Identification of an Endoplasmic Reticulum Membrane Protein Interacting with DNA Polymerase Beta by a Yeast Two-Hybrid Screen

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Base excision repair (BER) is a key pathway for maintaining genomic stability. A key enzyme in the BER pathway is DNA polymerase beta (polβ). It has been shown that more than 11% of breast, bladder, esophageal, colon, and gastric cancer samples studied so far exhibit polβ mutation. A truncated form of polβ, pol$\beta\Delta$ (exon 11 deletion), identified in a colon tumour sample, exhibited dominant negative activity. Using this pol$\beta\Delta$ as bait, we screened a HeLa cDNA library for any interacting protein(s) in the yeast two-hybrid (Y2H) system. Pol$\beta\Delta$ was cloned into a pGBKT7 vector (pGBKT7-pol$\beta\Delta$). pGBKT7-pol$\beta\Delta$ was transformed into the yeast strain AH109. Then the cDNA library was co-transformed into AH109/pGBKT7-pol$\beta\Delta$ and screened by the selection procedure. The yeast-purified plasmids were transformed into *Escherichia coli.* Plasmid DNA was isolated from the colonies, purified, digested with Sma I and Sal I, and the fragments were sequenced. Four positive clones were obtained. Out of these, three proteins were already known to interact with polβ (XRCC1, MGC5306, and AP endonuclease 1). The only member previously not known to interact with polβ was phosphatidylinositol glycosylase type S (PIGS). PIGS is a 64-kDa membrane protein, encoded in chromosome 17. The PIGS protein interacts also with wild-type polβ which was confirmed by co-immunoprecipitation and Western blot analysis. The role of the newly identified protein in the dominant negative function of the variant form of polβ remains to be seen.

Key words: Yeast Two-Hybrid, DNA Polymerase Beta, PIGS Gene

Introduction

DNA polymerase beta (polβ), a 39-kDa protein, is a key enzyme in the base excision repair (BER) pathway. It protects DNA from oxidative stress and alkylating agent-induced damage (Sobol *et al.*, 1996; Wood, 1996). Beside the roles of polβ in apoptosis, replication, homologous recombination, meiosis, and BER (Wilson *et al.*, 2000), polβ is involved early in the pathogenesis of Alzheimer's disease (Copani *et al.*, 2006). In addition, 40% of human colon cancer samples are known to bear polβ mutations (Starcevic *et al.*, 2004). Some of these mutants may lead to tumourigenesis (Wang *et al.*, 2007). One of these variant forms, with an 87-bp deletion, shows dominant negative (DN) activity (Bhattacharyya and Banerjee, 1997). During the investigation of the function of this variant form, we found that XRCC1 is responsible for the DN activity (Bhattacharyya and Banerjee, 2001). But when we studied the DN activity in the EM-9 cell line (deficient of XRCC1), we noticed the same DN function, indicating the involvement of another unknown protein in this activity. Hence, an attempt was made to screen a HeLa cDNA library in the yeast two-hybrid (Y2H) system to identify any new protein that may interact with polβ.

Materials and Methods

Construction of pol$\beta\Delta$ in the pGBKT7 vector

The pol$\beta\Delta$ (exon 11 deletion) construct was made in the DNA binding domain of the pGBKT7 vector from Clontech (Mountain View, CA, USA). The following primers were used to amplify the human pol$\beta\Delta$ insert in pCDNAI$_{neo}$: FP: 5′-GGTACCCGGGCATGAGCAAACGGAAGGC-3′, with a Sma I site at

the 5′ end of the sequence; and RP: 5′-GGGGT-CGACGAGCTCATTCGCTCCGGTCC-3′, with Sal I and Sac I sites at the 3′ end of the sequence. The PCR product of approximately 950 bp was cloned into the pCR2.1TOPO vector from Invitrogen (Bangalore, India) using chemically competent TOP10 cells, according to the manufacturer's instructions. The pol$\beta\Delta$ cDNA in pCR2.1TOPO was isolated from TOP10 cells, digested with Sma I and Sal I, and the fragments were separated by electrophoresis in an agarose gel. The pol$\beta\Delta$ insert was then bi-directionally cloned into pGBKT7, the activation domain vector from Clontech, at the Sma I and Sal I sites. The construct in pGBKT7 was then digested with the Sma I and Sal I restriction enzymes, and the correct sequence confirmed by sequencing.

Titer and amplification of cDNA library

The HeLa cDNA library constructed in the laboratory was titered and amplified. The library was directly plated on LB/amp plates at a density sufficient to allow near-confluence of the resulting colonies ($\sim$ 20,000 – 40,000 cfu per 150-mm plate). It was plated in such a way that the number of independent clones in the library was covered at least 2 – 3 times. The library was amplified to produce enough plasmid DNA to screen the library in yeast. We used 100 – 500 μg of plasmid DNA to screen $\sim 1 \cdot 10^6$ independent clones.

Yeast transformation

In preparation for library screening, the construct of pol$\beta\Delta$ in pGBKT7 was transformed into the yeast strain AH109 as follows: First, rich yeast medium containing yeast extract, peptone, and dextrose (YPD) was inoculated with the strain AH109. The cultures were grown overnight in a 30-°C incubator, with shaking. The following day, 300 mL of fresh YPD medium were inoculated with 2×30 mL of the overnight AH109 cultures and permitted to grow with shaking until the OD_{600} reached 0.4 – 0.6. The transformation protocol followed the manufacturer's instructions (Clontech). At least 0.1 mg of each plasmid DNA was mixed with herring testes carrier DNA (Clontech). Competent yeast cells re-suspended in 10 mM Tris-HCl, 1 mM EDTA, and 0.1 M lithium acetate, pH 7.5, were added to the plasmid DNA mixture, then a polyethylene glycol 4000/lithium acetate solution (Clontech) was added, and cells were incubated at 30 °C for 30 min, with shaking at 200 rpm. Dimethyl sulfoxide (DMSO) was added to a final content of 10 % (v/v), cells were heat-shocked at 42 °C for 15 min and permitted to recover in YPD medium for 1 h with shaking at 30 °C. Cells were centrifuged for 5 s at $16{,}000 \times g$, and the supernatant was removed. Cells were re-suspended in 500 μL of sterile distilled water, and 100 μL of cell suspension were plated onto the appropriate selection medium (trp$^-$). The construct was then assayed for β-galactosidase (β-gal) activity (indicating the expression of lacZ) to be certain that no auto-activation occurred without an interaction between two proteins. The transformed AH109 was then inoculated into selection medium minus tryptophan, and permitted to reach an OD_{600} of 0.4 – 0.6. Thereafter 50 μg of the HeLa cell cDNA library were sequentially transformed into AH109 containing pol$\beta\Delta$, according to the manufacturer's instructions. More than $2.5 \cdot 10^5$ cDNA library clones were screened. A medium stringency library screen was conducted with yeast cells plated onto selection medium lacking tryptophan, leucine, and histidine. The pGBKT7 vector has a tryptophan selection marker, the cDNA library has a leucine marker, and histidine autotrophy is an indicator of a protein-protein interaction in this Y2H system. Hence if a colony grows on medium lacking the above three amino acids, it has a good probability of being involved in a protein-protein interaction. However, false positive colonies can occur using this system. To eliminate these, additional tests were conducted, such as streaking the positive transformants from the tryptophan, leucine, and histidine plates onto medium lacking these three amino acids plus adenine, an additional selection marker indicating a possible protein-protein interaction with adenine autotrophy. These positive transformants were then assayed for β-gal activity. The positive colonies from this β-gal screen were subjected to polymerase chain reaction (PCR) using the primers from pGAPDH, the HeLa cDNA library vector. Some of the colonies containing an insert were cloned into pCR2.1TOPO, and then digested with EcoRI. Plasmids containing an insert were sequenced.

Identification of interacting proteins by Y2H

Plasmid DNA from the positive colony was isolated by adding 200 μL of lysis buffer containing 0.1 M Tris-HCl (pH 8.0), 50 mM EDTA, 1 % sodium dodecyl sulfate (SDS), 200 μL of phenol, and 100 μL of chloroform. The aqueous phase was separated by centrifugation at $16{,}000 \times g$ for 10 min. Two volumes

of 3 M ammonium acetate and ethanol were added to the aqueous (upper) phase. The sample was incubated at −70 °C for 1 h and centrifuged as above. The DNA was washed with 70% ethanol, air-dried, and re-suspended in 20 μL of Tris-EDTA buffer. The positive plasmids were transformed into *Escherichia coli.* To select for transformants containing only the AD/library plasmid, the bacteria were plated on Luria Bertani (LB) medium containing selective antibiotics. Then the plasmid was isolated, digested with *Hind III*, and analysed by agarose gel electrophoresis. After the inserts with variant patterns had been selected, they were sequenced and the obtained sequences were analysed by the BLAST program (Altschul *et al.*, 1997).

Synthesis and cloning of the new protein in the GFP vector

Total RNA from the HeLa cell line was isolated using the Trizol reagent (Invitrogen, Grand Island, NY, USA). From this RNA, first-strand cDNA was synthesized using the reverse transcriptase (RT)-PCR kit from Bioline (Taunton, MA, USA). PCR was performed using the forward primer 5′-TTATTATAAGCTTATGGCGGCCGCCG-3′ and the reverse primer 5′-TGACGCGAATTCTCAGTC-TGTCTTCTC-3′, and the product was cloned into the pEGFP-N1 expression vector at the Sac I and Pst I restriction sites, respectively. PCR conditions were as follows: 1 cycle at 95 °C for 2 min, 30 cycles at 95 °C for 30 s, 52 °C for 90 s, and 72 °C for 1 min, followed by one cycle at 72 °C for 5 min. The PCR product was run on an 1% agarose gel and visualized under ultraviolet light. Full-length phosphatidylinositol glycosylase type S (PIGS) cDNA was cloned into the pEGFP-N1 vector, full-length polβ and pol$\beta\Delta$ cDNA were cloned into the pcDNA3.1 vector, and the constructs were sequenced.

Transfection into the 19.4 cell line

The mouse embryonic fibroblast 19.4 (pol$\beta-/-$) cell line was obtained from Dr. Samuel H. Wilson (NIEHS, Research Triangle Park, NC, USA). The cell line was grown in Dulbecco's minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and grown at 37 °C in a humidified incubator under 5% CO_2. The GFP vector containing the PIGS sequence was transfected into the 19.4 cell line using the transfection kit Fugene 6 (Roche, Indianapolis, IN, USA). One μg of PIGS plasmid DNA, alone or together with 1 μg of pol$\beta\Delta$ DNA, was mixed with 100 μL of serum-free medium containing 3 or 6 μL of Fugene 6 and transfected into the 19.4 cell line. After 3 d, cells were transferred to a new plate and fresh medium containing neomycin (50 μg/mL) was added. Stable cell lines were prepared by growing cells in selective medium for one and a half month. Similarly, vector DNA alone and WT polβ and pol$\beta\Delta$ together were transfected into the 19.4 cell line. These sub-lines were named 19.4Δ, 19.4 WT, 19.4 WT/Δ, respectively.

In vitro co-immunoprecipitation and Western blot analysis

Protein-protein interaction between WT polβ/pol$\beta\Delta$ and PIGS was confirmed by this method. Cells (19.4Δ, 19.4 WT, 19.4 WT/Δ cell lines) were homogenized with 1× isosmotic homogenization buffer and centrifuged serially using the endoplasmic reticulum (ER) enrichment kit (Imgenex, San Diego, CA, USA) to remove nuclear, mitochondrial, and cell debris. Total ER fractions were prepared by centrifuging the supernatant at 90,000 × *g* for 60 min at 4 °C. The pellet thus obtained contained the total ER and was dissolved in lysis buffer containing 1% NP-40 and 20 mM Tris-HCl (pH 8.6). The lysate (250 μL) was mixed with 25 μL of protein A-conjugated agarose beads and incubated for 3 h at 4 °C. After centrifugation for 12 s at 12,000 × *g*, the supernatant was taken and incubated with 2 μg of the primary antibody (anti-GFP) for 1 h at 4 °C with gentle shaking. Then 25 μL of protein A-conjugated agarose were added, and the precipitate was collected after washing three times with washing buffer as described earlier (Wang *et al.*, 2004). Thereafter 50 μL of 2 × SDS sample buffer were added and heated to 100 °C for 3 min, supernatant was collected, and proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) according to the standard protocol (Wang *et al.*, 2004). The proteins were transferred to a nitrocellulose membrane and immuno-detected by Western blotting with anti-polβ primary antibody and HRP-conjugated anti-mouse IgG antibody according to the instructions of the supplier of the antibodies (Molecular Probes, Grand Island, NY, USA).

Bioinformatics

To predict possible interactions between the known proteins as well as unknown proteins, we used the

STRING 9.05 software (www.string-db.org). Phosphatidylinositol glycan anchor biosynthesis, class S, and polβ were entered as the protein of interest separately. *Homo sapiens* was selected as organism. The required confidence was set at high (0.70).

Results

Initial screening

In the yeast two-hybrid (Y2H) system, pol$\beta\Delta$ in the vector pGBKT7 was used as a bait to identify protein-protein interaction. By colony PCR, transformed AH109 clones, positive for pGBKT7-pol$\beta\Delta$, were confirmed. After a second selection process, more than 300 HIS3 positive clones were obtained. The HIS3 positive clones were screened again in SD/–Ade/–His/ –Leu/–Trp/X-α-Gal medium to screen for ADE2, HIS3, and MEL1 expression, respectively. This particular screening allows selection of the proteins that positively interact with pol$\beta\Delta$ with high stringency, thus resulting in fewer colonies by reduction of the number of false positives. Colony growth and blue colour indicate an interaction between the hybrid proteins.

Identification of PIGS gene by Y2H

Four positive cDNA clones were eventually isolated. Three of them encoded XRCC1, MGC5306, and AP endonuclease I (APEndoI), respectively, and the fourth one was found to contain the partial sequence of phosphatidylinositol glycosylase type S (PIGS) that was identified by the BLAST program (Altschul *et al.*, 1997) (Fig. 1a). PIGS is a membrane protein (Kazuhito *et al.*, 2001) here shown for the first time to interact with pol$\beta\Delta$. The pGBKT7 vector has a tryptophan selection marker, the cDNA library has a leucine marker, and protein-protein interaction was confirmed by expression of the histidine marker. Positive colonies were selected by Leu$^-$His$^-$Trp$^-$Adenine$^-$medium with the β-galactosidase (β-gal) expression marker (Fig. 1a). Positive colonies detectable by β-gal expression were identified by cloning in pCR2.1TOPO and sequencing using appropriate primers. pCL1, encoding a full-length GAL4 (β-galactosidase 4), and pGBKT7-53 encode fusions between the GAL4 DNA-BD (binding domain) and AD (activation domain), were used as positive controls (Fig. 1a) and selected against Trp1 and kanamycin in yeast and bacteria, respectively.

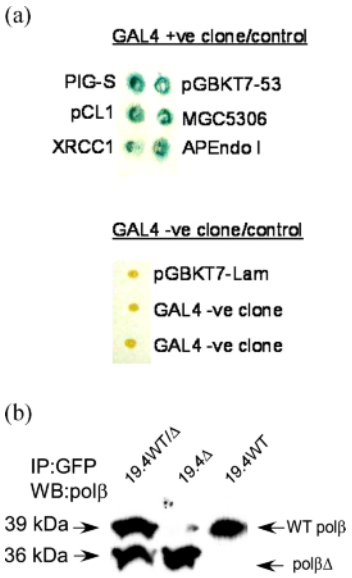

Fig. 1. (a) Interaction of WT polβ/pol$\beta\Delta$ and PIGS in the Y2H assay. In the upper panel, five positive controls were used: pGBKT7-53 (interacts with p53), MGC5306 (interacts with pol$\beta\Delta$), pCL1 (encodes full-length GAL4 protein), XRCC1 (known to interact with pol$\beta\Delta$), AP endonuclease I (known to interact with pol$\beta\Delta$). The lower panel represents a negative control (pGBKT7-Lamin C) and two negative clones. (b) Result of immunoprecipitation (IP)-Western blot analysis. Lane 1 shows two bands indicating the interaction of PIGS with both WT polβ and pol$\beta\Delta$. Lane 2 shows that the pol$\beta\Delta$ protein alone interacted with PIGS. Lane 3 shows that the WT polβ alone interacted with PIGS.

pGBKT7-Lam which encodes a fusion of the DNA-BD with human lamin C was used as negative control in this experiment (Fig. 1a). Both forward and reverse primers for PIGS cDNA were prepared using the published sequence. Then the full-length cDNA was made by RT-PCR using the above mentioned primer pair. Full-length human PIGS cDNA was cloned into the pEGFP-N1 vector.

Co-immunoprecipitation and Western blotting

The pEGFP-N1 vector containing the PIGS cDNA was co-transfected into the 19.4 cell line along with the pcDNA3.1 vector containing the WT polβ or pol$\beta\Delta$ cDNA, respectively, and transformed cell lines were selected against the proper antibiotic. The 19.4 cell line was the line of choice because it is polβ-deficient (Sobol *et al.*, 1996). Lysates of cells expressing PIGS-GFP/pol$\beta\Delta$ or PIGS-GFP/WT polβ were analysed by immunoprecipitation followed by Western blotting to

confirm the interaction of pol$\beta\Delta$ with the PIGS protein (anti-GFP mAb for IP, polβ primary antibody for immunodetection on Western blot). As only the PIGS cDNA was inserted in the GFP vector, and the other cDNAs were inserted in the pcDNA 3.1 vector, the immunoprecipitation by GFP antibody will selectively pull down PIGS-GFP along with associated protein(s). The result of this experiment indicates that the PIGS protein interacts with both the WT polβ and pol$\beta\Delta$ protein (Fig. 1b).

Discussion

Polβ is one of the smallest of the six mammalian DNA polymerases known so far. It is an evolutionarily highly conserved DNA repair protein which is essential for base excision repair (BER) function, and this 39-kDa protein is encoded by a single-copy gene and consists of a chain of 335 amino acids with two distinct functional domains of 8 kDa and 31 kDa (Wilson *et al.*, 2000). The gene is located on chromosome 8p12 (Wilson *et al.*, 2000). This region is frequently lost in prostate, breast, kidney, lung, bladder, and colon cancer, respectively. Previously, a total of 189 tumour samples along with 124 normal samples were screened for possible mutations within the polβ gene (Lundgren *et al.*, 1992; Muleris *et al*., 1996; Ochi *et al.*, 1986; Yaremko *et al*., 1995; Emi *et al*., 1992; Kovacs *et al*., 1987). More than 30% of the human tumours were found to have such mutations (Starcevic *et al*., 2004). More recently, two laboratories studied a total of 286 samples from human colon and ovarian cancers, and found polβ mutations in more than 56% of the samples (Donigan *et al*., 2012; Khanra *et al*., 2012a, b). Since polβ is a key enzyme in DNA repair, any perturbation in its expression or function can lead to increased mutation frequency and genomic instability (Cabelof *et al*., 2003). The polβ variants K289M and I260M have functional phenotypes that could be related to the etiology of human cancer (Lang *et al*., 2004; Dalal *et al*., 2005). The variant Val246 misincorporates nucleotides through altered DNA positioning in the active site (Dalal *et al.*, 2008). Expression of the E295K gastric carcinoma-associated polβ variant (Lang *et al*., 2007) interferes with BER in mouse cells and induces sister chromatid exchanges and cellular transformation (Dalal *et al*., 2008). An 87-bp deletion variant of polβ, found associated with breast, lung, colon, kidney, and prostate cancer, respectively, has been found to act as a dominant negative mutant (Bhattacharyya *et al*.; 1999, Chen *et al*., 2000). X-ray cross-complementing group 1 (XRCC1) protein is involved in the dominant negative activity of truncated polβ by forming a binary complex (Bhattacharyya and Banerjee, 2001). In order to determine whether any other protein(s) is (are) involved in this dominant negative action, we employed the Y2H system and a HeLa cDNA library.

Glycosylphosphatidylinositol (GPI) anchors many eukaryotic proteins to the cell membrane. The mammalian GPI transamidase is a complex of at least four subunits, GPI8, GAA1, PIGS, and PIG-T. Sequential addition of sugars and ethanolamine phosphates to phosphatidylinositol in the endoplasmic reticulum (ER), results in the synthesis of GPI (Udenfriend and Kodukula, 1995; Kinoshita and Inone, 2000). Preformed GPI is attached to proteins in the ER. Precursor proteins must have two signal peptides in order to be modified with a GPI. The first signal, *i. e.* the N-terminal signal peptide, is for translocation across the ER membrane, the second is the C-terminal anchor (GPI) attachment signal. The second signal peptide is recognized by the GPI transamidase, which cleaves the signal peptide from the protein and replaces it with GPI (Udenfriend and Kodukula, 1995; Sharma *et al*., 2000). Therefore, PIGS is an essential component of GPI transamidase and, in its absence, the carbonyl intermediate would not be generated. It is also possible, however, that lack of GAA1 or PIGS indirectly affects the signal recognition by causing a conformational change of the protein complex. It was reported that the GPI attachment signal peptide can be photo-cross-linked to a 60-kDa protein (Vidugiriene *et al.*, 2001). This can be GAA1 or PIGS. No information is presently available on the recognition of the other substrate, *i. e.* GPI. However, according to the STRING database [functional protein association network (Jensen *et al.,* 2009), www.string-db.org] there are eight predicted functional partners, namely glycosylphosphatidylinositol anchor attachment protein 1 (GPAA1), phosphatidylinositol glycan anchor biosynthesis, class K (PIGK), post-GPI attachment protein 1 (PIGAP1), phosphatidylinositol glycan anchor biosynthesis, class O (PIGO), plasminogen activator, urokinase receptor (PLAUR), phosphatidylinositol glycan anchor biosynthesis, class F (PIGF), phosphatidylinositol glycan anchor biosynthesis, class T (PIGT), and phosphatidylinositol glycan anchor biosynthesis, class U (PIGU) (Jensen *et al.,* 2009) (Figs. 2a, b, c). Figure 2a represents the evidence view, whereas Fig. 2b represents the confidence view according to which it was predicted that PIGS is strongly associated with PIGT, PIGK, PIGU, PIGO, and GPAA1, but in real-

ity the associations between PIGS and PIGT, GPAA1, and PIGU are weak (Kazuhito *et al*., 2001). Figure 2c represents the predicted (from experimental data, text mining data, or searches in different databases) action view. We did not find any suggested interaction between polβ and PIGS by using PIGS in the input form. Therefore, we put polβ in the protein input form and found eight proteins interacting with polβ. Figures 2d,

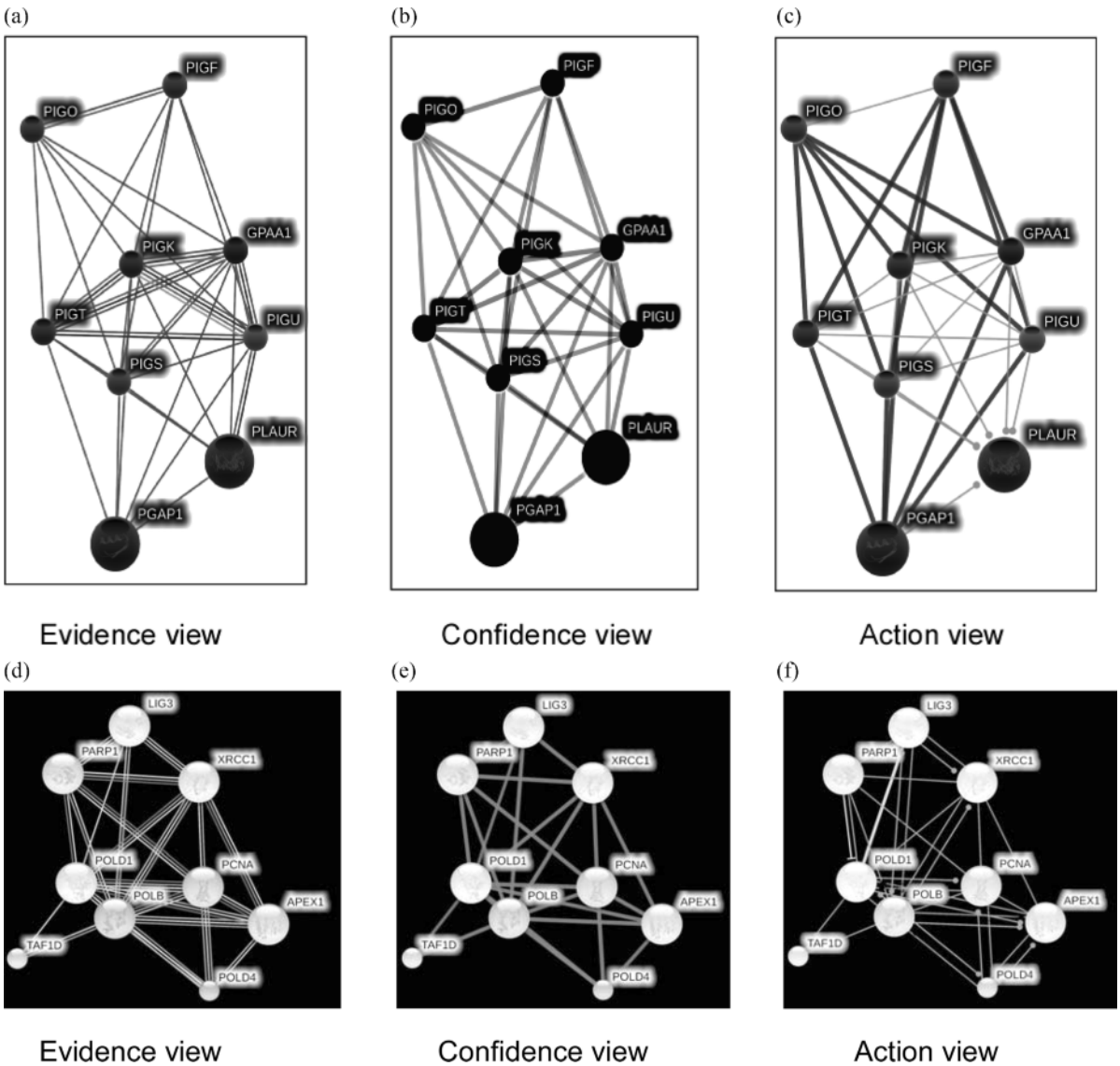

Fig. 2. Predicted functional partners of PIGS (a, b, c) and polβ (d, e, f) predicted by the STRING 9.05 program: (a) & (d) evidence view; (b) & (e) confidence view; (c) & (f) action view of PIGS and polβ, respectively. GPAA1, glycosylphosphatidylinositol anchor attachment protein 1 homologue; PIGK, phosphatidylinositol glycan anchor biosynthesis, class K; PIGAP1, post-GPI attachment protein 1; PIGO, phosphatidylinositol glycan anchor biosynthesis, class O; PLAUR, plasminogen activator, urokinase receptor; PIGF, phosphatidylinositol glycan anchor biosynthesis, class F; PIGT; phosphatidylinositol glycan anchor biosynthesis, class T; PIGU, phosphatidylinositol glycan anchor biosynthesis, class U; XRCC1, X-ray repair complementing defective repair in Chinese hamster cells; APEX1, APEX nuclease; POLD1, DNA polymerase delta 1; TAF1D, small nucleolar RNA, H/ACA box 32; LIG3, DNA ligase III; PCNA, proliferating cell nuclear antigen; PARP1, poly(ADP-ribose) polymerase 1; POLD4, DNA polymerase delta 4.

e, and f present the evidence, confidence, and predicted action view, respectively, for polβ interacting proteins. These proteins are: XRCC1 (X-ray repair complementing defective repair in Chinese hamster cells), APEX1 (APEX nuclease), POLD1 (DNA polymerase delta 1), TAF1D, (small nucleolar RNA, H/ACA box 32), LIG3 (DNA ligase III), PCNA (proliferating cell nuclear antigen), PARP1 [poly(ADP-ribose) polymerase 1]; POLD4 (DNA polymerase delta 4). Thus, we again did not find any suggestion of an interaction between polβ and PIGS. The probable reasons for this are the origins of the reference data mentioned above. In addition, the confidence score is predicted by a complex algorithm that finds and links the proteins from the same metabolic map in the KEGG database as evidenced by the different interacting proteins in case of polβ. These eight proteins are already known to interact with polβ. Therefore, the program could not find interaction between polβ and PIGS, as these two proteins belong to different metabolic pathways.

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