New Fast BiFC Plasmid Assay System for *in Vivo* Protein-Protein Interactions

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Key Words

Protein-Protein Interaction Assay Method • BiFC • POZ-domain

a highly efficient, sensitive and specific assay method for protein-protein interaction *in vivo*.

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Abstract

In this age of massive genetic and protein information, a fast and reliable method of studying *in vivo* protein-protein interactions is necessary. We have developed a novel system that can overcome limitations of existing assay methods. This new method adopts two existing systems for fast analysis of diverse protein-protein interactions. For rapid, large-scale cloning, we adopted the Gateway system and developed novel destination vectors containing YFP N-terminus (YN) or YFP C-terminus (YC) to visualize protein-protein interactions *in vivo* using bimolecular fluorescence complementation (BiFC). Using this system, we investigated molecular interactions among the three POZ-domain regulatory proteins mAPM-1, LRF, KLHL10 that belong to a subgroup of human POZ-domain proteins, and showed that the POZ-domains of mAPM-1, LRF and KLHL10 could form both homodimers and heterodimers. This new method is

Introduction

Cells carry out diverse biological functions through complex protein-protein interactions. Comprehensive determination of protein-protein interactions is important because it provides a framework for understanding both biological regulation through protein-protein interaction and the functional roles of many regulatory gene products. Various methodologies for analyzing protein-protein interactions have been developed; however, they have several limitations when studying large numbers of protein-protein interactions.

Most existing interaction assay methods have limitations with respect to cloning and protein expression. Cloning of genes is a time-consuming process and expression of functionally active recombinant proteins in a heterologous host, such as *Escherichia coli* or *Saccharomyces cerevisiae*, is often difficult. *In vitro* protein-protein assays, such as affinity chromatography,

pull-down assay, and protein chip can generate false-positive signal by electrostatic interactions, although *in vitro* protein-protein assay is often required [1, 2].

The yeast two-hybrid system is widely used as *in vivo* assay method [3]. However, if the interacting proteins are corepressor, repressor or contain a repressor domain, the interaction will not activate transcription and expression of the nutrition marker genes, and the molecular interaction can not be detected although the proteins interact with each other [4, 5]. Detection of weak interactions can also be difficult because of poor growth of yeast. Immunoprecipitation, another popular *in vivo* protein-protein interaction assay, requires highly specific antibodies that are often not available, and non-specific aggregations or precipitates can generate false-positive results [1, 2]. To overcome these problems, we developed an assay method which takes advantage of the Gateway system and bimolecular fluorescence complementation (BiFC) assay system.

The Gateway system enables convenient and efficient cloning of many genes into various vectors without restriction endonucleases and ligase treatment [6, 7]. The recently developed techniques of BiFC enable visualization of direct protein-protein interaction in intact cell. The BiFC assay is based on fluorescence generated by reconstitution of non-fluorescent molecules when they are brought together by specific interaction of fused proteins [8, 9].

The BTB/POZ (broad complex, tramtrack and bric-a-brac/poxvirus and zinc finger) domain is an evolutionarily conserved protein-protein interaction domain that is found at the N-terminus of many cellular regulators important in transcription, signal transduction, development, differentiation and oncogenesis [10-15]. Although some POZ-domain proteins are known to interact with other POZ-domains and non-POZ-domain proteins such as PLZF, Bcl-6, mSin3A, SMRT, N-CoR, HDAC, Sp1, and Cullin3 ubiquitin ligase [5, 15-18], their interaction partnerships and function of the interactions are largely unknown.

Using the new protein-protein interaction assay system, we analyzed the protein-protein interactions among the POZ-domain subgroup composed of mAPM-1 [19], LRF [20] and KLHL10 [21] and demonstrated that the POZ-domains formed both homodimers and heterodimers *in vivo*. The assay system is a highly efficient and sensitive compared with conventional protein-protein assay methods, and we were able to analyze the *in vivo* protein-protein interactions among the POZ-domains in 2-3 days.

Materials and Methods

Construction of plasmids pFPIA-YN, YC, and various pFPIA-POZ-YN or pFPIA-POZ-YC protein-protein interaction assay plasmids

pBiFC-YN, pBiFC-YC, pBiFC Jun-YN and pBiFC Fos-YC were kindly provided by Dr. Kerppola (University of Michigan, MI, USA). The reading frame A (RA) cassette (Invitrogen, CA, USA) was inserted into pBiFC-YFP N-terminus (a.a. 1-154, HindIII-EcoRV) and pBiFC-YFPC-terminus (a.a. 155-238, EcoRI-XhoI). We introduced cDNA fragments encoding nuclear localization signal of SV40 large T antigen (NLS, a.a. PPKRKV) [22] were introduced into the N-terminus of the above two vectors. FLAG Tag (a.a. YPYDVPDYA) and HA Tag (a.a. DYFLDDDK) were introduced into the C-terminus of YN and YC, respectively. The pFPIA system has new linker (YN, DPAFLYKVVVISVPVDSRGRSRIAT; YC, DPAFLYKVVVISRGTAARPACKIPNDLKQKVMNH) added downstream of NLS and in front of DNA region encoding YN or YC. Oligonucleotide primers used for PCR from BiFC-YN and BiFC-YC are as follows: pFPIA-YN forward, 5'-GAT CCT CGA GAC CAT GGC TCC TCC AAA AAA GAA G AGA AAG GTA CTT GCG GCC GCG AAT TCA-3'; pFPIA-YN reverse, 5'-GAT CGG GCC CTC ACT TGT CGT CAT CGT CTT TGT AGT CGG CCA TGA TAT AGA CGT T-3'; pFPIA-YC forward, 5'-GAT CAA GTT ACC ATG GCT CCT CCA AAA AAG AAG AGA AAG GTA CTT ATG GCC ATG GAG GCC-3'; pFPIA-YC reverse, 5'-GAT CTC TAG ATC AAG CGT AAT CTG GAA CAT CGT ATG GGT ACT TGTA CAG C TCG TCC AT-3'. Purified NLS-RA-YN-FLAG and NLS-RA-YC-HA PCR products were cloned into pcDNA3.0 using XhoI-ApaI and HindIII-XbaI to generate pFPIA(NLS)-YN and pFPIA(NLS)-YC, respectively. The pFPIA(NLS) Δ (Cm^R-*ccdB*)-YN and pFPIA(NLS) Δ (Cm^R-*ccdB*)-YC, which lack Cm^R and *ccdB* but still retain *attR1* and *attR2* sequences of pFPIA(NLS)-YN and pFPIA(NLS)-YC were prepared by BP and LR reactions of the annealed oligonucleotides (forward, 5'-GGG GAC AAG TTT GTA CAA AAAAGC AGG CTA CCC AGC TTT CTT GTA CAAAGT GGT CCC C-3'; reverse, 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA GCC TGC TTT TTT GTA CAAACT TGT CCC C-3' with pFPIA(NLS)-YN and pFPIA(NLS)-YC. The pFPIA(NLS) Δ (Cm^R-*ccdB*)-YN and pFPIA(NLS) Δ (Cm^R-*ccdB*)-YC were used as the negative control in protein-protein interaction assays.

To study molecular interactions among the member of the POZ-domain subgroup, mAPM-1 POZ-domain (a.a. 1-124), LRF POZ-domain (a.a. 1-129) and KLHL10 POZ-domain (a.a. 1-134) were prepared as PCR products flanked with *attB* sites. PCR cycling conditions were as follows: 94°C for 5 min and 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 sec, and 72°C for 10 min. Following oligonucleotide sets were used for amplification. mAPM-1 POZ-domain expression clone forward, 5'-GGG ACA AGT TTG TAC AAA AAA GCA GGC TCT GAC AAT GAC ATT GAT GAG-3'; reverse 5'-GGG GAC CAC TTT GTA CAAGAAAGC TGG GTC GCC TCC GGG CTC CAT GAT-3'. LRF POZ-domain expression clone, forward 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC TGC CGG CGG

CGT GGA CGG C-3'; reverse 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CTG CCG GTC CAG GAG-3'. KLHL10 POZ-domain expression clone, forward 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC TGA GAG CAC CGC-3'; reverse 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC ACA CAG CTC CGA CTT GAG-3'. The entry clones were generated by BP reaction using *attR* flanked POZ-domain PCR products and pDONR221 (Invitrogen, CA, USA). The entry clone was selected based on kanamycin resistance (positive selection) and loss of chloramphenicol resistance (negative selection). Because chloramphenicol resistance gene in the donor vector was replaced with the gene of interest by recombination, it was removed as by-product. The loss of chloramphenicol resistance was confirmed by growth test on a chloramphenicol LB agar plate and used as a negative selection marker. After positive and negative selection, selected entry clones were used for the preparation of mammalian POZ-domain expression clones by LR reaction. The expression clone was positively selected by ampicillin resistance and negatively by chloramphenicol resistance. The POZ-domains were located between NLS and YN or YC, and the YN and YC had C-terminal FLAG or HA tags, respectively. All BP reaction and LR reaction were performed according to manufacturer's protocol using BP clonaseTM Enzyme Mix and LR clonaseTM Enzyme Mix (Gateway system, Invitrogen, CA, USA).

For mammalian two-hybrid assays, PCR amplified cDNA fragments encoding POZ_{mAPM-1}, POZ_{LRF}, and POZ_{KLHL10} were cloned into pCMX-Gal4 and pCMX-VP16 plasmids.

To prepare recombinant GST-fused POZ_{mAPM-1} and POZ_{KLHL10}, PCR amplified cDNA fragments were cloned into pGEX4T1 (Amersham Biosciences, NJ, USA) plasmid. pGEX4T3-POZ_{LRF} plasmid was previously described [17]. All plasmid constructs were verified by sequencing.

Analysis of amino acid sequences homology of human BTB/POZ domain proteins

Amino acid sequences of virtually all POZ-domains were obtained from the BTB/POZ-domain database (<http://xtal.uhnres.utoronto.ca/prive/btb.html>) and analyzed by MacVector 7.2 Clustal W alignment program (Accelrys, CA, USA). The multiple alignment results were used to construct a phylogenetic tree based on distances between each pair of sequences by successive clustering (data not shown). Divergences of each base pair or evolutionary distance from multiple alignment results were calculated, and a phylogenetic tree was constructed using the Neighbor-Joining method. Evolutionary distances were calculated by uncorrected p-distance.

Cell culture and mammalian two-hybrid assays

African green monkey kidney cells (CV-1) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/ml streptomycin and penicillin (Invitrogen, CA, USA). Cells were plated into 6-well tissue culture plates. After the cells had grown for 12 hrs, they were transiently transfected with the pCMX-Gal4-POZ-domain (0.4 µg), VP16-POZ-domains plasmid (0.4 µg), pG5-Luc (0.2 µg) and pCMV-lacZ (0.2 µg) using Lipofectamine PlusTM

reagent (Invitrogen, CA, USA) in OPTI-MEM (Invitrogen, CA, USA). After 3 hrs, the cells were supplied with DMEM-10% FBS and allowed to grow for additional 12 hrs. Cells were harvested and lysed in 1x reporter lysis buffer (Promega, WI, USA). Luciferase reporter assays were performed using luciferase assay reagent (Promega, WI, USA) on a luminometer (EG&G Berthold, Germany). Luciferase activities were normalized with β-galactosidase activity and the protein concentration of the cell extracts was determined by the Bradford method. The assays were repeated at least three times.

Analysis of protein-protein interactions in vivo

CV-1 cells were maintained as described above. Cells grown on a glass-bottom dish (MatTek corporation, MA, USA) were cotransfected with a total of 1.2 µg of YN (0.6 µg) and YC (0.6 µg) fused protein expression plasmids using Lipofectamine PlusTM reagent (Invitrogen, CA, USA). Fluorescence was determined after 24 hrs. All fluorescent image analysis of cells transfected with YFP-POZ-domain expression plasmids was performed using LSD 510 confocal microscope (Carl Zeiss, Germany).

In vitro protein-protein interaction assay

pGEX4T1POZ_{mAPM-1}, pGEX4T3POZ_{LRF}, and pGEX4T1POZ_{KLHL10} expression plasmids were transformed into *E. coli* BL21 (DE3). To prepare recombinant GST-POZ-domain fusion proteins, *E. coli* BL21 (DE3) was induced to express the fusion proteins by incubating for 4 hrs with 0.5 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) at 37°C. The harvested *E. coli* were resuspended in HEMG buffer (40 mM HEPES, pH7.9; 100 mM KCl, 0.2 mM EDTA, 5 mM MgCl₂, 0.1% Nonidet P-40, 10% glycerol, 1.5 mM dithiothreitol) supplemented with 1 tablet/50 ml of a protease inhibitor mixture (Roche, Germany) and lysed by sonication. The supernatant of the lysate was applied onto a Glutathione-agarose 4B bead affinity column (Pepton®, Taejeon, Korea). [³⁵S]-methionine labeled POZ-domains were prepared using TnT Quick-coupled Transcription/Translation System (Promega, WI, USA). The [³⁵S]-methionine labeled proteins were incubated with either agarose-bound GST or GST-POZ-domain proteins of mAPM-1, LRF, KLHL10. The agarose-protein complex was washed with cold HEMG buffer, and the bound proteins were resolved by 12% SDS-PAGE and analyzed by autoradiography.

Western blot assays

Untransfected CV-1 cells and CV-1 cells transfected with pcDNA3.0, pFPIA(NLS)Δ(Cm^R-*ccdB*)-YN, pFPIA(NLS)Δ(Cm^R-*ccdB*)-YC, pFPIA(NLS)-YN, pFPIA(NLS)-YC, and other pFPIA(NLS)POZ-YN or -YC plasmids were harvested after 24 hrs and lysed by sonication to obtain total cell lysate. The lysates were separated on a 15% SDS-polyacrylamide gel and analyzed by western blot analysis using either anti-HA antibody (SantaCruz Biotechnology Inc, CA, USA) or anti-FLAG antibody (Sigma, MI, USA). Protein was detected using HRP (horseradish peroxidase)-conjugated anti-mouse secondary antibody (SantaCruz Biotech, CA, USA) and ECL reagents (Amersham Biosciences, NJ, USA).

Site-directed mutagenesis of ATG codon of NLS and generation of new ATG codon downstream of disrupted NLS

To prepare the YN or YC fusion expression constructs which do not have NLS at N-terminal, site-direct mutagenesis were performed. pFPIA(NLS) Δ (Cm^R-*ccdB*)-YN, pFPIA(NLS) Δ (Cm^R-*ccdB*)-YC, pFPIA-POZ_{LRF}-YN and pFPIA-POZ_{LRF}-YC vectors were mutated with following oligonucleotide primer sets to disrupt translation start ATG codon of NLS and to generate a new ATG at the downstream of disrupted NLS. FPIA(NLS)-YN, 1st round mutagenesis, YN forward, 5'-GCG GCC GCT CGAGACCTTGGC TCC TCC AAA AAA GA-3'; YN reverse, 5'-TCT TTT TTG GAG GAG CCA AGG TCT CGA GCG GCC GC-3'. 2nd round mutagenesis, YN forward, 5'-TCC AAA AAA GAA GAG AAT GGT ACT TGC GGC CGC GA-3'; YN reverse, 5'-TCG CGG CCG CA AGT A CCA TTC TCT TCT TTT TTG GA-3'. For FPIA(NLS)-YC, 1st round mutagenesis, YC forward, 5'-GAG ACC CAA GCT TAC CTT GGC TCC TCC AAA AAA GA-3'; YC reverse, 5'-TCT TTT TTG GAG GAG CCA AGG TAA GCT TGG GTC TC-3'. 2nd round mutagenesis, YC forward, 5'-AAA GAA GAG AAA GGT AAT TAT GGC CAT GGA GGC CC-3'; YC reverse, 5'-GGG CCT CCA TGG CCA TAA TTA CCT TTC TCT TCT TT-3'. The first mutagenesis changed ACCATG of NLS to ACCTTG, and second mutagenesis changed AGAAAG to AGAATG in YN-fusion expression constructs and CTTATG to ATTATG in YC-fusion constructs. After two round of mutagenesis, the translation start codon ATG is located downstream of disrupted NLS. The PCR mixture has 1 μ l of Ultra PFU Taq-polymerase (Stratagene, CA, USA), 5 μ l of 10x Ultra PFU Taq buffer, 100 ng of plasmid template, 125 ng of sense and anti-sense oligonucleotide primers and 4 μ l of dNTPs (2.5 mM). PCR cycling conditions were as follows: 94°C for 30 sec and 18 cycles of 94°C for 30 sec, 55°C for 1 min, 68°C for 8 min, and final extension reaction at 68°C for 10 min. After finishing the PCR, 20 μ l of the samples are digested with DpnI at 37°C for 1 hr and 10 μ l of the samples were transformed into *E. coli* DB3.1 to prepare mutated YN or YC fusion expression constructs plasmids.

Results

New protein-protein interaction assay plasmid vectors, pFPIA-YN and pFPIA-YC

We generated a new plasmid set that enables high speed cloning and visualization of protein-protein interactions *in vivo* based on the Gateway system and BiFC (Fig. 1). These plasmids were designated pFPIA: plasmids for fast protein-protein interaction assay. They commonly had a NLS, reading frame A, and FLAG or HA tags. The reading frame A contains *attR* sites, the *ccdB* gene and chloramphenicol resistance gene. High speed cloning is possible through *att* sites-specific recombination without treatment with restriction enzymes and DNA ligase. The *ccdB* gene products inhibit *E. coli*

Fig. 1. Diagram of the protein-protein interaction assay pFPIA plasmids, pFPIA(NLS)-YN and pFPIA(NLS)-YC. pFPIA plasmids have the unique features of both Gateway plasmids and BiFC plasmids incorporated into pcDNA3.0. To facilitate cloning of gene of interest downstream of nuclear localization sequence (NLS), *attR1*-Cm^R-*ccdB*-*attR2* DNA fragment amplified from pBiFC-ReadingframeA-YN or pBiFC-ReadingframeA-YC plasmid was placed in front of DNA fragment encoding YFP-N-terminus or C-terminus DNA fragment. YFP N-terminus (a.a. 1-154) and C-terminus (a.a.155-238) was tagged with FLAG or HA epitope, respectively. pFPIA, Plasmid for Fast Protein Interaction Assay; T7, T7 polymerase binding site; NLS, nuclear localization sequence of SV-40 large T antigen; *attR*, a site that recombine with *attL* sites and create *attB* sites; Cm^R, chloramphenicol resistance gene; *ccdB*, a gene which encodes a protein that interferes with *E. coli* DNA gyrase; YN, YFP N-terminus (a.a. 1-154); YC, YFP-C-terminus (a.a. 155-238); Sp6, Sp6 promoter; BGHpA, bovine growth hormone poly A site; SV40, SV40 promoter; Neomycin, neomycin resistance gene; ColE1, ColE1 ori; Ampicillin, ampicillin resistance gene; pCMV, CMV promoter.

Fig. 2. Comparisons of the three POZ-domains, mAPM-1, LRF, and KLHL10. (A) Structure comparison of three POZ-domain proteins, of mAPM-1, LRF, and KLHL10. (B) POZ-domain amino acid sequence alignment of LRF, mAPM-1, and KLHL10. Asterisks (*) indicate amino acids conserved among all three POZ-domains. Double dots (:) represent conserved amino acids by the two POZ-domains. Dots (.) indicate amino acids that are functionally similar. (C) Percentages of amino acids sequence homology among the POZ-domains. (D) Phylogenic relationship among the three POZ-domain proteins. POZ_{mAPM-1}, POZ_{LRF}, and POZ_{KLHL10} were very similar and classified into one subgroup upon phylogenic tree construction of the entire POZ-domain proteins by ClustalW program of Macvector 7.2.

DNA gyrase and inhibit growth of most *E. coli* strains such as DH5 α [23]. By BP and LR recombination reactions, while genes of interest were cloned into donor and destination vectors, *ccdB* and chloramphenicol genes were removed from the donor and destination vectors as by-products of recombination reactions. To ensure transfer of gene in each reaction, kanamycin resistance (for entry clone) or ampicillin resistance (for expression clone) is used as a positive selection marker and chloramphenicol resistance is used as a negative selection marker.

These positive and negative selection markers contribute to higher cloning efficiency of genes of interest [6]. Attachment of a NLS at the N-terminus was considered because detection of weak protein-protein interaction in whole cell could be difficult. Targeted expression of proteins in the nucleus could make the protein-protein interaction assay more sensitive. FLAG or HA-tags were used to monitor expression of two interacting proteins.

The POZ-domain is a highly conserved protein-protein interaction motif found in many regulatory proteins.

POZ-domain proteins are involved in many critical cellular processes through interaction with each other and with other regulatory proteins [10, 15]. The domain can form homomeric and heteromeric associations with other POZ-domains. Because most proteins play different roles depending on their partnerships, elucidation of complex protein-protein interaction network among the POZ-domain proteins may be crucial in understanding biological functions of the POZ-domain class proteins. Analysis of the amino acid sequence of virtually all of the human POZ-domains using ClustalW program of Macvector 7.2 revealed that they were classified into 31 subgroups. We selected a subgroup that contains mAPM-1 (mouse homolog of human APM-1, Genbank accession no. BC070424) [19], LRF (leukemia/lymphoma-related factor, ortholog of human FBI-1, Genbank accession no. AF086830) [20] and KLHL10 (mouse homolog of human KL10, Genbank accession no. AY495337) [21]. Analysis of structural features based on amino acid sequences showed that these proteins commonly have the POZ-domains at their N-terminal. LRF and mAPM-1 have four very similar zinc fingers, while

Fig. 3. Visualization of protein-protein interactions among the three POZ-domains by pFPIA system in CV-1 cells. (A) pFPIA(NLS)-POZ-YN and pFPIA(NLS)-POZ-YC were transfected into CV-1 cells in various combinations to investigate the molecular interaction of the POZ-domains. The three POZ-domains can interact and form homodimer or heterodimer depending on the expression plasmids transfected. (B) Both positive and negative controls of protein-protein interactions assays. CV-1 cells were transfected with pBiFC-YN/-YC set, pBiFC-Jun-YN/-Fos-YC set, pFPIA(NLS) Δ (Cm^R-ccdB)-YN/-YC set, or pCNA3.0 and yellow fluorescence was examined by microscopy. (C) Western blot analysis of various POZ-YN or POZ-YC fusion proteins expression in CV-1 cell lysate prepared from the control untransfected cells or the cells transfected with pCNA3.0 or other expression plasmids indicated. The molecular mass (kDa) of the specific protein bands are as follows. pBiFC-YN, 21 kDa; pBiFC-YC, 14 kDa; pBiFC-Jun-YN, 32 kDa; pBiFC-Fos-YC, 24 kDa; pFPIA(NLS) Δ (Cm^R-ccdB)-YN, 23 kDa; pFPIA(NLS) Δ (Cm^R-ccdB)-YC, 15 kDa; pFPIA-POZ_{mAPM-1}-YN, 32 kDa; pFPIA-POZ_{mAPM-1}-YC, 26 kDa; pFPIA-POZ_{LRF}-YN, 33 kDa; pFPIA-POZ_{LRF}-YC, 27 kDa; pFPIA-POZ_{KLHL10}-YN, 34 kDa; pFPIA-POZ_{KLHL10}-YC, 27 kDa.

KLHL10 has six kelch motifs (Fig. 2A). Amino acid sequence similarity among POZ_{mAPM-1}, POZ_{LRF}, and POZ_{KLHL10} is 49-70%, suggesting the possibility of molecular interactions (Fig. 2B-D). We investigated the

protein-protein interactions of these POZ-domains for validation of our high speed BiFC protein-protein interaction assay.

Fig. 4. Demonstration of specificity of the protein-protein interaction among the POZ-domains using the mutant POZ-domains in CV-1 cell. (A-C) The interactions between POZ-domain (pFPIA(NLS)POZ) and mutant POZ-domains (pFPIA(NLS) Δ POZ) were investigated by various combinations. They did not interact with each other and did not show non-specific association. Also the mutant POZ-domains neither interact with other mutant POZ-domain nor form homodimer by itself. (D) pBiFC-Jun-YN and pBiFC-Fos-YC set and pFPIA(NLS) Δ (Cm^R-*ccdB*)-YN and pFPIA(NLS) Δ (Cm^R-*ccdB*)-YC plasmid set were used as positive and negative control, respectively. (E) Western blot analysis of the mutant POZ-domain proteins expression in total cell lysate transfected with mutant POZ-YN or mutant POZ-YC expression plasmids. DIC, differential-interference-contrast microscopy image. Δ indicated the deletion of the 1-70 amino acids known to be important in protein-protein interaction.

Visualization of molecular protein-protein interactions among BTB/POZ domains in living cells

CV-1 cells were cotransfected with the plasmid mixture to investigate homodimerization and heterodimerization among the POZ-domains. Nuclear YFP fluorescence was examined by confocal microscopy. The negative controls pFPIA(NLS) Δ (Cm^R-*ccdB*)-YN and pFPIA(NLS) Δ (Cm^R-*ccdB*)-YC did not show fluorescence, while the positive controls pBiFC Jun-YN and pBiFC Fos-YC gave strong fluorescence. Jun-YN and Fos-YC were used as positive control because none of them exhibited fluorescence when expressed alone. And the specific molecular interaction between Jun and Fos and YFP fluorescence by molecular interaction between Jun and Fos were well documented [9].

The POZ-domains of mAPM-1, LRF and KLHL10

interacted as homodimers and heterodimers, however interactions involving KLHL10 were weak (Fig. 3A, B). Because fluorescence is generated by the molecular interaction, the intensity could depend on which part of YFP (YN or YC) was fused with which POZ-domain. We therefore fused the POZ-domains with alternative YFP fragments and observed fluorescence of similar intensity (data not shown). Western blotting assay with FLAG and HA antibodies showed that pFPIA(NLS) Δ (Cm^R-*ccdB*)-YN and pFPIA(NLS) Δ (Cm^R-*ccdB*)-YC were expressed in the cells (Fig. 3C). Expression of POZ-domains was also analyzed by western blot.

Based on the crystal structures of several POZ-domains, others have previously shown that α 1, β 1, B1, B2 and A1 parts of POZ-domain are important in dimeric interaction [14]. To provide negative controls for the

Fig. 5. Protein-protein interaction assays among the POZ-domain proteins by the two conventional methods. (A) Mammalian two-hybrid assay. CV-1 cells were cotransfected with various VP16 or Gal4 fusion plasmids and analyzed for reporter gene expression. POZ_{mAPM-1} and POZ_{LRF} interacted with each other and formed a homodimer and heterodimer, but POZ_{KLHL10} did not show significant interaction. (B) GST-fusion protein pull-down assay shows that POZ_{mAPM-1}, POZ_{LRF} and POZ_{KLHL10} could form homo- and heterodimers by interacting directly with each other. POZ_{KLHL10} strongly formed homodimers and heterodimers with other POZ-domains *in vitro*.

interaction assay, we prepared several POZ-domain expression plasmids with deletions at the regions critical for molecular interaction. YN or YC fused with the POZ-domain with this deletion did not give fluorescence, suggesting that mutated POZ-domains did not interact with other POZ-domain partners (Fig. 4).

Investigation of interaction using mammalian two-hybrid assay

Mammalian two-hybrid assay is frequently used to study protein-protein interactions in transfected cells. We examined whether the POZ-domain interaction data obtained by our newly developed method are in agreement with the mammalian two-hybrid assay. GAL4- and VP16-fused POZ_{mAPM-1}, POZ_{LRF}, POZ_{KLHL10} were prepared, and cotransfected into CV-1 cells in various combinations to detect molecular interaction among the POZ-domains. POZ_{mAPM-1} and POZ_{LRF} interacted and could form both homodimers and heterodimers, consistent with the data obtained by FPIA system (Fig. 5A). However, POZ_{KLHL10} did not show homodimeric and heterodimeric interactions. It appears that the molecular interactions involving KLHL10 may be too weak to give transcriptional activation. This may be due to low sensitivity of the mammalian two-hybrid assay methods compared with the developed FPIA system. Because the mammalian two-

hybrid assay is based on expression of a reporter gene, weak molecular interactions may not give sufficient transcriptional activation (reviewed in 21 and reference 12 there in). In addition, molecular interactions involving transcription repressors such as some POZ-domains cannot efficiently activate transcription of the reporter gene and can be difficult to detect.

Investigation of direct interactions among the POZ-domains by *in vitro* GST-pull down assay

To substantiate that the molecular interaction revealed by the FPIA system is generated by direct protein-protein interactions, we carried out GST-fusion protein pull-down assays. Recombinant GST, various GST-POZ-domains, and [³⁵S]-methionine labeled POZ-domain polypeptides were prepared and mixed in various combinations to investigate the molecular interaction among the POZ-domains. Pulldown of the GST-fusion protein-agarose complexes and SDS-PAGE/autoradiography analysis of the precipitates showed that the POZ_{mAPM-1} formed homodimers and also interacted with POZ_{LRF} and POZ_{KLHL10}, whereas the recombinant GST used as a negative control did not interact (Fig. 5B). The pull-down assay clearly shows direct protein-protein interactions among the POZ-domains of this particular subgroup that were not apparent in the mammalian two-

Fig. 6. Protein-protein interaction assay between the POZ_{LRF} proteins with no NLS attached in cytoplasm and nucleus. (A) Diagram of the pFPIA plasmids with no NLS, pFPIA(Δ NLS)-POZ_{LRF}-YN, pFPIA(Δ NLS)-POZ_{LRF}-YC, pFPIA(Δ NLS Δ Cm^R-ccdB)-YN, and pFPIA(Δ NLS Δ Cm^R-ccdB)-YC. These plasmids were generated by site-directed mutagenesis of the above plasmids. (B) Visualization of protein-protein interaction between the POZ_{LRF}-YN and POZ_{LRF}-YC, both with no-NLS attached in CV-1 cells. pFPIA(Δ NLS)-POZ_{LRF}-YN and pFPIA(Δ NLS)-POZ_{LRF}-YC were co-transfected into CV-1 cells to investigate the molecular interaction between the POZ-domains in cytoplasm. Also, pFPIA(Δ NLS Δ Cm^R-ccdB)-YN, and pFPIA(Δ NLS Δ Cm^R-ccdB)-YC were co-transfected as control. The POZ-domain of LRF interacted and formed a homodimer in both cytoplasm and nucleus. Δ indicated the disruption of NLS at N-terminus by site directed-mutagenesis.

hybrid assay but were detected by the FPIA assay. Overall, the newly developed FPIA method is fast, reliable, and sensitive. This assay can provide reliable protein-protein interaction information *in vivo* in just 3 days.

Protein-protein interaction between the POZ_{LRF} proteins with no NLS attached in cytoplasm and nucleus

Because the pFPIA(NLS)-YN and -YC plasmids were designed to express the YN- and YC-fusion proteins in the nucleus by having NLS of SV40 large T antigen at N-terminal, one may argue that the molecular interactions occurred in the nucleus may not necessary occur in other compartments of cell, such as cytoplasm. To investigate

the possibility, we generated the pFPIA(Δ NLS)-POZ_{LRF}-YN, pFPIA(Δ NLS)-POZ_{LRF}-YC, pFPIA(Δ NLS Δ Cm^R-ccdB)-YN and pFPIA(Δ NLS Δ Cm^R-ccdB)-YC plasmid constructs which have disrupted NLS by site-directed mutagenesis. We tested whether protein-protein interactions can be also analyzed in cytoplasm. The pFPIA(Δ NLS)-POZ_{LRF}-YN and pFPIA(Δ NLS)-POZ_{LRF}-YC were co-transfected into CV-1 cells. Also, as a control, pFPIA(Δ NLS Δ Cm^R-ccdB)-YN, and pFPIA(Δ NLS Δ Cm^R-ccdB)-YC plasmids were co-transfected. After 24 hrs of transfection, the fluorescence microscope examination showed that the POZ-domain of LRF interacted and formed a homodimer in both cytoplasm and nucleus. However, the

pFPIA(Δ NLS Δ Cm^R-*ccdB*)-YN, and pFPIA(Δ NLS Δ Cm^R-*ccdB*)-YC plasmids used as a control did not show molecular interaction (Fig. 6A). Our data suggest that the new pFPIA plasmid system can be used to investigate molecular interactions not only in nucleus but also in cytoplasm. Therefore, by selecting appropriate expression pFPIA plasmid set depending on the need of investigator, one can analyze the protein-protein interaction between proteins of interest in their normal physiological environment.

Discussion

The molecular mechanisms underlying biological processes can be best understood by dissecting the molecular network of protein-protein interactions. With the completion of the human genome project, there is an ever-increasing need for highly efficient *in vivo* protein-protein interaction assay methods to investigate gene function. Several methods have been developed to analyze protein-protein interaction, but due to their technical nature most of these have some limitations with respect to efficient large-scale detection of protein-protein interactions in living cells [1-2, 24-25]. Most methods require purified functionally active proteins and antibodies. Cloning the gene of interest can take several days and protein expression in a soluble form, purification, correct folding and translational modifications are often very difficult, if not impossible [1, 2]. In addition, *in vitro* protein-protein interactions have to be confirmed by *in vivo* assays because the proteins removed from their normal physiological condition can behave differently from the native forms. *In vivo* approaches, especially yeast two-hybrid assay, are widely used to screen protein-protein interactions. However, interactions that dependent on a posttranslational modification that does not occur in yeast will not be detected [1-2, 23]. Moreover, molecular interactions that lead to transcriptional repression or weak transcription activation cannot be detected and 30-50% false positive results were reported [4-5, 24-25]. Immunoprecipitation is one of the widely used *in vivo* protein-protein interaction assay. However, since target proteins have to be precipitated by antibodies, this method requires highly specific antibodies that are not always available, and experimental artifacts exist due to non-specific bindings of various proteins to antibodies that might never occur *in vivo* [1]. Finally, while the information gained from proteome-wide interaction maps is highly valuable, the generation of interaction map is

Fig. 7. Flow-chart of protein-protein interaction assay using pFPIA system. After preparation of the *attB* flanked PCR products of the genes of interest, expression clones can be prepared within 6 hrs by BP and LR reactions in an eppendorf tube. Entry clone is prepared by BP reaction between the PCR products and donor vector. And expression clones are created by LR reaction between the entry clones and pFPIA(NLS)-YN or pFPIA(NLS)-YC plasmid. The expression plasmids that include YN and YC fused genes are transiently transfected into mammalian cells and after 12 hrs, protein-protein interactions can be analyzed by observation of yellow fluorescence using fluorescence microscope.

time-consuming and requires extensive labor. In addition, assay results have to be confirmed by *in vivo* assay methodologies.

For these reasons, we developed an efficient *in vivo* protein-protein interaction assay system by adopting the Gateway system and BiFC. The power of the Gateway system is that BP and LR recombination mediated cloning can be performed within 6 hrs in a eppendorf tube and a high percentage (>95%) of the transformed *E. coli* contain the desired clone along with the *ccdB* gene and

chloramphenicol resistance gene that act as negative and positive selection markers, respectively [6, 27]. In addition, once genes of interest are selected, they can be transfected and subcloned into several other vectors at a time using an *in vitro* recombination reaction [6].

Our system also adopted BiFC to analyze protein-protein interactions in living cells. BiFC analysis does not require any information on the proteins and can be useful in studying any protein-protein interaction *in vivo*. By using standard fluorescence or confocal microscopy, interactions can be analyzed easily and staining of the cells with exogenous fluorescent molecules or dyes is not required [9, 28].

One limitation of BiFC is intrinsic association of fluorescence YFP fragments without specific interaction between proteins [28]. It is therefore necessary to confirm specificity of protein-protein interactions using mutant proteins with critical mutations in the protein-protein interaction interface [9, 28]. The generation of mutant proteins for all interesting proteins is time-consuming and not easy if the important interface to protein-protein interaction is unknown. Fortunately, in the FPIA system we developed, molecular interaction by spontaneous association of YN and YC was not detected. The possibility of false-positives due to forced nuclear expression of the YFP fusions was examined by mutant POZ-domains. The FPIA system therefore does not require generation of mutant protein for each protein of interest.

Compared with other protein-protein interaction assays, such as mammalian two-hybrid assay and GST pull-down assay, our FPIA assay method is very sensitive and highly efficient in detecting even weak protein-protein

interaction. The major advantages of the FPIA assay system is that protein-protein interaction can be examined in 3 days from gene cloning to interaction study (Fig. 7). This assay system requires only 3 steps: preparation of target DNA fragment with *att* sites at both 5' and 3' ends by PCR (2-3 hrs), BP and LR reaction mediated cloning into pDONR, pFPIA(NLS)-YN and pFPIA(NLS)-YC (6 hrs), transient transfection into cells (12 hrs), and finally microscopic observation.

Moreover, our new system can be applied to screen a cDNA library made with pFPIA(NLS)-YN or YC to isolate cDNAs encoding proteins that interact with the protein of interest. Firstly, one has to establish a stable cell line expressing YN or YC fused with the protein of interest. A cDNA library prepared in one of the pFPIA plasmids is transfected into these cells, and one simply needs to isolate cells that give fluorescence. After amplification of such cells, the corresponding cDNA can be isolated by PCR and sequenced.

The FPIA method may be one of the powerful methods available to investigate protein-protein interaction of structurally diverse proteins in many different cell types and organisms in normal physiological context.

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