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Detection of protein-protein interactions using nonimmune IgG and BirA-mediated biotinylation

Cai Huang[1,2] and **Ken Jacobson**[1,3]

[1]Department of Cell and Developmental Biology, University of North Carolina, Chapel Hill, NC, USA

[2]Markey Cancer Center and Department of Molecular and Biomedical Pharmacology, University of Kentucky, Lexington, KY, USA

[3]Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA

Abstract

Detection of protein-protein interactions in cells is crucial for understanding the biological functions of proteins, including their roles in signal transduction. However, current methods require specific antibodies both for immunoprecipitation and detection, making them expensive and sometimes unreliable. Here we describe protocols for protein-protein interaction assays that use nonimmune IgG-conjugated Sepharose to precipitate the IgG binding domain (ZZ) fused to the bait protein; the interaction partner is fused to Avitag and biotinylated by BirA so that it can be detected by a one-step blot with Dylight 680 streptavidin to detect the Avitag fusion protein. Since this method does not require specific antibodies and is inexpensive, sensitive, and reliable, it should be useful for detecting protein-protein interactions in cells.

Keywords

protein-protein interactions; BirA; streptavidin; ZZ domain

Protein-protein interactions contribute to a variety of biological processes, including signal transduction, tissue integrity, and force generation (1,2). An inexpensive and reliable method to measure protein-protein interactions would be of considerable utility. Currently, co-immunoprecipitations (Co-IP) are routinely used to detect protein-protein interactions in cells, where one antibody is used to isolate the bait proteins and another antibody is used to detect the interaction. Since antibodies are expensive reagents, qualities of antibodies change from lot to lot, and manufacturer claims may not be true (3), this method is neither economical nor reliable. Thus, a technique that does not depend on specific antibodies would be useful for protein-protein interactions assays.

Address correspondence to Cai Huang, Markey Cancer Center and Department of Molecular and Biomedical Pharmacology, University of Kentucky, Lexington, KY 40506, USA. cai-huang@uky.edu.

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The biotin/avidin (or streptavidin) system has numerous applications in modern biological studies because the interaction of biotin with avidin is one of the highest affinity interactions known in nature. Recently, the applications of this system have been greatly promoted by the discovery of BirA, an *Escherichia coli* biotin ligase that specifically conjugates biotin to a 15-amino-acid Avitag (GLNDIFEAQKIEWHE) (4,5). BirA-mediated biotinylation of Avitag fusion proteins has been used in protein purification (6), detection (7,8), and fluorescence imaging (9–12). Thus, BirA-mediated biotinylation, which can be easily detected by fluorescent streptavidin blot (designated as the Avitag-BirA system), has great potential application in detecting interacting Avitag fusion proteins in protein-protein interaction assays without using specific antibodies.

For protein-protein interaction assays in cells, a method is needed to isolate bait proteins. The ZZ domain, a synthetic IgG binding protein derived from tandem repeats of the B domain of protein A, was successfully used to replace protein A in antibody purification (13,14). It was also engineered to fuse with many different proteins and expressed as ZZ-tagged fusion proteins in diversified cell types, ranging from bacterium to mammalian cells (15–17). To date, no reports have suggested that the ZZ domain impairs the function of proteins fused to it, and ZZ fusion proteins can be easily purified by using IgG-Sepharose. Therefore, we proposed a novel method for protein-protein interaction assays in cells, in which inexpensive, nonimmune rabbit IgG-conjugated Sepharose beads can be used to precipitate the ZZ domain fusion protein (as bait); subsequently, fluorescent streptavidin can be used to detect the interacting Avitag protein that was biotinylated by BirA.

In this study, we have examined whether the Avitag-BirA system is useful for in vitro GST pulldown assays and whether the Avitag-BirA system, in combination with the ZZ domain purification technique (designated as the AviZZ system), can be used for protein-protein interaction assays in cells. Generic protocols for in vitro GST pulldown assays and protein-protein interaction assays in cells are schematically depicted in Figure 1, A and B, respectively.

Materials and methods

Reagents

Chinese hamster ovary (CHO)–K1 cells were from ATCC (Manassas, VA, USA). DMEM/F-12, FBS, G418, Lipofectamine, and Plus reagents were from Invitrogen (Carlsbad, CA, USA). Dylight 680 (DL680)–conjugated streptavidin was from Rockland (Gilbertsville, PA, USA). Biotin and CNBr-activated Sepharose 4B were from Sigma-Aldrich (St. Louis, MO, USA). pET21a-BirA was from Addgene (Boston, MA, USA; deposited by Alice Ting's lab at the Massachusetts Institute of Technology, Cambridge, MA, USA). pEGFP-Git1 and pEGFP-PIPKIγ were provided by Mark Ginsberg (University of California at San Diego, San Diego, CA, USA). pHM6-Tal1–433 was described previously (18). ImmunoPure Immobilized Protein A Plus and DL680 NHS ester were from Pierce (Rockford, IL, USA). Mouse paxillin cDNA was from Open Biosystems (Huntsville, AL, USA). Glutathione Sepharose and pGEX-6X-1 vector were from GE Heathcare Biosciences (Piscataway, NJ, USA). *Pfu* and Quick-Change mutation kit were from Agilent Technologies (Santa Clara, CA, USA). Protease inhibitor cocktail was from Roche Applied Science (Indianapolis, IN,

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USA). Nonimmune IgG was purified from pre-immune rabbit sera, which was from Genemed Synthesis (San Antonio, TX, USA), and conjugated to CNBr-activated Sepharose 4B according to the manufacturer's protocol.

Plasmid construction

To generate pEGFP-paxillin and pGEX-paxillin, DNA fragments encoding residues 2–557 of mouse paxillin were amplified by Turbo *Pfu*-based PCR using paxillin cDNA as template and 5′-AAAAAAGAATTCAGACGACCTCGATGCCCTG-3′ and 5′-AAAAAAGTCGACCTAGCAGAAGAGCTTCACGAAGCA-3′ as primers. The DNA fragments were digested with *Eco*RI and *Sal*I and, respectively, subcloned into pEGFP-C1 and pGEX-4T-3 vectors predigested with the same enzymes. To create pEGFP-BirA, DNA fragments encoding BirA were clipped from pET21a-BirA by *Eco*RI and *Xho*I digestion and then subcloned into pEGFP C2 that was predigested with *Eco*RI/*Sal*I. To construct AviTag paxillin (designated as pAvi-paxillin), synthetic AviTag-encoding DNA fragments 5′-CCGGTGCCACCATGGGTGGCGGTCTGAACGACATCTTCGAGGCTCAGAAAATCGAATGGCACGAAA-3′ and 5′-GATCTTTCGTGCCATTCGATTTTCTGAGCCTCGAAGATGTCGTTCAGACCGCCACCCATGGTGGCA-3′ were annealed and ligated with the larger fragment of *Age*I/ *Bgl*II-digested pEGFP-paxillin. To make pAvi-Git1, the DNA fragments encoding full-length Git1 were clipped from pEGFPGit1 by *Eco*RI and *Sal*I digestion and ligated with the larger fragment of *Eco*RI/*Sal*I-digested pAvi-paxillin. To generate Git1 fused with the IgG binding domain of protein A (designed as pZZ-Git1), DNA fragments encoding the ZZ domain were amplified by Pfu-based PCR using pEZZ18 as the template and 5′-ATATATACCGGTGCCACCATGGACAACAAATTCAACAAAGAACAACAAAACGCG-3′/5′-TTAATACTCGAGCTACTTTCGGCGCCTGAGCATCATTTAGC-3′ primers, digested with *Age*I and *Xho*I, and then ligated with the larger fragment of *Age*I/*Bgl*II-digested pEGFP-Git1. To construct pZZ-PIPKIγ and pAvi-PIPKIγ, DNA fragments encoding phosphatidylinositol 4-phosphate 5-kinaseγ (PIPKIγ) were clipped from pEGFP-PIPKIγ (on pEGFP-C2) by digesting with *Eco*RI/*Sal*I and were ligated with *Eco*RI/*Sal*I-digested pZZ and pAvi vectors (pZZ-Git1 and pAvi-paxillin were digested with *Eco*RI/*Sal*I to remove Git1 and paxillin), respectively. The resultant plasmids are not in-frame and were digested with *Xho*I/*Hin*dIII, treated with Klenow, and circularized to become in-frame pZZ-PIPKIγ and pAvi-PIPKIγ. To create pAvi-TH, DNA fragments encoding residues 2–433 of talin were amplified by *Pfu*-based PCR using talin cDNA as the template and 5′-TATATAGAATTCGTTGCGCTTTCGCTGAAGATTAGCATTG-3′/5′-ATATATCTCGAGTTACTGAAGGACTGTTGACTTTTTGGGAGACAC-3′ as primers, digested with *Eco*RI/*Xho*I, and subcloned into pAvi vector via *Eco*RI and *Sal*I sites. The resultant plasmid was not in-frame and was sequentially digested with *Eco*RI, treated with mung bean nuclease, cut with *Bgl*II, treated with Klenow, and circularized to become in-frame pAvi-TH. To generate pZZ-TH, DNA fragments encoding residues 2–433 of talin plus six histidine residues were amplified by *Pfu*-based PCR using pHM6-tal1–433 as template and 5′-ATATATCTCGAGTTGCGCTTTCGCTGAAGATTAGCATTGGG-3′/5′-TGAGTGGGTACCCTTAATGATGATGATGATGATGCACGTG-3′ as primers, digested with *Xho*I/*Kpn*1, and subcloned into pZZ vector via *Xho*I and *Kpn*1 sites.

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Cell culture and transfection

CHO-K1 cells were maintained in DMEM/F-12 medium containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. CHO-K1 cells were transfected with Lipofectamine Plus transfection reagent according to the manufacturer's protocol. Cells stably expressing EGFP-BirA were obtained by sorting enhanced GFP (EGFP) positive cells after G418 selection in the University of North Carolina Flow Cytometry Facility (Chapel Hill, NC, USA).

Protein interaction

GST pulldown assays—CHO-K1 cells stably expressing EGFP-BirA were transfected with Avitag plasmids. At 24 h post-transfection, cells were incubated with 500 µM biotin for 1 h and then lysed with Lysis Buffer A (50 mM Tris-HCl, pH 7.4, 1% NP40, 150 mM NaCl, 1 mM EDTA, and protease inhibitor cocktail). The lysates were cleared and incubated with glutathione Sepharose beads loaded with GST fusion proteins at 4°C for 2 h. The beads were washed with the lysis buffer four times and were resuspended in SDS sample buffer. Samples were analyzed by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in TBST buffer (10 mM Tris-HCl pH 7.5, 140 mM NaCl, 0.1% Tween 20) for 1 h, rinsed twice with TBST buffer, and then blotted with DL680-streptavidin (1:10,000) in TBST buffer containing 5% BSA for 1 h. The membrane was scanned with an Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, NE, USA).

Coprecipitation assays in cells—CHO-K1 cells stably expressing EGFP-BirA were transfected with a plasmid carrying a ZZ domain fusion protein and another plasmid encoding an Avitag fusion protein. At 24 h post-transfection, cells were incubated with 500 µM biotin for 1 h and then lysed with Lysis Buffer A. The lysates were cleared and incubated with rabbit IgG-Sepharose beads at 4°C for 2 h to precipitate the ZZ domain fusion protein. The beads were washed and analyzed by SDS-PAGE and Western blot as above. The Avitag protein that bound the ZZ domain fusion protein was detected with DL680-streptavidin, while the expression of the ZZ domain fusion protein was probed with DL680-rabbit IgG.

Results and discussion

To test whether the Avitag-BirA system would be feasible for in vitro GST pulldown assays (protocol depicted in Figure 1A), CHO-K1 cells stably expressing EGFP-BirA (CHOB cells) were transfected with pAvi-type I PIPKIγ, a talin-binding protein (19,20). At 24 h post-transfection, Avi-PIPKIγ was labeled with biotin by incubating the cells with biotin. Then we examined the binding of the biotinylated Avi-PIPKIγ expressed in CHO cells to GST-tal210-605, a talin fragment, and detected with DL680-labeled streptavidin. As shown in Figure 2A, the talin fragment GST-Tal210-605 strongly interacted with PIPKIγ, whereas GST-profilin had no association with PIPKIγ. The interaction of GST-paxillin with Git1, a paxillin binding protein (21), was also used to examine the performance of this method. Streptavidin-recognizing bands were detected in Avi-Git1-transfected cells but not in mock-transfected cells (Figure 2B), indicating that streptavidin specifically recognizes biotinylated

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Git1. Git1 bound paxillin, whereas profilin had no interaction with paxillin (Figure 2B). These results indicate that streptavidin can be used in conjunction with Avitag and BirA to replace antibodies when detecting protein-protein interactions in GST pulldown assays.

To compare the sensitivities between the Avitag-BirA system and conventional antibody detection, CHOB cells were transfected with Avi-PIPKIγ. The cells were treated with biotin, and the expression of Avi-PIPKIγ was detected by blotting with an anti-PIPKIγ antibody and DL680-streptavidin, respectively. As shown in Figure 2C, the Avitag-BirA system was approximately twice as sensitive as the anti-HA antibody detection. Three epitope tag antibodies including two anti-HA antibodies and one anti-GFP antibody were also compared with the Avitag-BirA system (data not shown). The Avitag-BirA system was significantly more sensitive than the conventional antibody detection, ranging from 1- to 18-fold.

Next, we tested whether the AviZZ system (protocol depicted in Figure 1B) could be used for protein-protein interaction assays in cells. Before we evaluated the full AviZZ system, we needed to test whether nonimmune IgG could be used to precipitate the ZZ-domain fusion protein (as bait) from cell lysates. To generate ZZ-PIPKIγ, DNA fragments encoding a 7-kDa, single copy of the tandem repeat sequence of the B domain of protein A (ZZ domain) were inserted at the N terminus of PIPKIγ. CHO-K1 cells were transfected with ZZ-PIPKIγ, and ZZ-PIPKIγ in lysates was precipitated by incubating with nonimmune rabbit IgG-conjugated Sepharose. As shown in Figure 3, A and B, ZZ-PIPKIγ was purified and efficiently enriched approximately 80-fold after IgG-Sepharose affinity pulldown. ZZ-tagged proteins were enriched 36-fold, on average, in precipitation assays using IgG-Sepharose. This efficiency is comparable to or better than antibodies commonly used for immunoprecipitation. Thus, nonimmune IgG-conjugated Sepharose can be used to isolate ZZ-tagged bait proteins in protein-protein interaction assays.

Next we asked whether DL680-streptavidin could be used to detect biotinylated Avitag proteins that bind the bait protein isolated by IgG-conjugated Sepharose. To this end, CHO-K1 cells that stably express BirA were transfected with Avi-PIPKIγ with or without ZZ-talin head (ZZ-TH), as indicated (Figure 4A). The cells were incubated with biotin to label Avi-PIPKIγ, and ZZ-TH was precipitated with IgG-Sepharose. The binding of Avi-PIPKIγ to ZZ-TH was detected using DL680-streptavidin. ZZ-TH strongly coprecipitated with Avi-PIPKIγ, whereas Avi-PIPKIγ was not detected when empty vector was transfected instead of ZZ-TH (Figure 4A, lane 1) or when IgG-Sepharose was replaced with Sepharose alone for isolation (Figure 4A, lane 4). Cotransfection of ZZ- PIPKIγ and Avi-TH also generated similar results (Figure 4B).

We further examined this method by testing the coprecipitation of paxillin and Git1. CHOB cells were transfected with Avi-paxillin with or without ZZ-Git1 cotransfection, as indicated (Figure 4C). The interaction of Avi-paxillin to ZZ-Git1 was analyzed and detected by using Dylight680 streptavidin as described above. ZZ-Git1 bound Avi-paxillin, except in cases where ZZ-Git1 was not expressed (Figure 4C, lane 1), or Sepharose was used in the precipitation (Figure 4C, lane 4). Thus, Avitag, in combination with BirA and the ZZ domain (designated as AviZZ system), constitutes an inexpensive and sensitive method for protein-protein interaction assays in cells.

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Finally, we compared the efficiency of the AviZZ system to that of the conventional Co-IP for protein-protein interaction assays. CHOB cells were transfected with Avi-TH plus wild-type (WT) ZZ-PIPKIγ or Avi-TH plus ZZ-PIPKIγ W647F, a mutant that is deficient in talin binding; PIPKIγ WT and the mutant were precipitated with IgG-Sepharose, and the interaction of TH with PIPKIγ was detected with DL680-streptavidin. At the same time, CHO-K1 cells were transfected with His-tagged TH plus EGFP-PIPKIγ WT or His-tagged TH plus EGFP-paxillin (as a control), and His-tagged TH was precipitated with an anti-polyHis monoclonal antibody; the interaction of PIPKIγ with TH was detected with an anti-GFP polyclonal antibody. As shown in Figure 4D, the interaction reflected by the blots detected by the AviZZ system was >5-fold stronger than that detected by the conventional immunoprecipitation. Several other commercial antibodies were also tested for Co-IP, but these antibodies were unable to precipitate any proteins, even though the manufacturers claimed that they could be used for immunoprecipitation. The higher efficiency of the AviZZ system is probably due to both the high efficiency of the ZZ-tagged protein precipitation by nonimmune IgG and the high sensitivity of the Avitag-BirA system.

The AviZZ system uses low-cost nonimmune IgG for precipitation and fluorescent streptavidin for one-step detection, whereas conventional immunoprecipitation assays require expensive specific antibodies for both precipitation and detection. Also, the nonimmune IgG provides efficient precipitation of the ZZ domain fusion proteins, while many commercial antibodies are not efficient for immunoprecipitation, and the quality of specific antibodies is variable (3). Furthermore, the AviZZ system is more sensitive than conventional antibody/antigen-based detection, because of the high affinity of the biotin-streptavidin interaction. The system can be easily adapted for other applications, such as ubiquitination assays. This method requires protein overexpression and therefore cannot be used for endogenous protein-protein interaction studies. Another drawback is that the ZZ domain (7 kDa) is larger than most other epitope tags. That said, the AviZZ system provides a low-cost, reliable, and sensitive method for protein-protein interaction assays in cells.

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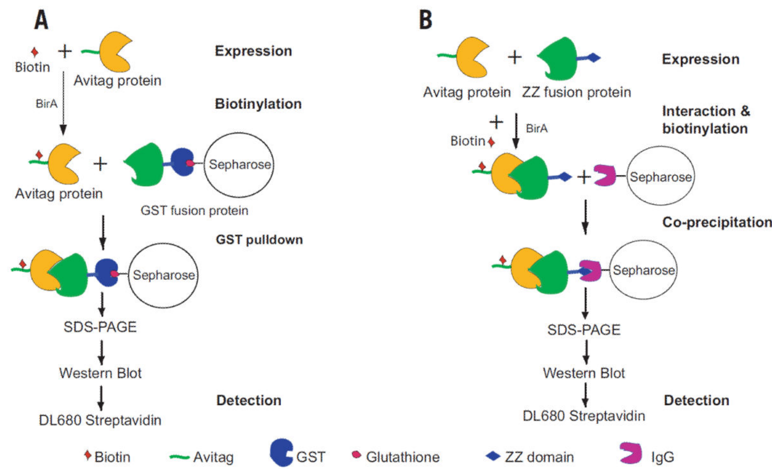

Figure 1. Scheme depicting the principle of protein-protein interaction assays using the Avitag-BirA system

(A) CHOB cells were transfected with a plasmid encoding an Avitag protein. After biotin treatment, the Avitag protein was biotinylated by BirA. The cells were lysed, and the Avitag protein in cell lysates was pulled down with glutathione Sepharose preloaded with a GST fusion protein. After SDS-PAGE and Western blotting, the interaction of the biotinylated Avitag protein with the GST fusion protein was detected in one step by blotting with DL680-streptavidin. (B) A plasmid encoding a ZZ domain fusion protein was cotransfected with a plasmid expressing an interacting Avitag protein into CHOB cells. After biotin treatment, the Avitag protein was biotinylated by BirA. The cells were lysed, and the ZZ domain fusion protein in cell lysates was precipitated with IgG-Sepharose. After SDS-PAGE and Western blotting, the interaction of the biotinylated Avitag protein with the ZZ domain fusion protein was detected in one step by blotting with DL680-streptavidin.

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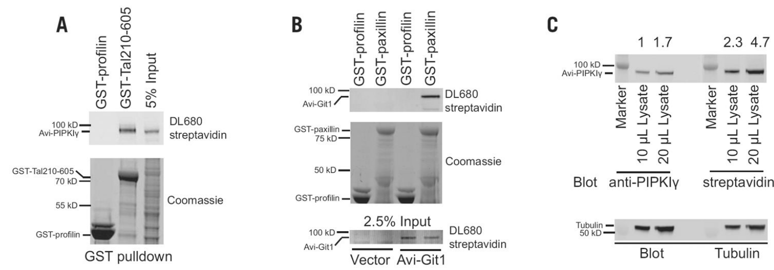

Figure 2. PIPKIγ-talin and paxillin-Git1 interactions were used to examine the potential of the Avitag-BirA system for in vitro GST pulldown assays

(A) PIPKIγ pulldown with a GST talin mutant. CHOB cells were transfected with Avi-PIPKIγ and treated with biotin. The lysates were incubated GST-profilin or GST-Tal210–605 immobilized on glutathione Sepharose beads. The Avi-PIPKIγ binding was detected by blotting with DL680-streptavidin. (B) Git1 pulldown with GST-paxillin. The CHOB cells were transfected with Avi-Git1 or empty vector and treated with biotin. The lysates were incubated GST-profilin or GST-paxillin immobilized on glutathione Sepharose beads. The Avi-Git1 binding was detected by blotting with DL680-streptavidin. (C) Sensitivity comparison between the Avitag-BirA system and the conventional antigen/antibody detection. The CHOB cells were transfected with Avi-PIPKI and treated with biotin. Cell lysates were applied to SDS-PAGE and Western blotting, and Avi-PIPKIγ was detected by an anti-PIPKIγ polyclonal antibody and DL680-streptavidin, respectively. Densities of protein bands were measured using ImageJ as described previously (18); the relative densities are shown on the top of the figure.

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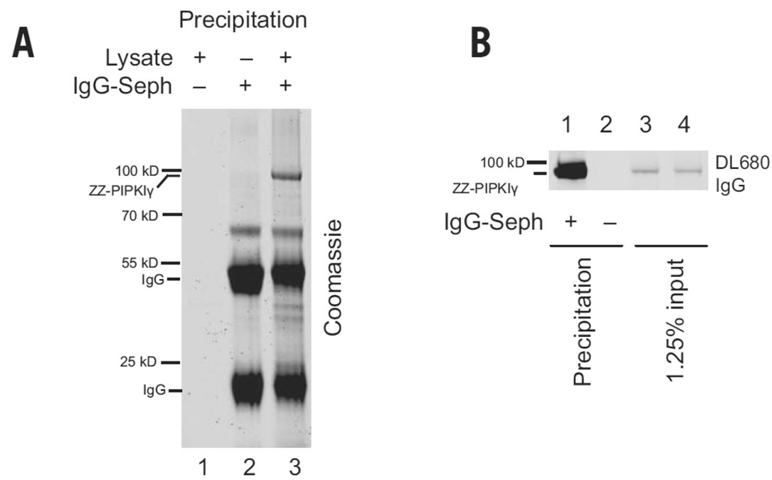

Figure 3. Precipitation and enrichment of ZZ domain fusion proteins by IgG-Sepharose

(A) ZZ-PIPKIγ was used to test the feasibility of IgG-Sepharose for precipitation. CHO-K1 cells were transfected with ZZ-PIPKIγ, and cell lysates were incubated with Sepharose (lane 1) or IgG-Sepharose (IgGSeph) beads (lane 3). The beads were washed and analyzed with SDS-PAGE. IgG-Sepharose beads that were not incubated with cell lysate were run as a control. Leaky protein bands from IgG-Sepharose are shown in lanes 2 and 3. (B) IgG-Sepharose enriches ZZ-PIPKIγ from cell lysates. Cell lysates containing ZZ-PIPKIγ were incubated with IgG-Sepharose (lane 1) and Sepharose beads alone (lane 2). The beads were washed, and ZZ-PIPKIγ bound on the beads was detected by probing with DL680-streptavidin after SDS-PAGE and Western blotting. Cell lysates (1.25%) used in lane 1 and lane 2 were shown in lane 3 and lane 4, respectively. Lane 1 shows that considerable enrichment from lysates is achieved.

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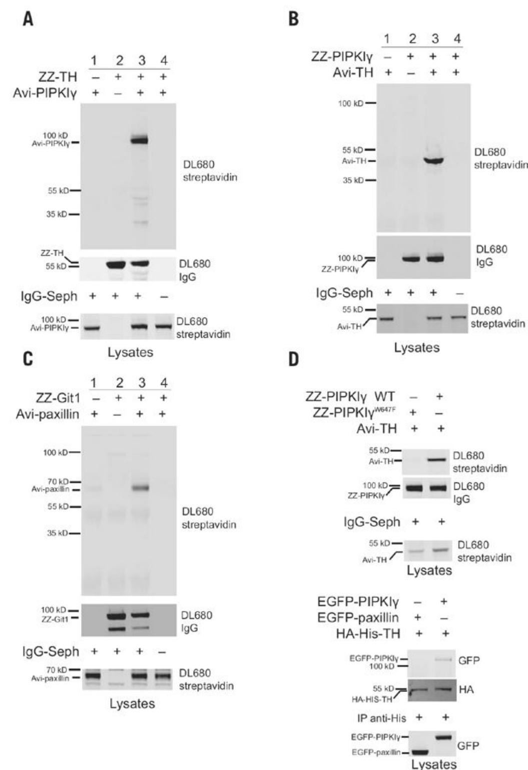

Figure 4. PIPKIγ-TH and Paxillin-Git1 interactions were used to examine the potential of the AviZZ system for protein-protein interaction assays in cells

(A) The AviZZ system was examined by testing the coprecipitation of Avi-PIPKIγ and ZZ-TH. ZZ-TH was cotransfected with Avi-PIPKIγ or empty vector into CHOB cells as indicated. After biotin treatment, ZZ-TH was precipitated with IgG-Sepharose (lanes 1–3) or Sepharose beads (lane 4). The beads were analyzed by SDS-PAGE and Western blotting. The interaction of Avi-PIPKIγ with TH was detected by blotting with DL680-streptavidin (upper panel). ZZ-TH precipitated by IgG-Sepharose was detected with DL680-conjugated nonimmune IgG (middle panel), and the expression of Avi-PIPKIγ in cell lysates was probed with DL680-streptavidin (lower panel). (B) The AviZZ system was examined by testing the coprecipitation of Avi-TH and ZZ-PIPKIγ. ZZ-PIPKIγ was cotransfected with Avi-TH or empty vector into CHOB cells as indicated. After biotin treatment, ZZ-PIPKIγ was precipitated with IgG-Sepharose (lanes 1–3) or Sepharose (lane 4). (C) The generality of AviZZ system was further investigated by testing the coprecipitation of Avi-paxillin and ZZ-Git1 in CHOB cells as described in panel A. (D) The efficiency of the AviZZ system compared with that of the conventional co-immunoprecipitation assays. Upper panel, co-precipitation of Avi-TH and ZZ-PIPKIγ by using the AviZZ system. The experiment was performed as described in panel B. Lower panel, co-immunoprecipitation of His-TH and EGFP-PIPKIγ. His-TH (pHM6-Tal1–433, expressing talin head with HA and His double tags) was cotransfected with EGFPPIPKIγ or EGFP-paxillin (as a control) into CHO-K1 cells as indicated. His-TH was immunoprecipitated with an anti-polyHis monoclonal antibody. The interaction of EGFP-PIPKIγ with His-TH was detected by blotting with an anti-GFP polyclonal antibody.