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A streamline proteomic approach for characterizing protein-protein interaction network in a RAD52 protein complex

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Abstract

Large-scale identification of protein-protein interactions (PPIs) in functional complexes represents an efficient route to elucidate the regulatory rules of cellular functions. While many methods have been developed to identify the PPIs associated with particular target/bait protein in complexes, little information is available about the interaction relationships among all components in a complex. Here, we have established a strategy of integrating proteomic identification of complex components with mammalian two-hybrid screening of their binary relationships to achieve information content of both breadth (*i.e.*, identifying all potential interacting partners of the protein of interest) and depth (*i.e.*, detailed mapping of the physical interactions of a subset of the identified and functionally related proteins) in characterizing protein complexes. In the initial phase of quantitative proteomic analysis of this streamline, the proteins that specifically complex with the target/bait protein were pulled down by immunoprecipitation and identified by mass spectrometry (MS)-based “dual-tagging” quantitative proteomic approach. In the second phase of in-depth characterizations of binary relationships, the physical interactions of a subset of functionally closely related complex components are mapped by mammalian two-hybrid assay. The screening for binary relationships of complex components not only serves as a validation of the first phase of proteomic identification, but also further deepens the understanding of the protein complex of interest. With this streamlined approach, we studied the protein complexes that are associated with a DNA recombination protein RAD52. In the initial phase, multiple proteins both known and unknown to interact with RAD52 were identified by the “dual-tagging” proteomic method. In the second phase, a complex protein-protein interaction network, which may play important roles in coordinating the activity of DNA repair with that of cell division, was defined by the mammalian two-hybrid assay.

Keywords

DNA repair; mammalian two-hybrid; mass spectrometry; protein complex; protein-protein interactions; interaction network; proteomics; RAD52

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Introduction

Protein-protein interactions play important roles in regulating signal transduction and other cellular pathways in all living organisms. Thus, identification and characterization of multiprotein complexes including complex components and their interacting relationships involving in cellular pathways are critical steps for elucidating the regulatory mechanisms in different physiological processes in cells. There are many ways to investigate protein complexes and protein-protein interactions, and each method has its own strength and drawbacks.¹ Traditionally, highly purified protein complexes can be obtained by liquid chromatography through multiple columns and multiple steps of purification.² The drawback of this method is that it requires a large quantity of starting materials and is usually time-consuming. Yeast two-hybrid screening is now commonly used to identify the interactions of possible gene products.³ The strength of the method is that the screening can be performed in the high throughput and systematic manner.^{4, 5} However, there are some well-appreciated caveats to the method, including high level of false-positives and false-negatives in identifications.⁶ At protein level, tandem affinity purification (TAP) has been widely used in the isolation and purification of protein complexes at close to natural level. This method was originally designed to identify protein complexes in yeast,⁷ and was later modified for mammalian cells.^{8, 9} However, its applications in mammalian cells are not as successful as in yeast. One of the factors that is causing problems in applying this method in mammalian cells is the existence of untagged version of the endogenous target/bait protein which could lead to low yield of the protein complex isolated through TAP tagged bait.⁸ In addition to the conventional biochemical methods, various proteomics-based methods have also been developed in the past years to address these concerns.^{10, 11}

By integrating stable-isotope labeling, affinity purification, and mass spectrometry, we have previously developed a quantitative “dual-tagging” proteomic approach that allows unambiguous identification of protein complexes from cells after a single step affinity pull-down under mild purification conditions.^{12–14} In this design, the bait protein is epitope-tagged for affinity isolation of the complex (epitope-tagging), and in parallel the whole proteome of the cells expressing the epitope-tagged bait at physiological relevant levels is labeled with stable isotope-enriched heavy amino acids (isotope-tagging). In mass spectrometric measurements, the heavy amino acids incorporated in the cellular proteins provide “in-spectra” quantitative markers so that those proteins showing the increases in their abundances bound to the bait can be unambiguously identified after single-step affinity purification. Because this method does not require multiple steps of binding and washing, it substantially increases the chances of identifying the proteins that weakly associate with the target protein in a complex. Nonetheless, as other affinity-based methods, such a profiling experiment can only provide a list of target/bait-specific interacting proteins without the information about the physical interaction relationships or binary interactions among the complex components in a network.

To not only identify target-specific interacting partners but also reveal their interaction network in a complex, here we present an integrated strategy of quantitative proteomics for complex component identification with mammalian two-hybrid assay for establishing their

interaction relationships. Using this approach, we studied the protein complexes that associated with DNA recombination protein RAD52. In the initial phase, multiple RAD52-interacting proteins both known and unknown were identified by our “dual-tagging” proteomic method. In the second phase, a complex protein-protein interaction network, which may play important roles in coordinating the activity of DNA repair with that of cell division, was defined by the mammalian two-hybrid assay.

Materials and Methods

Plasmids, Stable Cell Line and Cell Culture

Human RAD52 coding sequence was in-frame cloned into the XhoI and BamHI sites of plasmid pLXSP with a c-Myc tag at the N-terminus. Plasmid pLXSP was derived from plasmid pLXSN (BD Biosciences, NJ) by replacing the *Neo^r* gene with a *Puro^r* gene. The construct was transfected into human kidney 293T cells (ATCC, VA) using the calcium-phosphate method, and the cells were selected in D-MEM (Invitrogen, CA) supplemented with 10% FBS, 1% penicillin and streptomycin, and 1.2 µg/ml puromycin (Sigma, MO). The expression of c-Myc-tagged RAD52 was confirmed by Western blotting. Whereas the parental 293T cells (control cells) were cultured in regular unlabeled D-MEM medium, the cells expressing c-Myc-tagged RAD52 (c-Myc-RAD52 cells) were maintained in the D-MEM containing leucine-*d*₃ to isotope label the proteome.

Protein Purification

Equal numbers of parental control cells and the c-Myc-RAD52 cells (5×10^8 cells/each) were harvested, and washed twice with cold PBS. Total cellular protein was recovered by incubating the control and c-Myc-RAD52 cells respectively in 5 packed cell pellet volumes of lysis buffer (10 mM Hepes-NaOH, pH7.9, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, and 1% NP-40) supplemented with protease inhibitor cocktail (Roche, IN) and phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM sodium fluoride and 10 mM β-glycerophosphate) on ice for 30 min, and douncing 20 times on ice. After centrifugation (30,000g for 15 min at 4°C), 4 N NaCl were added to the cleared lysate to a final concentration of 150 mM. The protein extract from each cell population was incubated with 150 µl of anti-c-Myc agarose beads (Sigma, MO) at 4°C for 2 hours with end-to-end rotation. The beads were then washed 4 times (4 ml each time) with wash buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, and 0.1% NP-40) plus the protease inhibitor cocktail and phosphatase inhibitors. The bound proteins were eluted with a buffer containing 100 mM ammonium hydroxide, and 1 M NaCl, pH 11.75. The eluted proteins from two cell populations were then mixed, concentrated with trichloroacetic acid precipitation, and separated on a 4–20% SDS-PAGE gel. After staining with coomassie brilliant blue, the entire lane of the gel was cut into 25 slices for LC-MS/MS analysis.

LC-MS/MS Analysis and Database Searching

In-gel digestion, LC-MS/MS analysis, and database searching were performed as described previously.^{12, 15} Database (NCBIInr, 2004-01-20), searching program (Mascot, version 2.0), and the parameters used for database searching were the same as described previously.¹²

Mammalian Two-hybrid Analysis

Vectors encoding Gal4 DNA binding domain (BD), and transcription activation domain (AD) were from a mammalian two-hybrid assay kit (BD Bioscience, NJ). The Gal4 GFP reporter plasmid was kindly provided by Dr. Toshi Shioda (Massachusetts General Hospital Cancer Center). The coding sequences of the two genes of interest were in-frame inserted into the BD and AD vectors respectively, and the two constructs were co-transfected into 293T cells with the Gal4 GFP reporter plasmid by calcium phosphate method (2 µg each plasmid in a 60 mm plate). The negative control was performed by co-transfection of 293T cells with the Gal4 GFP reporter plasmid, and the BD and AD constructs in which the BD and AD were fused to two proteins that do not interact. The expression of GFP was analyzed by flow cytometry.

Flow Cytometry Analysis

The exponentially growing cells were harvested, and washed once with PBS. GFP fluorescence was measured by using a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences, NJ). 10,000 events were recorded for each measurement. Four independent sample preparations were performed for each data point.

Results

The Strategy for Characterizing Interaction Network in a RAD52-associating Complex

Our two-phase, streamlined strategy for mapping complex protein-protein interactions is illustrated in Figure 1A, and the “dual tagging” proteomic method is shown in Figure 1B. In the first phase of the two-tiered approach, a “dual tagging” proteomic method is used to identify all the proteins that complex with the bait protein RAD52. In the second phase, a subset of functionally closely related complex components is chosen, and the physical interactions are mapped by the mammalian two-hybrid assay.

Multiple Proteins Were Identified to Interact with RAD52 in a Functional Complex

Using the “dual tagging” proteomic procedures (Figure 1B), we isolated the protein complexes that associated with RAD52 from human kidney 293T cells. Of the total of 385 proteins identified, 28 proteins were selectively enriched with the anti-c-Myc beads by a factor of at least 2.5 (Table 1). Reproducibility was assessed according to the method described previously.¹² The selectively enriched proteins were distributed in several biologically functional categories (Table 1). RPA14 is a subunit of RPA, which has been shown to play important roles in DNA replication and DNA repair.¹⁶ Actin is an abundant protein, and is known to play vital roles in the cytoskeleton. Together with profilin I, which may be predominantly associated with monomeric actin in cells,¹⁷ actin has been shown to play important roles in cytokinesis.^{18, 19} The 14.3-3 proteins have multiple biological functions, including DNA repair.²⁰ Protein oxidation has been shown to be highly related to DNA damage, so the identifications of the three proteins related to protein oxidation/reduction (peroxiredoxin I, thiol-specific antioxidant protein, and Horf6) suggest that RAD52 may play important roles in the oxidative stress-mediated DNA damage and repair.

Mammalian Two-Hybrid Screening Validates the Accuracy of the Proteomic Data, and Further Establishes a RAD52 Protein Complex Whose Components Span from DNA Repair, to DNA Replication, to Cytokinesis

It has been known that the RAD52 epistasis group consists of RAD51, RAD52, and RPA. RPA contains three members, RPA70, RPA32, RPA14, and the three members form a trimeric structure.¹⁶ Part or all of the three subunits of RPA have been shown to interact with RAD52 in yeast²¹ and mammalian cells.^{22, 23} RPA was also shown to interact with RAD51 through RPA70.²⁴ RAD52 has been shown to interact directly with RAD51.^{25, 26} Actin is known to interact with profilin I, and the two proteins form a heterodimer.²⁷ HSP70 is a chaperone protein. Interestingly, it has been reported that HSP70 interacts specifically with an actin-profilin I heterodimer, but not with individual actin or profilin I.²⁸ The proteomic data obtained in the present study together with the previously reported results suggest that RAD52, actin, profilin I, RAD51, HSP70, and members of RPA protein (RPA70, RPA32, and RPA14) may form a complex protein-protein interaction network in cells. In order to explore this possibility, we decided to use mammalian two-hybrid assay to systematically determine the binary protein-protein interactions for all the related factors, including the three proteins that were not identified by our proteomic method in the present study: *i.e.*, RAD51, RPA70, and RPA32.

As demonstrated in Figure 2, multiple protein-protein interactions reported previously were re-confirmed by our mammalian two-hybrid approach in the present study, including the interactions of actin-profilin I (Figure 2B),²⁷ RAD51-RPA70 (Figure 2E),^{24, 29} and the trimeric structure of RPA (RPA70, RPA32, and RPA14)¹⁶ (Figure 2E). The confirmations of multiple previously reported protein-protein interactions served as a good validation of our two-hybrid approach. There were four cases of inconsistency between our mammalian two hybrid data and the previously reported results. Compared with the respective controls, the protein pairs of RAD52-RPA70, RAD52-RPA32, RAD52-RPA14, and RAD52-RAD51 failed to induce significantly higher levels of GFP in the present study (Figure 2A). However, those protein pairs have been shown by other research groups to interact with each other.^{21–23, 25, 26} The weak GFP fluorescence intensity for the RAD52-RPA70, RAD52-RPA32, and RAD52-RPA14 interactions were possibly caused by the interference of the N-terminus of RPA, which was observed in yeast.²¹ Noticeably, while the interactions that are consistent with the published results (*i.e.*, the pairs of actin-profilin I, RPA70-RAD51, RPA70-RPA14, RPA32-RPA14),^{16, 24, 27, 29} all induced strong GFP expressions (Figure 2B, and 2D), the inconsistent protein pairs induced very weak GFP expression (Figure 2A). These results suggest that special cautions need to be taken to interpret the mammalian two-hybrid data when only low levels of reporter gene expression are achieved (for both potential false-positive and false-negative results). In addition to the previously reported interactions, we also identified multiple new interactions among the components in this protein complex. Most noticeably, members of RPA, which are known to interact with RAD52,^{21–23} were found to interact with β -actin, and RAD51 was found to interact with both β -actin and profilin I (Figure 2). These specific interactions clearly indicate that the identification of actin as a component of RAD52 complex by our proteomic approach (Table 1) is valid, though actin is an abundant cellular protein. Most importantly, the interactions between RAD51/RAD52/RAP, and β -actin/profilin I have linked the activities of DNA

repair with those of cytokinesis. We have summarized our binary protein-protein interaction data in Figure 3, which demonstrates that these eight proteins form a complex protein-protein interaction network.

Immunoprecipitation and Western Blot Analyses further Validate Parts of the Proteomic and Mammalian Two-Hybrid Results

Consistent with the quantitative proteomic data (Tables 1), Western blotting showed that RPA14 and HSP70 were co-precipitated with RAD52 (Figure 4A). As actin, HSP70 is abundant cellular protein. The immunoprecipitation/Western results here reassured that HSP70 is a genuine component of RAD52 complex, but not a contamination. We also explored the potential reasons causing the inconsistency between our proteomic data and the previously reported results. For example, RAD51 has been known to interact with RAD52.^{25, 26} However, RAD51 was not in the list of our proteomic proteins identified (Table 1). When large amounts of starting materials (same as used for proteomic analysis) were used for immunoprecipitation, and longer exposure times (20 min) were used in Western blotting, we did detect a weak RAD51 band (Figure 4B). Thus, we assume that the failure of detecting RAD51 as a RAD52 interacting protein in our proteomic analysis might result from low quantity of RAD51 in the immunoprecipitate.

Discussion

In this study, we have used a strategy of integrating proteomic identification with mammalian two-hybrid assay to analyze the RAD52 protein complex. Using a quantitative proteomic strategy (Figure 1B), we identified multiple proteins associating with RAD52 (Table 1). Most of the proteins identified in the present study have not been reported previously. The identified proteins are distributed in several different biologically functional areas (Table 1). We then picked up the proteins that function in DNA repair, DNA replication, and cell division for further analysis: the data from literature and the proteomic results in the present study suggest that the proteins function in these areas are to interact with each other to form a complex protein-protein interaction network. We performed mammalian two-hybrid assays to systematically analyze the binary protein-protein interactions for a set of closely related factors, including RAD52, RAD51, β -actin, profilin, HSP70, and three members of RPA protein.

In the mammalian two-hybrid assays, in addition to confirming the already reported protein-protein interactions, we identified multiple new interactions that have not been observed previously by other approaches. Among these interactions identified, the interactions that link DNA repair to cytokinesis are of particular interesting. The two-hybrid results demonstrate that RPA (which is known to interact with RAD52) interact with β -actin, and RAD51 interacts with both β -actin and profilin I (Figures 2 and 3). After the anaphase of mitosis in cell division, a contractile ring forms in the middle of the elongated cells. When the ring constricts, the elongated cell will be cleaved into two daughter cells.¹⁹ Actin is a major component of the contractile ring, and profilin I has been shown to play an important regulatory role in the formation of the ring.¹⁸ Profilin I is an essential protein for cell survival and cell division, and Pfn1-null mice are not viable.³⁰ In fission yeast

Schizosaccharomyces pombe, cells harboring Pfn1-null gene cannot form the contractile ring, are arrested in the cell cycle at cytokinesis, and are not viable.³¹ On the other hand, profilin I was also found to play a role in DNA metabolism. For example, partial loss of profilin I expression correlates with the tumorigenic phenotype, suggesting that profilin I functions as a tumor suppressor.³² The dual functions of profilin I in both cytokinesis and DNA metabolism, and the physical interactions between the important proteins functioning in DNA repair (RAD51 and RAD52) and the essential proteins functioning in cytokinesis (actin and profilin I) (Figures 2 and 3), suggest that DNA repair and cytokinesis may be two functionally inter-connected and inter-regulated cellular processes. Consistent with this notion, it was recently reported that BRCA2 deficiency impairs the completion of cell division by cytokinesis, and the BRCA2-deficient tumors might be caused by the abnormal cytokinesis activities.³³ BRCA2 is an important DNA repair protein, and is required for the repair of the DSBs by recombination.³⁴ Given the high fidelity of DNA repair, DNA replication in cells, and separation of DNA and other cellular materials into two daughter cells, eukaryotic cells may have evolved molecular mechanisms that coordinate the activities of DNA repair with those of cytokinesis, so the cells containing un-repaired DNA or other genetic errors will not be passed into daughter cells. If this mechanism truly exists, the RAD52 protein complex defined in the present study, which contains the proteins functioning the DNA repair, DNA replication, and cytokinesis, may be one of the important components of this coordination machinery.

Proteomic analysis is powerful in identifying interacting partners of the protein of interest, but not good for detailed mapping of the physical interactions of proteins. On the other hand, the two-hybrid system is useful for analyzing detailed physical interactions of proteins, but not good for screening binding partners of the protein of interest at the proteome level. By coupling the two methods together, we have demonstrated in this study that information content of satisfactory breadth (*i.e.*, identifying all potential interacting partners of the protein of interest; Table 1) and depth (*i.e.*, detailed mapping of the physical interactions of a subset of the identified, functionally related proteins; Figures 2 and 3) on protein complex of interest can be obtained simultaneously.

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Figure 1.

Integration of “dual tagging” proteomic identification and mammalian two-hybrid screening for mapping complex protein-protein interactions. A, a two-phase, streamlined strategy. In the first phase of quantitative proteomic analysis, the proteins that complex with the bait protein are identified. In the second phase of in-depth characterizations, a subset of functionally closely related complex components is chosen, and the physical interactions are mapped by mammalian two-hybrid assay. B, the schematic of the “dual-tagging” proteomic approach for identifying proteins that interacting with RAD52. The parental cells are cultured in regular unlabeled medium (green dish), and the cells stably expressing c-Myc-RAD52 are grown in the leucine- d_3 containing medium (red dish). Equal numbers of unlabeled and labeled cells are harvested, lysed, and incubated with equal amount of anti-c-Myc beads. Proteins eluted from the beads are mixed and separated by SDS-PAGE, digested with trypsin, and the resulting peptides analyzed by mass spectrometry. The relative intensity of the paired peak reflects the binding profiles of the parental protein to the bait protein RAD52.

Figure 2.

The binary protein-protein interactions among RAD52, RAD51, β -actin, profilin I, HSP70, RPA14, RPA32, and RPA70 detected by mammalian two-hybrid assays. To determine the protein-protein interactions, two genes of interest were first in-frame fused to Gal4 DNA binding and transcription activation domain respectively. After transfection of the two constructs with a GFP reporter plasmid, the expression of GFP was measured by a flow cytometer. Proteins X and Y are two proteins that are known to do not interact, and used as a negative control. Each bait and prey fusion protein with empty vector were also tested to serve as additional controls. * denotes that the interaction that has been reported previously by other research groups. # denotes that the interaction was newly identified in this study. Each data point is the average of the measurements of three to four independent sample preparations.

Figure 3.

The eight proteins form a complex protein-protein interaction network. The blue line (

) indicates that the interactions induced weak GFP expression (1.2 – 2.0 fold of the highest fluorescence intensity of the negative controls). Red line (

) indicates that the interactions induced strong GFP expression (>2 fold of negative control). The interactions that were reported previously, but not identified by mammalian two-hybrid assays in the present study are also included as weak interaction (see text for details).

Figure 4.

Immunochemical validation of the proteomic and two-hybrid data. Proteins from parental cells as well as c-Myc-RAD52 cells were immunoprecipitated (IP) with anti-c-Myc beads, and the immunoprecipitated proteins were analyzed by Western blotting (WB). The co-immunoprecipitation of RPA14 and HSP70 with RAD52 (7×10^7 cells/each) (A), and RAD51 with RAD52 (5×10^8 cells/each) (B) is shown.

TABLE 1

List of the proteins identified to be associated with RAD52 by a dual-tagging proteomic procedure

Category	Accession no.	Protein name	H : L ratio ^a	S.D.	No. of peptides
Mitosis, DNA replication and repair	gi 863018	recombination protein RAD52	28.4	5.8	10
	gi 4506587	replication protein RPA14	19.8	4.8	3
	gi 999511	profilin I	13.1	0.4	2
	gi 28336	β-actin	12.9	3.3	8
	gi 4507953	14-3-3 ζ	7.2	0.8	2
	gi 5803225	14-3-3 ⅆ	10.9	6.1	2
Heat shock proteins	gi 31542947	heat shock 60kDa protein 1	8.1	0.7	5
	gi 462325	Heat shock 70 kDa protein 1	2.5	0.3	14
	gi 5729877	heat shock 70kDa protein 8 isoform 1	2.7	0.7	7
	gi 72219	heat shock protein 90 α	13.1	2.4	4
Oxidation related	gi 4505591	peroxiredoxin 1	7.5	1.5	3
	gi 438069	thiol-specific antioxidant protein	11.7	3.5	2
	gi 3318841	Horf6	14.2	1.2	2
Translation factors	gi 62896605	eukaryotic translation elongation factor 1 α	7.8	2.4	3
	gi 4503481	eukaryotic translation elongation factor 1 γ	3.6	0.1	3
	gi 1085404	translation elongation factor eEF-1 δ	2.6	0.1	2
Carbohydrate, and energy metabolisms	gi 4557032	lactate dehydrogenase B	16.8	1.2	2
	gi 31645	glyceraldehyde-3-phosphate dehydrogenase	6.4	NA	1
	gi 180555	creatine kinase-B	7.3	1.4	3
	gi 4503571	enolase 1	9.8	1.6	2
	gi 28595	aldolase A	24.7	NA	1
Ribosomal proteins	gi 4506679	ribosomal protein S10	3.4	NA	2
	gi 4506695	ribosomal protein S19	3.2	1.3	4
Others	gi 10863927	peptidylprolyl isomerase A	12.8	2.2	3
	gi 1065361	ADP-ribosylation factor 1	3.6	NA	1

Category	Accession no.	Protein name	H : L ratio ^a	S.D.	No. of peptides
	gi 5174477	α-tubulin	2.9	0.3	6
	gi 25470890	DAZ associated protein 1 isoform a	4.3	NA	2
	gi 34335134	SEC13-like 1 isoform b	4.6	NA	2

^aHeavy : Light isotope ratio calculated as the ratio of leucine-*δ*3-labeled peptide to unlabeled peptide.