEC62* was fused to the 3′ end of the N-terminal fragment of the four split-Trp systems, allowing for the expression of $^{44}N_{trp}$-Sec62p, $^{53}N_{trp}$-Sec62p, $^{187}N_{trp}$-Sec62p, and $^{204}N_{trp}$-Sec62p. *SEC63* was fused to the 5′ end of the corresponding C-terminal fragments, allowing for the expression of Sec63p-$^{44}C_{trp}$, Sec63p-$^{53}C_{trp}$, Sec63p-$^{187}C_{trp}$, and Sec63p-$^{204}C_{trp}$.

To monitor the interaction between Sec62p and Sec63p, *trp1* yeast strains expressing pairs of matching N_{trp}-Sec62p and Sec63p-C_{trp} fusion proteins were spotted on selective plates lacking tryptophan (Figure 6). Strains coexpressing $^{44}N_{trp}$-Sec62p/Sec63p-$^{44}C_{trp,}$ $^{187}N_{trp}$-Sec62p/Sec63p-$^{187}C_{trp}$, and $^{204}N_{trp}$-Sec62p/Sec63p-$^{204}C_{trp}$ were able to grow on plates lacking tryptophan at 23°C but not at 30°C. Only small colonies were detected after 7 days for $^{187}N_{trp}$-Sec62p/Sec63p-$^{187}C_{trp}$ and after 10 days for $^{204}N_{trp}$-Sec62p/Sec63p-$^{204}C_{trp}$, whereas strains coexpressing $^{44}N_{trp}$-Sec62p/Sec63p-$^{44}C_{trp}$ grew significantly faster. No growth at all was observed for strains expressing $^{53}N_{trp}$-Sec62p/Sec63p-$^{53}C_{trp}$. To verify that the observed complementation of tryptophan auxotrophy is a result of the interaction between the Sec62p and Sec63p moieties of the fusion proteins, we fused the C-terminal fragments of split-Trp^{44} and split-Trp^{187} to the cytoplasmic site of Ste14p (Figure 5B). Ste14p is a membrane protein of the ER that is known to interact with neither Sec62p nor Sec63p (Figure 5B) [5]. No growth on plates lacking tryptophan was observed when matching pairs of Sec62p and Ste14p fusion proteins were coexpressed at 23°C or 30°C for 10 days (Figure 6). The cellular amount of Ste14p-$^{44}C_{trp}$ is roughly 2- to 3-fold lower than the amount of Sec63p-$^{44}C_{trp}$ as determined by Western blotting (data not shown). Since this relatively small effect cannot account for the clear growth difference between the strains expressing either $^{44}N_{trp}$-Sec62p/Sec63p-$^{44}C_{trp}$ or $^{44}N_{trp}$-Sec62p/Ste14p-$^{44}C_{trp}$, we conclude that the $^{44}N_{trp}$-Sec62p/ Sec63p-$^{44}C_{trp}$ interaction signal is specific.

Our data demonstrate that, in particular, split-Trp^{44} is well suited for the detection of protein-protein interac-

(B) Structure of tPRAI with the substrate analog 1-(O-carboxyphenylamino)-1-deoxy-D-ribulose-5-phosphate (rCdRP) [37]. Residues corresponding to the cleavage sites found in the circular permutated variants of split-Trp are highlighted (split-Trp^{44}↔tPRAI^{34}, split-Trp^{53}↔tPRAI^{43}, split-Trp^{187}↔tPRAI^{166}, split-Trp^{204}↔tPRAI^{183}). Residues shown in yellow correspond to the amino acids deleted in split-Trp^{204}.

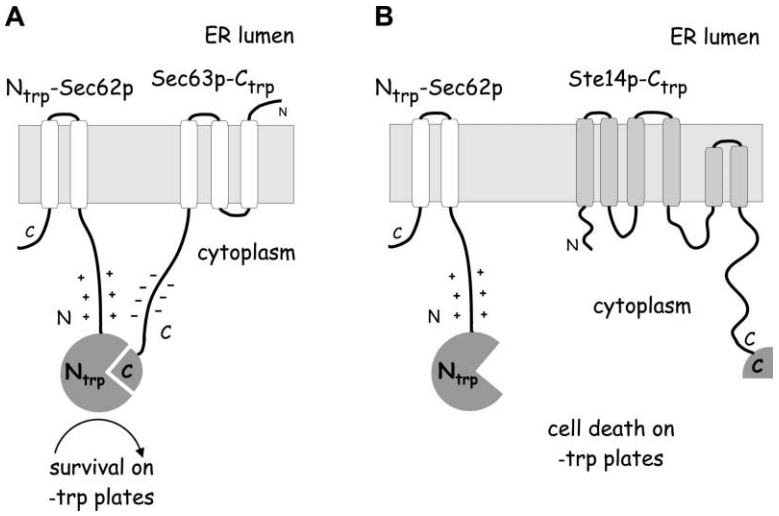

Figure 5. Analysis of the Interaction between Sec62p and Sec63p Using the Split-Trp System

(A) N_{trp} is fused to the N terminus of Sec62p, and C_{trp} is fused to the C terminus of Sec63p, resulting in N_{trp}-Sec62p and Sec63p-C_{trp}, respectively. The linker between the cytosolic domains of Sec62p and Sec63p and the corresponding Trp1p fragments consists of six residues. The known interaction between the positively charged cytosolic N-terminal domain of Sec62p and the negatively charged C-terminal tail of Sec63p should lead to the reconstitution of active Trp1p and complementation of tryptophan auxotrophy [33].

(B) Coexpression of N_{trp}-Sec62p with Ste14p-C_{trp}, a further membrane protein of the ER, which does not interact with Sec62p, should not lead to the formation of a functional Trp1p and the complementation of tryptophan auxotrophy [5].

tions between membrane proteins. Interestingly, yeast cells coexpressing $^{44}N_{trp}$-Sec62p and Sec63p-$^{44}C_{trp}$ require lower growth temperatures for the complementation of tryptophan auxotrophy than the cells expressing the corresponding C1 and C2 coiled-coil fusions. This effect might be due to a more favorable orientation of the N- and C-terminal Trp1p fragments in the antiparallel coiled coil than in the Sec62p/ Sec63p complex.

In conclusion, we have used directed evolution to convert N-(5′-phosphoribosyl)-anthranilate isomerase into a split-protein sensor. In coupling the interaction of cytosolic and membrane proteins to a simple growth assay, the split-Trp system possesses all of the necessary features to complement already existing systems to measure and screen for new protein interactions. Future experiments will focus on the use of split-Trp in identifying partners of medically relevant targets. Furthermore, the evolutionary approach introduced here should also be applicable to other enzymes. By generating novel split-protein sensors that are based on proteins functioning in the matrix of the mitochondrium, the peroxisome, or the lumen of the secretory path, our approach might help to overcome the lack of techniques to measure protein interactions in the interior of these organelles. Finally, the analysis of the different split-Trp pairs that either spontaneously assemble into a functional $(\beta/\alpha)_8$-barrel or need to be fused to interacting proteins to yield folded protein supports the hypothesis

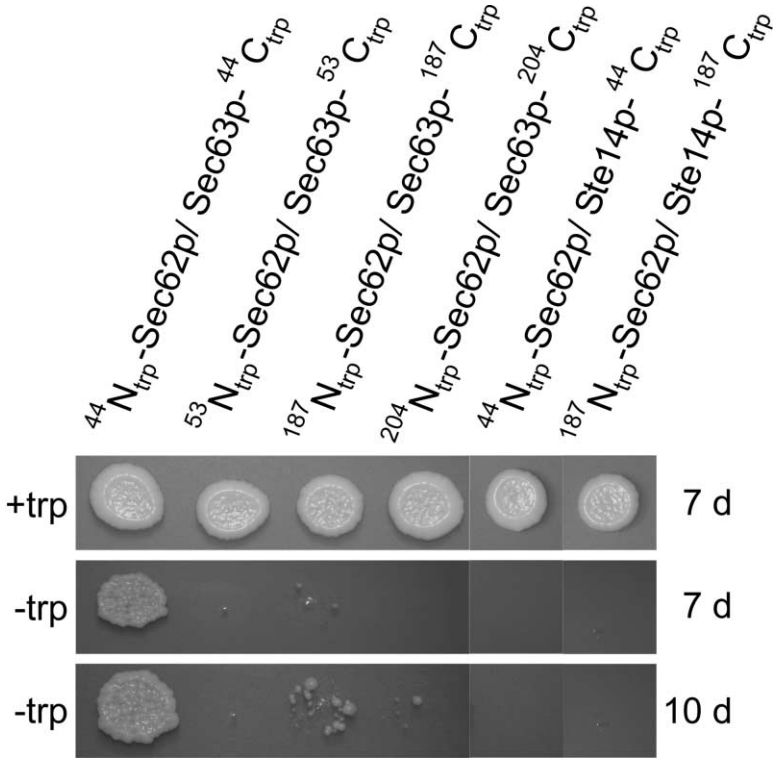

Figure 6. Split-Trp Interaction Assay of Sec62p and Sec63p

A colony of EGY48 cells coexpressing N_{trp}-Sec62p with Sec63p-C_{trp} or Ste14p-C_{trp} was suspended in 1 ml water, and 5 μl was spotted on copper-containing medium with or without tryptophan (trp). Cells coexpressing $^{44}N_{trp}$-Sec62p/Sec63p-$^{44}C_{trp}$ complement tryptophan auxotrophy, as indicated by their growth after 4 days at 23°C. Large colonies were visible after 7 days of incubation, whereas only small colonies were observed for cells expressing $^{187}N_{trp}$-Sec62p/Sec63p-$^{187}C_{trp}$. No or only very small colonies were observed for cells coexpressing $^{53}N_{trp}$-Sec62p/Sec63p-$^{53}C_{trp}$ or $^{204}N_{trp}$-Sec62p/Sec63p-$^{204}C_{trp}$, respectively. No growth was observed for cells coexpressing $^{44}N_{trp}$-Sec62p/Ste14p-$^{44}C_{trp}$ or $^{187}N_{trp}$-Sec62p/Ste14p-$^{187}C_{trp}$ even after 10 days of incubation at 23°C.

that a large N-terminal subdomain of Trp1p is an important intermediate in the folding of the $(\beta/\alpha)_8$- barrel.

Significance

Most biological processes are controlled by protein-protein interactions, and split-protein sensors have become one of the few available tools for the characterization and identification of protein-protein interactions in living cells. Here, we introduce a combinatorial approach for the generation of new split-protein sensors and apply it to the $(\beta/\alpha)_8$-barrel enzyme N-(5′-phosphoribosyl)-anthranilate isomerase Trp1p from *Saccharomyces cerevisiae*. These so-called split-Trp protein sensors were capable of monitoring the interactions of pairs of cytosolic and membrane proteins. The direct read-out through complementation of tryptophan auxotrophy qualifies the split-Trp system for high-throughput applications in yeast and bacteria. In addition, the introduced combinatorial approach should be able to generate split-protein sensors of almost any protein, thereby yielding tailor-made sensors for different applications.

Experimental Procedures

Chemicals, yeast nitrogen base, and amino acids were purchased from Fluka AG or Sigma-Aldrich AG. Enzymes for recombinant DNA work were purchased from MBI Fermentas or New England Biolabs. Antibodies were purchased from Babco (monoclonal anti-HA antibody) and Sigma-Aldrich AG (anti-mouse-HRP antibody conjugate). Microcon PCR columns were purchased from Millipore. Oligonucleotides were ordered from Microsynth GmbH, Switzerland.

Identification of Fragmentation Sites in Trp1p

Using PCR, the *TRP1* gene of *Saccharomyces cerevisiae* was rearranged to start with residue 63. The former start codon was fused to the stop codon via a linker sequence containing a unique AvrII restriction site (Figure 1). This rearrangement was performed to avoid unwanted isolation of wild-type gene in the subsequent selections. At the same time, a HindIII restriction site was introduced via the PCR primers at the newly generated N and C termini. The rearranged gene was first inserted into a high-copy plasmid and, after amplification of the vector DNA, cut out with HindIII. The rearranged gene was then incubated with T4 DNA ligase at 16°C for 14 hr at a DNA concentration of 0.14 mg/ml, leading to the formation of circular DNA as well as dimers and higher oligomers. After inhibition of the ligase at 65°C for 20 min and desalting of the solution using a microcon PCR column, the ligation products were incubated with DNaseI (~1.2 units/mg DNA) in 50 mM Tris-HCl (pH 7.5), 1 mM $MnCl_2$ at 25°C for 6 min. The activity of the DNaseI was determined immediately before the digestion in small test reactions. The DNaseI reaction was quenched by phenol extraction and ethanol precipitation. After incubation of the digested DNA with T4 DNA ligase and T4 polymerase to repair nicks, gaps, and to flush the ends of the fragments, DNA fragments corresponding to the size of the original gene were isolated by gel electrophoresis. These fragments were ligated into a pRS316-based yeast expression vector that was cleaved with HpaI and dephosphorylated [34]. In the resulting vector, the gene encoding the C-terminal half of Trp1p is fused to a gene encoding for a Flag tag, a polypeptide C1, and a five-residue linker sequence, and it is expressed under the control of the P_{CUP1}-promoter. The gene coding for the N-terminal half of Trp1p is fused to a gene encoding for a six-residue linker sequence, a polypeptide C2, and a HA tag. It should be noted that due to the blunt-end cloning step, the majority of the clones are out of frame with one or both of the polypeptides that form the coiled coil or inserted in a wrong orientation into the plasmid. C1 and C2 are two peptides that associate into an antiparallel coiled coil [22]. The sequences of the peptides C1 and C2, including epitope tag and linker, are as follows: C1, MDYKDESGQALEKELAQNEWELQALEKELAQLEKEL QAGSGSG; C2, GGSGSGQALKKKLAQLKWKLQALKKKNAQLKKKL QAGSYPYDVPDYAAFL.

After transformation in XL1Blue, resulting in a library of about 3×10^4 independent clones, bacteria were scratched from the plate, and plasmids were isolated and linearized with AvrII. To insert a terminator for the C-terminal fragment and a promoter for the N-terminal fragment, a DNA fragment was constructed by PCR consisting of the *CYC1* terminator, a geneticin resistance gene, the P_{GAL1}-promoter, and flanking regions of about 50 base pairs at the 5′ and 3′ ends homologous to the original N and C termini of Trp1p. The linearized vector (0.3 μg) and the PCR fragment (3 μg) were then cotransformed in chemically competent EGY48 (MATα *ura3 trp1 his3* 6*lex*Aop-*LEU2*) and plated on medium lacking uracil but containing geneticin (500 μg/ml) to select for the insertion of the PCR fragment into the linearized vector through homologous recombination. The homologous recombination also suppressed the predominant isolation of *TRP1* genes that were cut near the original N or C termini. After 3 days of incubation at 30°C, approximately 1600 colonies were isolated and subsequently replica plated on plates lacking uracil and tryptophan but containing geneticin (250 μg/ ml), galactose (2%), and $CuSO_4$ (0.1 mM). After replica plating, 45 colonies were able to complement tryptophan auxotrophy. Approximately half of those 45 colonies required the presence of galactose and $CuSO_4$ to grow on plates lacking tryptophan, and 11 of these clones were then analyzed by DNA sequencing (Figure 2). Six of the eleven clones were out of frame with C2. These frame shifts resulted in the replacement of C2 in split-Trp^{135} and split-Trp^{170} with a peptide of 66 residues possessing the sequence TVDLDQVRHLRRSWRSL SGNCKLLRRRMPSLRRSSRLEVTHTMFQITLHFYKSTSRGGPVP SFCSL and in split-Trp^{180}, split-Trp^{198}, split-Trp^{203}, and split-Trp^{204b} with a peptide of 10 residues possessing the sequence (E/Q)RWIW IRSGT. In Split-Trp^{44}, the mutation Gly8Cys was introduced during the fragmentation procedure. However, the influence of this mutation seems to be of minor importance, as the deletion of the first ten amino acids still allowed split-Trp^{77} to complement tryptophan auxotrophy (Figures 2 and 3). For split-Trp^{44}, split-Trp^{53}, split-Trp^{187}, split-Trp^{204}, and split-Trp^{77}, the sequence encoding N_{trp}-C2 was deleted from the plasmid using BglII and SalI and replaced with a PCR fragment encoding only the corresponding N_{trp} fragment. The resulting constructs were controlled by sequencing and retransformed into EGY48.

Construction and Characterization of Membrane Protein Fusions

The gene of *SEC62* was amplified by PCR from yeast EGY48 genomic DNA and combined by overlap extension PCR with the N-terminal fragments of split-Trp^{44}, split-Trp^{53}, split-Trp^{187}, and split-Trp^{204}, yielding $^{44}N_{trp}$-*SEC62*, $^{53}N_{trp}$-*SEC62*, $^{187}N_{trp}$-*SEC62*, and $^{204}N_{trp}$-*SEC62* [35]. The four N_{trp}-*SEC62* PCR products were isolated by gel electrophoresis and ligated in a pRS315-derived expression vector (*LEU2*) under the control of the P_{CUP1}-promoter [34]. The genes of *SEC63* and *STE14* were amplified by PCR from yeast EGY48 genomic DNA and combined by overlap extension PCR with the DNA coding for the C-terminal fragments of split-Trp^{44}, split-Trp^{53}, split-Trp^{187}, and split-Trp^{204}, yielding *SEC63*-$^{44}C_{trp}$, *SEC63*-$^{53}C_{trp}$, *SEC63*-$^{187}C_{trp}$, *SEC63*-$^{204}C_{trp}$, *STE14*-$^{44}C_{trp}$, and *STE14*-$^{187}C_{trp}$ [5, 35, 36]. The different *SEC63*-C_{trp} and *STE14*-C_{trp} PCR products were isolated by gel electrophoresis and ligated into a pRS316-derived vector (*URA3*) under the control of the P_{CUP1}-promoter [34]. All Sec63p and Ste14p fusions contained a HA tag fused to the C terminus of Trp1p. The linker between the cytosolic domains of Sec62p, Sec63p, and Ste14p and the corresponding Trp1p fragments consists of six residues (GGSGSG). Functionality of the N_{trp}-Sec62p fusion proteins was confirmed by complementation of the temperature-sensitive yeast strain RSY529 (MATα his4 leu2-3,112 ura3-52 sec62-1) [33]. Cells expressing the N_{trp}-Sec62p fusion proteins were able to grow at the permissive temperature of 37°C, whereas control cells not expressing a functional copy of Sec62p did not grow. Expression of Sec63p and Ste14p fusion proteins was verified by immunoblotting using antibodies against the HA tag.

For the growth assays, EGY48 yeast cells were cotransformed

with the plasmids encoding matching pairs of N_{trp}-Sec62p and Sec63p-C_{trp} or Ste14p-C_{trp} fusion proteins. A colony was suspended in 1 ml water, and 5 μl was spotted onto selective medium lacking leucine, tryptophan, and uracil but containing 0.1 mM $CuSO_4$ to induce protein expression from the P_{CUP1}-promoter. The plates were incubated at 23°C and 30°C for 4–10 days to observe the growth behavior of the different constructs.

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