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Protein interaction platforms: visualization of interacting proteins in yeast

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Abstract

Here we describe the protein interaction platform assay, a method for identifying interacting proteins in *Saccharomyces cerevisiae*. This assay relies on the reovirus scaffolding protein μ NS, which forms large focal inclusions in living cells. When a query protein is fused to μ NS and potential interaction partners are fused to a fluorescent reporter, interactors can be identified by screening for yeast that display fluorescent foci.

A variety of methods have been developed to screen for interacting proteins in living cells, including yeast two-hybrid (Y2H) and protein-fragment complementation assays^{1,2}. We recently demonstrated a complementary approach: the protein interaction platform (PIP) assay, in which we generate inclusions that serve as platforms for assembling interacting proteins in living mammalian cells. Here, we investigated whether this assay could be adapted to the yeast *S. cerevisiae*, in which (unlike in mammalian systems) plasmids for expressing proteins are readily maintained.

PIP relies on the reovirus scaffolding protein μ NS to form distinctively large focal cytoplasmic inclusions in mammalian cells^{3,4}. The C-terminal one-third of μ NS (μ NS residues 471–721) formed inclusions when expressed in *S. cerevisiae* (Fig. 1a). These inclusions did not perturb yeast growth, and mutations in the putative zinc-hook motif of μ NS that disrupt the formation of platforms in mammalian cells⁵ also disrupted their formation in yeast (Fig. 1b).

The basic design of PIP as adapted to yeast involved fusing one protein (A) to μ NS and a second protein (B) to GFP, so that if A and B interact, fluorescent green inclusions are visible in individual yeast cells (Fig. 1c,d). We created a series of recombination-based destination vectors using Gateway technology (Invitrogen) to facilitate the transfer of open reading frames such that they are fused to μ NS at their N termini and expressed from either a constitutive (*GPD*) or an inducible (*GALI*) yeast promoter. Many Gram-negative bacterial pathogens, including *Shigella* spp. and *Salmonella* spp., directly inject virulence proteins, called effectors, into host cells via specialized secretion systems. Some effectors require chaperones for delivery to the bacterial secretion apparatus. *Shigella flexneri* encodes four chaperones and at least 30

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AUTHOR CONTRIBUTIONS

A.M.S., M.F.M. and A.O.A. performed and analyzed the experiments. A.M.S., M.L.N. and C.F.L. designed the experiments. M.L.N. and C.F.L. wrote the manuscript.

effectors. Three of these chaperones (IpgA, IpgC and IpgE) bind to at most two effectors each^{6–8}, whereas the fourth (Spa15) was known to interact with at least five effectors at the start of this work^{9–11}. We used PIP to study the interactions between *S. flexneri* chaperones and effectors.

We first tested whether PIP could detect known interactions between the *S. flexneri* effectors IpaB and IpaC and their cognate chaperone IpgC (ref. 12). Fluorescent platforms were visualized with co-expression of either effector with IpgC, regardless of which protein was fused to μ NS (Fig. 2a), but not with co-expression with the other chaperones (Supplementary Fig. 1a), and regardless of whether expression occurred from a constitutive or inducible promoter (Supplementary Fig. 1b). To confirm that IpaB and IpaC colocalized with IpgC in the inclusions, we co-expressed μ NS-IpgC fused to CFP with either IpaB or IpaC fused to YFP. As expected, we observed platforms that contained both fluorescent fusions (Supplementary Fig. 2b).

We next tested for interactions between the *S. flexneri* effectors IcsB, IpgD and IpgB2 (ref. 13), which are toxic to yeast, and their respective chaperones IpgA, IpgE and Spa15. An interaction between IpgD and IpgE was observed regardless of which protein was fused to μ NS, whereas an interaction between IcsB and IpgA was seen only when IcsB was fused to GFP and IpgA to μ NS (Fig. 2b). In the case of IpgB2, a severely toxic effector¹³, expression of the GFP-fused proteins was detected poorly, if at all, thus precluding the detection of fluorescent inclusions. Nevertheless, we were able to detect an interaction between a nontoxic mutant of IpgB2 (IpgB2 W62A; ref. 14) and Spa15.

To investigate the general applicability of PIP to detect inter-actions with proteins that are toxic to yeast, we identified three *Salmonella typhimurium* effectors (SopE1, SopE2 and SigD) whose expression markedly inhibited yeast growth (Supplementary Fig. 2). The cognate chaperone for each of these effectors is known^{15,16}. We detected all three of these chaperone-effector interactions via PIP (Supplementary Fig. 3). Thus, PIP is applicable to study interactions involving toxic proteins as long as the toxicity does not prevent detection of the fluorescent proteins.

We next investigated whether PIP could detect interactions between each of the four *S. flexneri* chaperones and the remaining 19 effectors encoded on the *S. flexneri* virulence plasmid. No new interactions with IpgA, IpgC or IpgE were detected (Supplementary Fig. 4), but Spa15 interacted with 9 of the 19 effectors (Fig. 3). In 6 of these cases we detected the interaction regardless of which protein was fused to μ NS. In most of the positive cases, the majority of the GFP-fused protein localized to the platforms; fluorescent signal in the cytosol was greatly diminished. In some cases, though, we observed a single small fluorescent focus in each cell while the majority of GFP-fused protein remained more broadly distributed, as when expressed on its own. This phenomenon occurred several times when a strong interaction for the chaperone-effector pair was observed in the complementary direction—for example, upon co-expression of μ NS-Spa15 with GFP-IpaH1.4 or GFP-IpgA with μ NS-IcsB, and upon co-expression of μ NS-IpgC or μ NS-IpgE with several of the GFP-effectors such as GFP-OspC2 and GFP-OspC3. We suspect that these single small foci represent weak interactions.

For 10 of the 19 *S. flexneri* effectors, we did not detect interactions with any of the 4 chaperones, irrespective of whether μ NS was fused at their N termini (which could sterically hinder chaperone binding) or whether YFP was fused at their C termini (Supplementary Fig. 5). Our inability to detect an interaction for these effectors, and for OspD2 and IpgB2 W62A, which only displayed an interaction when fused to GFP, was not due to an inability of these proteins to form μ NS-based inclusions. By testing each protein as a μ NS-CFP fusion, we found that, with the exception of IpaJ and OspD2, whose expression was undetectable, and OspG, which

did not form inclusions, all of these proteins formed μ NS-based inclusions when expressed in yeast (Supplementary Fig. 6).

To ascertain whether the protein interactions detected in PIP represented biologically functional interactions, we investigated which effectors were dependent on Spa15 for their secretion by the *S. flexneri* type III secretion system (Supplementary Fig. 7). In Δ *spa15* *S. flexneri* strains, we observed a complete loss of secretion of six of the ten effectors (OspB, OspC1, OspD1, OspD2, IpgB1 and IpgB2) and a moderate loss of secretion of three others (IpaA, OspC2 and OspC3) that interact with Spa15 in PIP (Supplementary Fig. 7). Endogenously expressed IpaA and OspC3 were previously observed to be absent from culture supernatants of a Δ *spa15* *S. flexneri* strain¹⁰. The leaky secretion that we observed with these three effectors is thus presumably due to their over-expression in our system. In the Δ *spa15* *S. flexneri* strain, we observed little if any decrease in secretion of IpaH1.4 (Supplementary Fig. 7), although it was seen to interact with Spa15 in PIP. There is previous evidence that at least one member of the IpaH family of effectors requires Spa15 for secretion¹¹. Thus, the weak Spa15-IpaH1.4 interaction that we detect may indeed represent a physiological one. The ten effectors that were not seen to interact with Spa15 in PIP did not have altered secretion in the Δ *spa15* *S. flexneri* strain (Supplementary Fig. 7). Thus, PIP is both sensitive and specific for detection of Spa15-interacting effectors.

We also tested the ability of the GAL4-based Y2H assay to detect interactions between the 200 *S. flexneri* chaperone-effector pairings tested with PIP. Not surprisingly, six toxic effectors inhibited growth in this assay; these effectors were not further studied in Y2H. Whereas PIP detected interactions between 14 effectors and their chaperones, Y2H detected only 10 such interactions, all of which overlapped with interactions detected in PIP (Supplementary Table 1). Furthermore, whereas PIP detected interactions in both directions in 9 of the 14 cases, Y2H detected the interaction in both directions in only 4 of the 10 cases.

PIP detected two chaperone-effector interactions involving non-toxic proteins that were missed by Y2H. In one of these cases (IpaB-IpgC), the proteins had been previously reported to interact in Y2H, but only when fragments of IpaB were used as bait¹². With two additional effector-chaperone pairs (IpaA-Spa15 and OspC3-Spa15), we were able to detect interactions in both directions in PIP, but in only one direction in Y2H. In both of these cases, a Y2H interaction had previously been reported in the complementary direction, but only when fragments of OspC3 or IpaA were studied¹⁰. The most likely explanation for the differences in detecting these three interactions is that in the case of Y2H, the configuration of interacting proteins must be such that the GAL4 domains are correctly juxtaposed to reconstitute a functional transcription factor. Thus, another advantage of PIP is that a positive readout requires only the recruitment of interacting proteins to the same cellular foci; the specific geometry of the interaction is likely to be less important.

Although PIP has yet to be adapted to select for interacting proteins, automated microscopy is increasingly available and could be used to screen for fluorescently tagged proteins whose localization is altered by a μ NS-fused query partner. This approach is especially feasible for proteome-wide screens in yeast, given the availability of yeast strains that express one of at least 75% of the endogenous open reading frames fused to GFP (ref. 17). Given the high conservation of proteins involved in basic cellular processes among all eukaryotes, the approach should provide insights into systems other than yeast.

Additional modifications to PIP could be made such that the readout is amenable to fluorescence resonance energy transfer and interactions detected via plate assay or by using fluorescenceactivated cell sorting. Although PIP has now been optimized for yeast, it was

originally devised for mammalian cells^{3,4} and should be easily adapted to a variety of cell types and organisms.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemethods/>.

ONLINE METHODS

Plasmids

With the exception of the previously described low copy plasmids that were used to express the GFP-*Shigella* effector fusions¹³, the majority of plasmids in this study were created via the Gateway (Invitrogen) site-specific recombination system¹⁸. To create *Shigella flexneri* entry clones, genes encoding each of the effectors and chaperones were first amplified by a nested polymerase chain reaction such that they were flanked by *attB* sites (Supplementary Table 2). In addition to an *attB* site, a Shine-Dalgarno sequence was introduced upstream of each open reading frame (ORF). We created open (lacking a stop codon) and closed versions of each *S. flexneri* ORF. The genes encoding wild-type and mutant alleles of μ NS were also amplified such that they were flanked by *attB* sites. The nested PCR involved the use of gene-specific primers in the first round of PCR. A universal 5' primer and gene-specific primers were used in the second round of PCR. The amplified genes were then introduced into pDNR223 to create Gateway entry vectors (Invitrogen) via BP reactions (Invitrogen). Each insert was sequence verified and subsequently transferred to a variety of Gateway destination vectors (Invitrogen) via LR reactions (Invitrogen). The *S. typhimurium* entry clones were provided by the Pathogen Functional Genomic Resource Center at the J. Craig Venter Institute.

The yeast GFP- μ NS (residues 471–721) expression plasmids were constructed by transferring genes encoding wild-type and mutant alleles of μ NS (residues 471–721) from pDNR223 into pAG415GAL-eGFP-*ccdB*. In order to create μ NS fusion proteins via the Gateway recombination system, the gene fragment encoding amino acids 471–721 of μ NS was PCR amplified such that it was flanked by *NheI* sites and was introduced into the *SpeI* sites of either pAG416GAL-*ccdB* or pAG415GPD-*ccdB*. Each of the designated *S. flexneri* ORFs (closed configuration) was then transferred from the pDNR223 clones into this destination vector. For the colocalization studies, IpaB and IpaC were fused to YFP at the amino termini by transferring each ORF to pAG416GAL-YFP-*ccdB*. In order to create CFP- μ NS fusion proteins, the genes encoding CFP and μ NS (residues 471–721) were “sewn” together via PCR and introduced into the *SpeI* site in pAG415GAL-*ccdB*. The *S. flexneri* carboxy YFP fusion protein expression plasmids were constructed by transferring each ORF in the open configuration into pAG413GAL-*ccdB*-YFP. All of the pAG yeast expression plasmids were generous gifts from S. Alberti and S. Lindquist (Massachusetts Institute of Technology)¹⁹ and are currently available through <http://www.addgene.org/>. The Y2H clones were also constructed via Gateway technology using BD and AD destination plasmids, which were gifts from D. Hill and M. Vidal (Dana Farber Cancer Institute, Harvard Medical School)²⁰.

The bacterial Flag-tagged clones were also made by Gateway recombination assays. In this case, pDSW206 (ref. 21) (ColE1 origin of replication, ampicillin resistance) was modified such that it had a Gateway cassette inserted into the *SmaI* site in its polylinker upstream of a 3× Flag tag inserted between the *XbaI* and *HindIII* sites. Genes encoding each of the *S. flexneri* effectors in the open configuration were transferred into this vector to create the IPTG-inducible Flag-tagged effectors. The *spa15* complementation plasmid was created by transferring *spa15* into pNG162 (p204promotor (IPTG-inducible), pSC101 origin of

replication, spectinomycin resistance)²² after transferring the Gateway cassette into pNG162 at the SmaI site in its polylinker.

PIP assays

Each of the yeast expression plasmids that encodes one of the fluorescent fusion proteins was transformed into S288C MAT α , and the plasmids encoding the μ NS fusion proteins were transformed into S288C MAT α . To create a complete matrix of the 152 chaperone-effector pairings, the strains that conditionally express each fluorescent fusion protein were mated with the strains that conditionally express each of the μ NS fusion proteins. This was conducted in a 96-well array format. Once the diploids were generated, each diploid strain was grown overnight in a sterile 96-well round bottom tray. Each well contained 200 μ l of synthetic complete medium lacking histidine and uracil (SC-HU) plus 2% (wt/vol) raffinose as a carbon source. In the morning, the plates were spun down, the 'old' medium was removed by aspiration and the yeast pellets were resuspended in 200 μ l of fresh (SC-HU 2% raffinose) medium. After 2 h, 15 μ l of each well was transferred to a fresh 96-well bottom tray that contained 140 μ l of SC-HU plus 2% raffinose and 2% galactose. Yeast were grown under these inducing conditions for 3–4 h, at which time they were transferred to a glass bottom 96-well plate and visualized using a Nikon TE300 microscope with Chroma Technology filters. Images were captured digitally using a black and white Sensys charge-coupled device (CCD) camera and IP LAB software (Scanalytics). Color figures were assembled by separately capturing signals with each of the appropriate filter sets and digitally pseudocoloring the images using Adobe Photoshop. Scale bars were inserted after the acquisition of the images. The bars represent approximately 2 μ m—half the length of a haploid yeast. The bars are consistently scaled throughout Figures 1–3.

Yeast growth assays

Growth phenotypes of yeast that conditionally express the *S. typhimurium* proteins were assayed in 96-well plates. Individual yeast transformants were inoculated into each well of a 96-well plate containing non-inducing selective synthetic medium supplemented with 4% (wt/vol) glucose. Saturated cultures were transferred to non-inducing selective synthetic solid medium, 2% (wt/vol) glucose using a 3.18-mm-diameter 96-pinner tool (V&P Scientific, Inc.) on a Biorobot 3000 (Qiagen). After one to two days, the yeast spots on the solid plates were transferred to fresh liquid 96-well plates using a 1.58-mm-diameter 96-pinner tool and incubated for 16–18 h to grow to an optical density at 600 nm (OD₆₀₀) of 0.3–0.4. The liquid cultures were next transferred with a 1.58-mm-diameter pinner to inducing selective synthetic liquid medium containing 4% (wt/vol) galactose in 96-well plates ("induction plates"). All incubations were done at 30 °C and in the absence of agitation. All of the amino acid mixes for the yeast synthetic dropout media were purchased from MP Biomedicals.

Y2H assays

The Y2H expression plasmids were introduced into MaV103 and MaV203, and the Y2H assays were performed in a 96-well format as previously described^{20,23}. In this case, the two-hybrid selection was conducted on medium lacking leucine, tryptophan and histidine, supplemented with 30, 40 or 50 mM 3-amino-1,2,4-triazole. Growth was scored after 3 d of growth at 30 °C.

S. flexneri deletion strains

The Δ *spa15* *S. flexneri* 2457T strain was constructed using the λ red recombination system²⁴. Deletion of *spa15* was limited to removing amino acids 4–71, as removal of the entire coding frame resulted in a severe inhibition of secretion, presumably due to polar effects on the downstream operon resulting from loss of translational coupling.

***S. flexneri* secretion assays**

The pDSW206-based plasmids encoding each of the IPTG-inducible Flag-tagged effectors were transformed into wild-type and *AspA15 S. flexneri* 2457T. To determine whether the Flag-tagged effectors were secreted by the *S. flexneri* strains, overnight cultures grown in TCS (trypticase soy) broth were back-diluted 1:100 into 2 ml of TCS broth. The diluted cultures were grown for 2 h until they reached an OD₆₀₀ of ~0.5. At this time 1 mM IPTG was added to the cultures. After another 30 min, the bacteria were resuspended in 2 ml of phosphate-buffered saline containing 10 µM Congo red (Sigma) and incubated for an additional 30 min. All incubations were conducted at 37 °C. After this incubation, the bacteria were centrifuged and the pellets were saved for the whole-cell lysates. The supernatant was then subjected to a second centrifugation step to ensure that few *S. flexneri* were present in the supernatant fraction. Proteins present in the second supernatant were precipitated by the addition of TCA (trichloroacetate). Proteins were analyzed by western blot analyses with antibodies to Flag (Sigma), antibodies to isocitrate dehydrogenase and to IcsA (the latter two were generous gifts from the laboratory of M. Goldberg at Massachusetts General Hospital, Harvard Medical School).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Development of PIP assay in yeast. (a,b) Yeast cells expressing wild-type (a) or mutant (b) alleles of GFP-μNS at 3–4 h post-induction of fusion protein expression. The left panel in a is a fluorescence image; the right panel shows simultaneous imaging of phase and fluorescence illumination. (c,d) Schematic and representative images of PIP. When fused to μNS, protein A is recruited to large platforms. If protein B does not interact with protein A (c), then the localization pattern of protein B is unaltered in the presence of μNS–protein A. Alternatively, if protein B interacts with protein A (d), then a protein B–GFP fusion appears as fluorescent foci. Scale bars are approximately 2 μm.

Figure 2.

Detection of previously characterized chaperone-effector interactions via PIP. Each image is representative of yeast expressing designated effectors (e) and chaperones (c). The effectors and chaperones are fused to either GFP or μNS as designated. (—) indicates an absence of chaperones or effectors in these cells. **(a)** Yeast expressing nontoxic effectors IpaB and IpaC with or without their cognate chaperone IpgC. The image of GFP-IpgC (alone) is duplicated. **(b)** Yeast expressing toxic effectors and their respective chaperones, as indicated. IpgB2* refers to the mutant protein IpgB2 W62A. Images in the first three rows in **b** represent 3-s exposures normalized to display pixels visible over a defined linear range, thus demonstrating relative levels of expression of these effectors. Scale bars are approximately 2 μm.

Figure 3.

S. flexneri effector-chaperone interactions by PIP. Yeast co-expressing the designated effectors and chaperones were visualized at 3–4 h post-induction of fusion protein expression. The first column shows yeast expressing each effector fused to GFP. The second through fifth columns show yeast co-expressing each effector fused to GFP along with each of the four chaperones fused to μ NS. The last column shows yeast co-expressing the effectors fused to μ NS and Spa15 fused to GFP. Scale bars are approximately 2 μ m.