One- plus Two-hybrid System, a Novel Yeast Genetic Selection for Specific Missense Mutations Disrupting Protein/Protein Interactions*

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To facilitate analysis of protein/protein interaction interfaces, we devised a novel yeast genetic screening method, named the “one- plus two-hybrid system,” for the efficient selection of missense mutations that specifically disrupt known protein/protein interactions. This system modifies the standard yeast two-hybrid system to allow the operation of dual reporter systems within the same cell. The one-hybrid system is first used to select the intact interacting partner (prey), resulting in the positive selection of informative missense mutants from a large library of randomly generated mutant alleles. Then in a second screening step, interaction-defective prey mutants for a given protein are selected using the two-hybrid reporter system among the isolated missense mutants. We used this method to characterize the interactions between unliganded nuclear receptors (NRs) and the conserved motif within the bipartite NR interaction domains (IDs) of the NR corepressor (N-CoR) and identified the specific residues of N-CoR-IDs required either generally for optimal NR binding or to interact with a particular NR. This efficient and rapid method should allow us to quickly analyze a large number of interaction interfaces. Molecular & Cellular Proteomics 6:1727–1740, 2007.

Protein/protein interactions are critical to almost all biological processes. Therefore, identification of potential protein/protein interactions is believed to be an essential step in understanding the molecular mechanism of a given cellular event. Yeast two-hybrid systems are standard methods widely used to identify novel protein/protein interactions and to analyze the structure-functional relationships of known interactions between two proteins (1–3). This system was first devised by Fields and Song (1) on the basis that transcription activation domain. In the yeast two-hybrid system, the first protein of interest is expressed as a DBD fusion (“bait”), whereas the second protein of interest is expressed as a hybrid protein with an activation domain (“prey”). Interaction between bait and prey leads to the assembly of the two-hybrid proteins onto promoter-binding sites for the DBD, resulting in the induction of the reporter gene by the functionally reconstituted activator protein.

Although yeast two-hybrid methods are suitable for the sensitive detection of protein/protein interactions in vivo, these interactions need to be tested in the relevant biological system. For such functional analyses, identification of missense mutations that specifically disrupt the interaction with a given partner (loss-of-interaction mutants) is very helpful for determining the functional significance and molecular basis of the interaction. Modified yeast two-hybrid systems, known as “reverse two-hybrid” or “split hybrid” systems, have been developed to rapidly isolate mutant prey proteins that are specifically defective in the interaction with a potential partner (4–7). The “reverse two-hybrid system” uses a URA3 reporter gene as a counterselective marker that, as with the yeast two-hybrid system, is activated by bait/prey interactions. In this system, expressed Ura3p inhibits growth of the cells on medium containing 5-fluoroorotic acid, which Ura3p converts into a toxic compound (5). In the “split hybrid system,” the two-hybrid interaction of two proteins results in the expression of the TetR repressor. TetR subsequently binds to the tet operators, blocking expression of the HIS3 reporter gene and preventing yeast growth in medium lacking histidine (7). Thus,

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1 The abbreviations used are: DBD, DNA-binding domain; NR, nuclear receptor; N-CoR, nuclear receptor corepressor; SMRT, silencing mediator for the retinoid and thyroid hormone receptor; LBD, ligand-binding domain; AF2, activation function 2; RD, repression domain; ID, interaction domain; CoRNR, corepressor nuclear receptor; GBD, Gal4 DNA-binding domain; N1, N-CoR-ID1; N2, N-CoR-ID2; S1, SMRT-ID1; S2, SMRT-ID2; RAR, retinoic acid receptor; TR, thyroid hormone receptor; RXR, retinoid X receptor; LXR, liver X receptor; PPAR, peroxisome proliferator-activated receptor; UAS, upstream activating sequence; 3AT, 3-amino-1,2,4-triazol; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; HA, hemagglutinin; HEK, human embryonic kidney.
both systems are specifically designed for positive selection of interaction-defective mutants using specific counterselection markers, and they can be used to identify events that dissociate protein/protein interactions (8).

Despite the advantages of these modified two-hybrid methods in selecting non-interactors, they have two major technical obstacles, which prohibit a wider usage of these methods. First, they were not designed to positively select for informative missense mutations among interaction-defective alleles isolated by split hybrid or reverse two-hybrid screening. In the split hybrid system, for example, the prey protein is expressed as a triple fusion between the VP16 activation domain and β-galactosidase to allow identification of uninformative mutations (truncation) as white colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) plates, corresponding to a negative color selection for the missense mutants (7). In the reverse two-hybrid systems, several alternative strategies have also been used to isolate non-interacting full-length alleles by adding easily detectable C-terminal fusions such as green fluorescent protein (9–11) or an epitope tag (12) to prey proteins. In these systems, interruption of the prey by an uninformative mutation is indicated by green fluorescence or immunoblot analysis for the epitope. Accordingly these methods cannot positively select the missense mutants. Because greater than 97% of counterselected non-interacting alleles are expected to contain uninformative mutations (6, 7, 13), isolation of the small portion of full-length alleles from a large library of mutant alleles remains a major technical hurdle. Second, it has been reported that a high background of false positives (more than 65%) is generally produced during the first counterselection step of reverse two-hybrid screening (12, 13). This might be due to the spontaneous inactivation of the counterselection system itself, including the loss of marker gene function or bait plasmid. All these observations strongly suggest that negative selection of full-length allele (detectable C-terminal fusion) after counterselection of non-interactor (reverse two-hybrid screening) is not an effective strategy to isolate specific missense alleles from a randomly generated mutant library.

Recently Gray et al. (13) reported an alternative method designed to generate a full-length, high coverage allele library based on in vitro recombinational cloning and positive selection of full-length clones in Escherichia coli. In this method, mutagenized prey proteins are expressed as a C-terminal fusion of the kanamycin resistance gene to confer antibiotic resistance in E. coli to full-length clones. Accordingly this system requires two in vitro recombinational cloning steps and generation of a full-length allele library in E. coli prior to isolating non-interacting mutants using the yeast reverse two-hybrid system. In addition to this technical complication, more than 60% of non-interactors recovered from reverse two-hybrid screenings have turned out to be false positives. All these technical problems explain why we still need a more rapid and efficient system for the positive selection of full-length alleles as well as a simple manipulation method for allele library generation.

Many nuclear receptors (NRs) function as ligand-regulated transcription factors. NRs control numerous critical biological events, including development, growth, differentiation, and homeostasis (14–16). In the absence of ligand, the apo form of the NR recruits transcriptional corepressor proteins, such as the nuclear receptor corepressor (N-CoR) and silencing mediator for the retinoid and thyroid hormone receptor (SMRT), to repress the transcription of target genes (17, 18). N-CoR and SMRT are modular proteins that contain three independent autonomous repression domains (RDs) and two separate NR interaction domains (IDs) located at their N-terminal and C-terminal regions, respectively (19, 20). IDs have been shown to directly interact with the ligand-binding domains (LBDs) of the apo form of non-steroid receptors as well as antagonist-bound steroid receptors (21–25).

As the molecular determinants required for NR interactions by corepressors, the corepressor nuclear receptor (CoRNR) box and the related extended helix motif of the consensus sequence LXI(I/H)LXX(L/I), where X is any amino acid, have been identified within IDs of N-CoR and SMRT (26–28). Perissi et al. (28) have suggested that the corepressor motif adopts a three-turn α-helix, as compared with the two-turn helix NR-interacting motif (LXLL) of coactivators, and interacts with specific residues in the LBD pocket that largely overlap with those residues required for binding to the coactivator LXLL motif. Interestingly NRs have the ability to distinguish the IDs of N-CoR and SMRT for their specific interactions, determining corepressor preference (N-CoR versus SMRT) or the ID preference (ID1 versus ID2) of a given NR (29, 30). For example, TR preferentially recruits N-CoR via its specific interaction with ID3, which is absent in SMRT (31, 32), and the orphan NR RevErb selectively interacts with N-CoR-ID1 (30). In contrast, the retinoic acid receptor (RAR) binds to ID1 of SMRT, whereas the AF2 deletion form of the retinoid X receptor (RXRΔAF2) and liver X receptor (LXR) interact exclusively with the ID2 of corepressors (30, 33). Although it is quite likely that some residues within or outside of the extended helix motifs may contribute to these specificities, the molecular basis of this selective and specific binding of IDs of N-CoR and SMRT to different NRs is not fully understood.

Here we report a novel yeast genetic method, the “one-plus two-hybrid system,” that efficiently selects for missense mutations that specifically disrupt a known protein/protein interaction. This system consists of dual reporter systems in which a one-hybrid system is first used for a positive selection of full-length alleles from a randomly generated mutant library and a two-hybrid reporter system is used for the second screening of interaction-defective mutants among the isolated missense mutants. In a first demonstration of this method, we successfully identified the specific amino acid residues of N-CoR-IDs that are either generally required for optimal NR binding (general determinants) or involved in the preferential interaction with a particular NR (specific determinants).
was performed using the Mn2+-mediated PCR mutagenesis method (42) with 30 rounds of PCR (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s) using Taq polymerase and pRS424UB42-N1-GBD or pRS424UB42-N2-GBD as templates in the presence of 0.1 mM MnCl2. Two oligonucleotides were designed as universal primers for mutagenic PCR of the prey gene: forward primer (oligo-SF), 5’-CC AGC TTC TTG CTG AGT GGA GAT G’-3’; reverse primer (oligo-SR), 5’-CGG TTT TTG TTT GGA GCA GCA C-3’, corresponding to an N-terminal portion of GBD. The mutagenic PCR products obtained with these primers commonly contained about 100 bp of flanking region at each end with sequence identities to the gap plasmid prepared by EcoRI/BamHI digestions of pRS424UB42-GBD.

One- plus Two-hybrid Screening—To construct the mutant cell library of randomly mutated N1 or N2, we used a single step method based on the in vivo gap repair (43). Each of the mutagenic PCR products (1 µg) was co-transformed with the gap plasmid (4 µg) into strain YOK400 carrying the pSH18-34 reporter as well as the bait plasmid pRS325LexA-RAR or pRXPLAF2. His+ transformants were obtained after a 4-day incubation at 30 °C on glucose medium containing 10 mM 3AT and lacking histidine. More than 1000 transformants were picked onto plate medium containing X-gal but lacking histidine, and the yeast colonies showing a white or weak blue color were isolated from the wild-type blue colonies. These candidates were retested for color phenotype by streaking on X-gal plates and subjected to the liquid β-galactosidase assay for the selection of non-interacting mutants based on quantitative data. Prey vectors were rescued from the mutant candidates and individually transformed into the EGY48 strain expressing LexA-NR (for the test of two-hybrid interaction) as well as the EGY-LG strain containing the pLGSD5 plasmid (UASgal4-LacZ reporter) to check for intact GBD (one-hybrid test). Prey plasmids that still conferred blue color in the one-hybrid test and white color in the two-hybrid test were chosen as the final mutant candidates and subjected to DNA sequencing to identify the mutational site(s).

Preparation of Whole Cell Extracts and Immunoblot Analysis—All protein manipulations were carried out at 4 °C in the presence of protease inhibitors (1 mM PMSF, 2 mM benzamidine, 5 µg/ml leupeptin, 5 µg/ml pepstatin). Yeast protein extracts were prepared by cultivating the cells in the appropriate medium to midlog phase followed by glass bead disruption as described previously (44). Preparation of the whole cell extract from transiently transfected HEK293 cells was performed as described previously (45). Protein concentrations of the whole cell extracts were determined using a Bradford protein assay kit (Bio-Rad). Equal amounts of protein samples (40 µg for the yeast extract and 80 µg for the mammalian cell extract) were separated on either 10% or 12% SDS-polyacrylamide gels and transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences). Membranes were probed with monoclonal antibodies against LexA (sc-7544; Santa Cruz Biotechnology), polyclonal antibodies against GBD (sc-510; Santa Cruz Biotechnology), and monoclonal antibodies against hemagglutinin (HA) (12CA5) to detect the expression levels of the target proteins. The blots were developed with the Amersham Biosciences ECL kit according to the instruction manual.

In Vitro GST Pull-down Assay—pGEX4T-1 derivatives expressing the wild-type and N1 or N2 mutants were introduced in E. coli strain DH5α. GST alone or GST-fused proteins were overexpressed by induction for 3 h in the presence of 0.25 mM isopropyl β-D-thiogalactopyranoside and purified with the use of glutathione-agarose beads (Promega) according to the manufacturer’s instructions. NR proteins were synthesized by in vitro translation of pcDNA3HA-based NR constructs using the TNT transcription-coupled translation system (Promega). The radiolabeled NR proteins were added to similar
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amounts of GST or GST-fused proteins (2–4 μg) bound to glutathione-agarose beads pre-equilibrated with buffer A (50 mM Tris-HCl (pH 7.9), 5% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1 × protease inhibitor, 0.01% Nonidet P-40, 150 mM KCl) in a final volume of 250 μl. The beads were washed three times in the same buffer, and the bound radiolabeled proteins were analyzed by SDS-PAGE followed by autoradiography.

Cell Culture and Transient Transfection Assay—HEK293 cells were grown in 24-well plates with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 24 h and transiently transfected with the appropriate set of reporter and expression plasmids using SuperFect reagent (Qiagen). The total amounts of expression vectors were kept constant by adding appropriate amounts of pcDNA3HA. After 24 h, the cells were harvested and assayed for luciferase activity as described previously (35). The results from triplicate samples were averaged and normalized to LacZ and 2-AZ activity to control for transfection efficiency.

RESULTS

One- plus Two-hybrid Screening Strategy—Potential protein/protein interactions identified by the yeast two-hybrid assays or other in vitro approaches need to be further tested in relevant biological systems. Isolation of mutant proteins specifically altered in their ability to interact with a potential partner can facilitate analysis of the functional importance and biological significance of a given protein/protein interaction. Accordingly, we developed the one- plus two-hybrid system by combining the modified one-hybrid system with the standard yeast two-hybrid system (1). Basically, this one-hybrid selection system is different from the conventional system in that it is designed not to identify proteins harboring the desired DNA binding activity but to select for full-length alleles using C-terminal fusions of the GBD from the randomly generated mutant allele library (see below). This results in the rapid identification of specific missense mutations that disrupt the association of the two interacting proteins. For this screening system, we designed new cloning vectors and a yeast strain to operate the dual reporter systems for one-hybrid and two-hybrid assays (Fig. 1). As with the canonical yeast two-hybrid system, the bait protein of interest (X) is expressed as a LexA fusion, whereas the prey protein (Y) is expressed as a triple fusion between the B42 activation domain and the GBD. Strain YOK400 was constructed as a host strain for the dual reporter system, allowing us to monitor one-hybrid and two-hybrid interactions in the same cells as shown in Fig. 1. YOK400 strain, a derivative of strain EGY48, contains dual reporter genes: the yeast HIS3 gene driven by the UASgal promoter, which acts as the chromosomal reporter for one-hybrid interactions of the prey-GBD fusion, and the LacZ gene under the control of a tandem array of lexA operators, which acts as the episomal reporter for the standard two-hybrid interactions.

The basic strategy of the one- plus two-hybrid system is shown in Fig. 1. If X and Y form a protein-protein complex, B42-Y-GBD is recruited to the episomal lexAop-LacZ reporter and generates blue yeast colonies on X-gal plates as in the standard yeast two-hybrid system. The plasmid DNAs used for transfection included the Gal4-TK-LUC luciferase reporter (200 ng/well), the pSV-β-gal control plasmid, pcMX-Gal4N-RAR or -RXRαAF2, and the pcDNA3HA-N1 or -N2 wild type or mutants for the dominant negative assay as indicated in the figure legends.

**Fig. 1.** One- plus two-hybrid system strategy. The bait protein (X) is expressed as a LexA fusion, whereas the prey protein (Y) is expressed as a triple fusion between the B42 activation domain and the GBD. The protein/protein interaction between X and wild-type Y brings the B42 activation domain to the episomal LexA-driven LacZ reporter and generates blue yeast colonies on X-gal plates as in the standard yeast two-hybrid system. A, the chromosomal reporter that directs expression of HIS3 via upstream Gal4-binding sites requires the intact B42-Y-GBD fusion protein for cell survival in the absence of histidine (His+ phenotype; positive in the one-hybrid selection). Cells containing constructs with specific missense mutations in Y generate the functional B42-Y-GBD fusion and have a strong His+ phenotype, which serves as the first positive selection for full-length alleles (upper panel). Among these His+ cells, non-interacting Y mutants can be selected simply by isolating white yeast colonies again growing on X-gal/His− medium (two-hybrid selection), which serves as the second color selection for loss of interaction (lower panel). B, any nonsense or frameshift mutations in Y will lead to the lack of functional GBD and concurrent cell death on medium lacking histidine (His− phenotype; negative in the one-hybrid selection).
of functional GBD and concurrent cell death at the first step one-hybrid selection for the His\textsuperscript{+} phenotype (Fig. 1B). In summary, this novel system comprises dual reporter systems in which the modified one-hybrid system is used for the first positive selection of full-length alleles from a randomly generated mutant library and the two-hybrid reporter system is used for the second screening for loss-of-interaction prey mutants among the isolated missense mutants.

**Interaction Profile of NRs with Corepressor-IDs in the Yeast Two-hybrid Assay**—First we investigated the interaction pattern between IDs of corepressors and various NRs in the yeast two-hybrid assay to confirm the biological significance of studying NR/corepressor interactions in yeast. The small fragments containing the extended helix motif of either ID1 (N1) or ID2 (N2) of N-CoR were used as prey in the two-hybrid interaction (Fig. 2A). As shown in Fig. 2B, N1 and N2 correspond to 60-amino acid polypeptides in which the conserved helix motifs involved in NR interaction are centrally located. Similarly the equivalent regions of ID1 and ID2 of SMRT (called S1 and S2, respectively) were utilized to test their interactions with various NRs. All ID fragments were inserted between the B42 and GBD regions of the pRS424UB42-GBD vector to use as prey proteins in the two-hybrid interaction assay as well as in the mutant screening assays, whereas the NR baits were expressed as LexA fusions.

Previously it has been shown in a mammalian two-hybrid interaction assay that both ID1 and ID2 of N-CoR bind to various NRs but with different binding specificities (26, 28, 30, 32). To confirm these results, yeast two-hybrid assays were performed to examine the ID preference (ID1 versus ID2) or corepressor preference (N-CoR versus SMRT) of NRs in their interactions with corepressors. At first, we checked the strengths of these interactions on X-gal plates to evaluate the quality of the interactions during actual screening (Fig. 2C). Consistent with previous reports, interactions of the corepressor-ID with TR\textalpha and RAR\textalpha were much stronger than those of RevErb, PPAR\gamma, and especially RXR. In the case of RXR, we used the RXR\textalpha mutant lacking the AF2 domain (RXR\textalphaΔAF2) because deletion of the AF2 domain dramatically increases the ability of RXR to interact with N-CoR in vitro and in vivo (46). TR\textalpha and RAR\textalpha bound to both ID1 and ID2 of corepressors but interacted more strongly with ID1 (Fig. 2C). RXR\textalphaΔAF2 and PPAR\gamma interacted primarily with N2 and S2 with similar strengths (i.e. no corepressor preference). In contrast, RevErb showed strong interaction with N1 and S1 but not with N2 or S2 (Fig. 2C). In the quantitative assays for these interactions, we observed a pattern of NR/corepressor interactions similar to those obtained in the X-gal plate assay (Fig. 2, C and D). These results indicate an apparent linear relationship between X-gal plate and β-galactosidase assays under our experimental conditions that would explain the significant differences (more than 10-fold) in the interactions of the corepressor-ID with RAR and RXR\textalphaΔAF2 in a liquid β-galactosidase assay (Fig. 2D). In particular, we observed a clearer...
corepressor preference of some NRs in the quantitative assay; the TR showed obvious N-CoR preference, and N-CoR interactions with RevErb and PPAR were stronger than those of SMRT (Fig. 2D). However, unlike the previous observation, RAR interacted somewhat better with N-CoR than with SMRT in the yeast system (29, 30). From these results, we concluded that the specific interaction profiles between the IDs of corepressors and the NRs described in the mammalian system can be recapitulated in yeast.

**Control Experiments for One- plus Two-hybrid Screening**—We used the one- plus two-hybrid screening system to identify loss of interaction mutants between RARα and N-CoR-ID1 (N1) and between RXRαΔAF2 and N-CoR-ID2 (N2). Because these two examples correspond to the strongest and the weakest interactions among the ID/NR interactions we tested (Fig. 2C), it provides a means to validate the broad scope of our system for detecting protein/protein interactions with a wide range of binding affinities. Before the actual screening, a series of preliminary control experiments were performed to functionally test the bait and prey fusion proteins and the utility of the constructor constructs. We introduced the B42-N1-GBD or B42-N2-GBD prey plasmids into the EGY-LG strain carrying the episomal UAS<sub>GAL</sub>-LacZ reporter (pLGSD5) to functionally test the triple fusion by checking for the blue color phenotype on X-gal plates. The absence of autonomous transcriptional function by LexA-RAR or -RXRΔAF2 was also tested in strain EGY48 by checking for the white phenotype on X-gal plates.

Strain YOK400 expresses a very low level of His3p even in glucose medium (repressive condition). To establish the growth conditions that minimize the basal level of growth for the YOK400 strain and permit the growth of cells expressing only the intact triple fusion prey, we determined the optimal concentration of 3AT, a competitive inhibitor of His3p, for the actual screening. Strain YOK400 was transformed with plasmids expressing B42-N1-GBD, B42-N2-GBD, or B42 alone, and the resulting transformants were grown in a series of synthetic glucose/His− medium containing different concentrations of 3AT (0, 5, 10, and 20 mM). In the presence of 10 mM 3AT, the growth of yeast cells expressing B42-N1-GBD or B42-N2-GBD was barely affected, whereas the growth of cells expressing the B42 domain alone was completely inhibited, indicating that 10 mM was the optimal 3AT concentration for the positive selection of the intact prey fusion (His<sup>+</sup> phenotype).

**Validation for the Enrichment of Full-length Clones after One-hybrid Selection**—To verify how effectively our one-hybrid system eliminates truncation mutants and enriches for full-length alleles, a randomly mutagenized allele library for the N2 fragment was constructed in the YOK400 strain. We adopted a PCR-mediated random mutagenesis and gap repair-recombination method to generate the mutant cell library in one step, which dramatically accelerates the entire screening process (42, 43). Mutagenic PCR products of N2 were generated using specific primers corresponding to portions of the B42 and GBD regions in the presence of 0.3 mM MnCl<sub>2</sub> and co-transformed along with the linearized gap plasmid into strain YOK400. The introduced mutagenic PCR products served as templates for copying into the gap plasmid by the in vivo gap repair system of Saccharomyces cerevisiae (43). The transformants were selected on minimal medium plates containing histidine, and 600 transformants were patched onto duplicate medium plates containing histidine or onto plates lacking histidine but containing 10 mM 3AT. Among 600 transformants, only 92 colonies (15%) were not viable on medium plates lacking histidine (His<sup>−</sup> phenotype) under these conditions. Plasmids were successfully rescued from 50 colonies displaying the His<sup>−</sup> phenotype and from 43 colonies showing the His<sup>−</sup> phenotype. All the plasmids were retransformed into the EGY-LG strain to confirm the one-hybrid interaction (intactness of prey) and subjected to DNA sequencing.

Fig. 3 shows the overall distribution of the mutations detected within the mutagenized open reading frame (102 amino acids) of the N2 fragment partially flanked by B42 and GBD. Among the 50 clones conferring the His<sup>−</sup> phenotype, 27 clones had single or double substitution mutations (including five silent mutations), and 23 clones were identified as wild type (Fig. 3A). The mutations were uniformly distributed, and the mutation frequency was 2.3 mutations/kb of DNA under these PCR conditions. Importantly there were no nonsense or frameshift mutations among the 50 clones recovered from the colonies showing the His<sup>−</sup> phenotype. Conversely 42 of the 43 clones conferring the His<sup>−</sup> phenotype had nonsense or frameshift mutations. Among these mutations, 24 and 10 mutations were single base pair deletions and insertions, respectively, and eight mutations were nonsense mutations (Fig. 3B). However, one clone from 43 colonies showing the His<sup>−</sup> phenotype (2%) turned out to be a substitution mutant (false positive). The prey for the false positive clone was found to be intact by retransforming the clone into EGY-LG and YOK400 strains, suggesting that the false positive clone might be due to spontaneous inactivation of the HIS3 reporter system in this cell. All these results clearly demonstrate that our modified one-hybrid system designed for the selection of full-length alleles effectively eliminates uninformative nonsense or frameshift mutations that constitute the bulk of the unwanted mutations generated by random mutagenesis.

**Screening of N-CoR-ID Mutants Defective in Their Interactions with RAR or RXRΔAF2**—We selected 0.1 mM MnCl<sub>2</sub> for error-prone PCR for the actual screening because multiple point mutations were generally introduced at higher MnCl<sub>2</sub> concentrations (Fig. 3 and data not shown). The randomly mutagenized N1 and N2 DNA fragments were generated in the presence of 0.1 mM MnCl<sub>2</sub> and co-transformed along with the linearized gap plasmid into strain YOK400 harboring the LexA-RAR or -RXRΔAF2 plasmids and the episomal two-hybrid reporter (lexA<sub>op</sub>-LacZ) plasmid. As a control, mutagenic PCR products generated with or without 0.3 mM MnCl<sub>2</sub>...
were also co-transformed. The transformants were grown in synthetic glucose medium containing 10 mM 3AT but lacking histidine for the positive selection of intact prey fusions. Among the surviving transformants, non-interacting mutants could be easily selected by isolating white colonies on X-gal plates.

Table I shows the results of screening for interaction-defective mutants of N1 and N2. As predicted, in both cases, the data revealed a gradual increase in the mutation rate (as inferred from the occurrence of white colonies) with increased concentrations of MnCl₂ in the PCR. Consistent with this, the total number of transformants decreased with the introduction of PCR products generated under increased MnCl₂ concentrations, confirming the elimination of uninformative mutations in this step. In the control transformations, all of the tested colonies transformed with the gap plasmid alone were blue in color. In addition, the number of colonies transformed by the gap plasmid alone was very small compared with the number co-transformed with PCR products (less than 0.1%), and all were blue in color, suggesting they were generated by the incompletely digested supercoiled plasmid. These observations enabled us to conclude that our system works as designed.

For the isolation of N1 mutants, we isolated 84 white yeast colonies.

**Table I**

| Transforming DNAs          | N1 | Mutation rate (%) | N2 | Mutation rate (%) |
|---------------------------|----|-------------------|----|-------------------|
| Transforming DNAs         |    | Total no. of      |    | Total no. of      |
|                           |    | transformants     |    | transformants     |
|                           |    | (no. of white/no. |    | (no. of white/no. |
|                           |    | picked)           |    | picked)           |
| Gap only                  | 6  | 2.24 × 10³        | 4  | 1.76 × 10³        |
| Gap + PCR products (0.1 mM MnCl₂) | 1.52 × 10³ | 3.0 (84/2816)³ | 1.00 × 10³ | 3.2 (39/1223)³ |
| Gap + PCR products (0.3 mM MnCl₂) | 0.76 × 10³ | 10.0 (7/70) | 0.58 × 10³ | 14.0 (10/72) |
| B42-N1(or N2)-GBD         | 5.1 × 10³ | 0 (0/107)         | 4.2 × 10³ | 0 (0/80)         |

*For the small scale test, 200 ng of gap plasmid and 800 ng of PCR products were generally used for co-transformation.

*Data from the actual screening experiment, which was scaled up 5-fold over that of the small scale test.

*One microgram of supercoiled prey plasmid was transformed for the control experiment.
The identified N-CoR-ID mutants

The mutational sites and the changed amino acids found in the isolated N1 and N2 mutants defective in interactions with RAR and RXRΔAF2, respectively, are shown. The first Leu of the extended helix motif (LXX(I/H)XXX/I/L) is denoted as +1, and the residues corresponding to the CoRNR motif are indicated in bold. The number of each mutant allele recovered from the screening is shown in parentheses.

| N-CoR-ID1 | Changed amino acid (no. of rescued) | N-CoR-ID2 | Changed amino acid (no. of rescued) |
|-----------|-------------------------------------|-----------|-------------------------------------|
| Leu+1     | His (4), Arg (2), Pro (4)           | Glu+2     | Val (1)                             |
| His+4     | Pro (1)                            | Ile+5     | Ala (1), Asn (1), Thr (2), Val (1)  |
| Ile+5     | Asn (3), Thr (6)                   | Arg+6     | Gly (2)                             |
| Cys+6     | Arg (1), Ser (1)                   | Ala+8     | Thr (3), Val (1)                    |
| Gln+7     | Pro (1)                            | Leu+9     | Ile (1)                             |
| Ile+8     | Asn (2)                            | Met+10    | Pro (1)                             |
| Ile+9     | Phe (2), Thr (6)                   |           |                                     |
| Phe+13    | Ser (4)                            |           |                                     |

...colonies via color selection on X-gal plates among the 2816 transformants that survived in the first positive screening for missense mutations. Among these, the prey plasmids were rescued from 42 candidates and individually retransformed into EGY48 expressing LexA-RAR and EGY-LG to confirm two-hybrid and one-hybrid (intactness of prey fusion) interactions, respectively. A total of 36 candidates showing white color in the two-hybrid assay and blue color in strain EGY-LG were subjected to DNA sequencing. Similar results were obtained with the N2 mutant screening. We checked 1223 yeast transformants for loss of interaction and identified 34 white candidates. Among these, 17 completely white candidates were selected and subjected to DNA rescue. Through sequential confirmation and DNA sequencing, 14 final candidates were isolated as N2 mutants. Almost all of the mutants had a single point missense mutation. Although some mutants harbored double mutations, there was no nonsense or frameshift mutation. The double mutants were subjected to PCR-based site-directed mutagenesis to construct single point mutants (C+6S and I+8N for N1 where the first Leu of the extended helix motif LXX(I/H)XXX(I/L) is denoted as +1). Identification of Diverse N-CoR Mutants Defective in RAR or RXR Interactions — Table II shows the mutational sites and the changed amino acids found in the isolated N1 and N2 mutants defective in interactions with RAR and RXRΔAF2, respectively. In the case of N-CoR-ID1, almost all of the identified amino acid residues required for RAR interaction were located within the extended helix motif (Leu+1, Ile+5, Cys+6, Ile+8, Ile+9). Interestingly residue Phe+13 was also identified as an absolute requirement for the RAR interaction, although it is located downstream of the core motif. Among these residues, Cys+6 appeared to act as a minor determinant for RAR binding because the C+6S mutant had significant binding activity (Fig. 4A). Although proline substitution mutants were also isolated as the sole mutants for His+4 and Gln+7 residues, we did not regard them as informative because proline is known to act as a helix breaker (data not shown). Importantly most of the N1 mutations were recovered multiple times, and more than one amino acid change was observed at Leu+1 (to His, Arg, or Pro), Ile+5 (to Asn or Thr), and Ile+9 (to Phe or Thr), indicating that the screen was carried out under near saturating conditions. In the case of the N2 mutants, although the number of final mutants was somewhat smaller than for N1, we could identify specific residues located within the extended helix motif (Glu+2, Ile+5, Arg+6, Ala+8, and Leu+9) as determinants of the RXR interaction (Table II and Fig. 4B). Among these, changes to multiple amino acids were observed at residues Ile+5 (to Ala, Asn, Thr, or Val) and Ala+8 (to Thr or Val). In addition to these, an M+10P mutant was also isolated but was regarded as uninformative as described above. In conclusion, using our unbiased genetic selection system, we successfully identified multiple, informative N-CoR-ID mutations that disrupt NR interactions and found that almost all of them are located within the conserved NR interaction motif of the N-CoR as predicted.

The General and Specific Determinants of N-CoR-IDs for NR Interactions — Next we examined the binding patterns of the selected N1 and N2 mutants with various target NRs using the conventional yeast two-hybrid assay (Fig. 4). As shown in Fig. 4A, the interaction of N1 with TR or RevErb is absolutely dependent on residues Leu+1, Ile+5, Ile+8, and Ile+9 of N1 because a mutation in any of these residues resulted in a complete loss of reporter gene activation. Interestingly the residue Cys+6, which acts as a minor determinant for RAR binding, was required for interactions with TR and RevErb. Conversely the F+13S mutation abolished the RAR interaction completely, whereas it failed to severely affect the interaction of N1 with TR or RevErb, indicating that Phe+13 is a minor component for TR or RevErb binding but absolutely required for RAR binding (Fig. 4A). These phenomena cannot be attributed to differences in the expression levels of the B42-GBD forms of the N1 mutants (Fig. 4C). Taken together, these results enabled us to define N1 residues Leu+1, Ile+5, Ile+8, and Ile+9 as general determinants and Cys+6 and Phe+13 as specific determinants of N-CoR-ID1 for NR interactions.

We also evaluated the interactions of N2 mutants with RAR, TR, and PPARγ in addition to RXR in the yeast two-hybrid...
The mean activity was measured as described in the legend to Fig. 2D. The mean ± SE values are presented on the y axis. The binding strength of wild-type N1 or N2 to each NR was set to 100%. C and D, expression levels of the indicated N1 (C) or N2 (D) proteins. Immunoblot analysis was performed on whole cell extracts prepared from EGY48 strains used in the yeast two-hybrid assay (A and B). The expression levels of LexA-NRs (RAR (A) and RXRΔAF2 (B) for loading control) and the indicated prey proteins (B42-GBD fusions) were examined using specific antibodies against LexA and GBD, respectively. WT, wild type.

assays (Fig. 4B). Intriguingly we observed a similar, but not identical, binding pattern relative to that of N1. First we consistently observed strong and general effects on NR binding by substitutions at Ile+5 and Arg+6, indicating that these residues are general determinants of NR binding by N-CoR-ID2. In contrast, mutations at residues Glu+2 and Ala+8 showed differential effects on NR binding depending on the NR member. This was shown by the observation that the E+2V and A+8T mutants were able to interact to some extent with TR, RAR, and PPARγ but not with RXRΔAF2 (Fig. 4B). Interestingly all of the tested NRs except for TR showed greatly reduced interactions with the L+9I mutant, although Leu or Ile is generally found in this location of corepressors. We consider the residue Leu+9 to be a general rather than a specific determinant because another substitution mutant of this residue (L+9A) has been reported to have a complete and general defect on NR binding (28). The expression level of each N2 mutant was also confirmed by Western blot analysis (Fig. 4D).

In Vitro Interactions of N-CoR-ID Mutants with Various NRs in GST Pulldown Assays—To verify the molecular determinants for N-CoR/NR interactions, we investigated the in vitro interactions between N-CoR-ID mutants and NRs using GST pulldown analysis (Fig. 5). We prepared NRs as 35S-labeled proteins by in vitro translation and examined their binding to GST-fused N1 or N2 derivatives. As shown in Fig. 5A, N1 mutants carrying a substitution at residue Leu+1, Ile+5, Ile+8, or Ile+9 displayed no detectable interactions with the tested NRs, confirming the yeast two-hybrid data identifying these residues as general determinants of ID1/NR interactions (Fig. 4A). Moreover the F+13S mutant partially interacted with TR and RevErb but not with RAR, defining the Phe+13 residue as a specific determinant for NR binding. Inconsistent with the yeast data, however, the mutation at residue Cys+6 had only a partial or negligible effect on interactions with TR and RevErb, suggesting that the Cys+6 residue acts as minor determinant of the NR interaction in vitro (Fig. 5A).

Unfortunately we could not detect any significant interaction between GST-fused N2 proteins and RAR or PPARγ in the analysis for N-CoR-ID2 (data not shown). In the case of RXRΔAF2 and TR, N2 binding was absolutely dependent on residues Ile+5 and Arg+6, again defining these residues as general requirements for the N-CoR-ID2/NR interaction. In contrast, the mutations at residues Glu+2, Ala+8, and Leu+9 showed a negligible effect (Glu+2) or partial defects (Ala+8 and Leu+9) in TR binding as well as significant defects in the RXRΔAF2 interaction, which is roughly consistent with the yeast two-hybrid data (Figs. 4B and 5B). Because it was also reported that LXR interacts with ID2 of corepressors (33), we performed GST pulldown analysis to examine the interactions of LXR with the isolated N2 mutants. As shown in Fig. 5B, similar bindings were observed between RXRΔAF2 and LXRα and between TR and LXRβ. This was shown by the fact that both LXR isoforms had residues Ile+5 and Arg+6 as general requirements for N2 interactions, whereas residues Glu+2, Ala+8, and Leu+9 acted as either minor or specific determinants for N2/LXR interactions depending on the LXR isoform. Collectively both the in vivo and in vitro results consistently indicated that residues Leu+1, Ile+5, Ile+8, and Ile+9 of N1 and Ile+5, Arg+6, and Leu+9 of N2 are commonly required for optimal NR binding (general determinants), whereas residues Cys+6 and Phe+13 of N1 and Glu+2 and Ala+8 of N2
are differentially important for NR binding, depending on the specific NR member (specific determinants).

**Functional Tests of the Isolated N-CoR-ID Mutants in Mammalian Cells**—To establish the functional properties of the individual N-CoR-ID mutants in the relevant biological system, we tested their effects on NR function in mammalian cells. Because the predominant role of corepressors is associated with the repressive function of unliganded NRs, we examined the effects of overexpression of N-CoR-ID fragments on NR-mediated repression of a reporter gene (dominant negative effect). In a control experiment, we transiently co-transfected pCMXGal4-RAR and the luciferase reporter gene driven by the upstream Gal4-binding site (Gal4-TK-LUC) into HEK293 cells. As expected, Gal4-RAR showed a 2.5-fold repression of reporter gene expression, and this repressive effect was reversed by the overexpression of the wild-type N1 and N2 to each NR are set to 100%. WT, wild type.

levels of the N1 or N2 mutants (Fig. 6, C and D). All these results clearly demonstrate that N-CoR-ID mutants isolated by the yeast one- plus two-hybrid system show identical functional properties in a mammalian system.

**DISCUSSION**

Protein/protein interactions observed in the yeast two-hybrid system do not guarantee that the inferred interactions are events of physiological relevance. To reveal the functional significance and molecular basis of the interaction, identification of missense mutations that specifically disrupt the interaction with a given partner (loss-of-interaction mutants) is essential. For this purpose, we developed a novel mutant screening method, the one- plus two-hybrid system. This method is a genetic selection system based on a modification of the canonical yeast two-hybrid system for the efficient isolation of specific missense mutations that disrupt given protein/protein interactions. In a first demonstration of this method, we identified the specific amino acid residues within N-CoR-IDs that are generally required for NR binding or specifically required for interacting with a particular NR.

**Molecular Determinants of N-CoR-IDs for NR Interactions**—Two NR interaction motifs of N-CoR share the consensus sequence LXX(I/H)XXX(I/L), which has amphipathic properties. These motifs are predicted to adopt an extended helix by comparison with the known LXXLL motif of coactivators (28). Moreover, some specific residues within or flanking the extended helix motifs (Cys+6 and Phe+13 for ID1 and Glu+2 and Ala+8 for ID2) were found to be involved in
A Novel Genetic Selection for Full-length Allele

Fig. 6. Dominant negative effects of N-CoR-ID mutations on NR-mediated transcriptional repression. A and B, HEK293 cells were transfected with the Gal4-TK-luciferase reporter alone or in combination with pCMX-Gal4-RARα (A) or -RXRAF2 (B) plasmid (200 ng/well) and the pcDNA3HA vector (200 ng/well) expressing the wild type (WT; black bar) or indicated mutants (hatched bars) of N1 (A) or N2 (B) fragments. Luciferase activities were measured and normalized as described under “Materials and Methods” and represented as the -fold repression over the value obtained with the reporter alone. The results are the mean ± S.E. values obtained from at least three independent experiments performed in triplicate. C and D, expression levels of N1 (C) or N2 (D) derivatives were examined by immunoblot analysis of whole cell extracts prepared from untransfected (−) or transfected cells with the use of monoclonal anti-HA antibody. Nonspecific signals generated by the anti-HA antibody served as loading controls.

The extended helix motif of N-CoR-ID1 is not currently available, the extended helix motif of N-CoR-ID1 (+1LADHICQIL+9) is thought to form a three-turn helical conformation. It has been proposed that residues Leu+1, Ile+5, and Ile+9 of ID1 are on the same face of the helix so that they can make core contact with a common structure imbedded in NR-LBDs (28). We have classified Leu+1, Ile+5, Ile+8, and Leu+9 as the general determinants for the interaction of ID1 with NRs. Consistent with our findings, these residues are conserved within either the extended helix motif (Leu+1, Ile+5, and Leu+9) or the CoRNR motif (Ile+5, Ile+8 and Leu+9) of N-CoR-ID1, reinforcing the general importance of these residues for NR binding. It is unexpected that the Phe+13 residue of ID1 was identified as a specific determinant for NR binding because it lies far outside the extended helical motif. Interestingly Phe+13 is conserved among N-CoR-IDs of different species but not among SMRT-IDs (Tyr+13), suggesting a role for this residue in the N-CoR preference shown by target NRs.

Although the crystal structure of the NR-ID1 complex is not currently available, the extended helix motif of N-CoR-ID1 (+1LADHICQIL+9) is thought to form a three-turn helical conformation. It has been proposed that residues Leu+1, Ile+5, and Ile+9 of ID1 are on the same face of the helix so that they can make core contact with a common structure imbedded in NR-LBDs (28). We have classified Leu+1, Ile+5, Ile+8, and Leu+9 as the general determinants for the interaction of ID1 with NRs. Consistent with our findings, these residues are conserved within either the extended helix motif (Leu+1, Ile+5, and Leu+9) or the CoRNR motif (Ile+5, Ile+8 and Leu+9) of N-CoR-ID1, reinforcing the general importance of these residues for NR binding. It is unexpected that the Phe+13 residue of ID1 was identified as a specific determinant for NR binding because it lies far outside the extended helical motif. Interestingly Phe+13 is conserved among N-CoR-IDs of different species but not among SMRT-IDs (Tyr+13), suggesting a role for this residue in the N-CoR preference shown by some NRs.

The extended helix motif of N-CoR-ID2 (+1LEDIIRKAL+9) is thought to represent the C-terminal extension form of the CoRN2R2 motif (LEDII). Among these, we identified Ile+5, Arg+6, and Leu+9 residues as general determinants for NR binding, whereas Glu+2 and Ala+8 proved to be specific determinants. In the crystal structure of the PPARα-LBD complexed with the antagonist GW6741 and a SMRT-ID2 peptide (47), the extended helix motif (+1LADHICQIL+9) of SMRT-ID2 forms a three-turn helix in which Leu+1, Ile+5, and Leu+9 are aligned on the same face of the helix to form the core hydrophobic interaction with the receptor. Considering the near sequence identity between the extended helix motifs of N-CoR-ID2 and SMRT-ID2, this observation supports our conclusion that the residues Ile+5 and Leu+9 of N-CoR-ID2 are of general importance in NR binding. In particular, our screening did not find any mutations at residue Leu+1 probably because of the small number of N2 mutants available, although Leu+1 has been shown by others to be a general determinant for NR binding (26, 28, 47). We generated an L+1R substitution mutant by site-directed mutagenesis and identified this residue as an absolute requirement for the binding of all tested NRs (data not shown).

It is intriguing that residue Arg+6 of N-CoR-ID2 was identified as being a general requirement for NR binding in contrast to the NR-specific role of Cys+6 in N-CoR-ID1. The crystal structure of the PPARα-SMRT-ID2 complex reveals that residue Arg+6 of S2 forms a strong intramolecular hydrogen bond with Asn-303 located in helix 4 of the PPARα-LBD (47). Consistent with this, the Asp-295 residue of RXR, which is equivalent to Asn-303 of PPARα, is also required for optimal interaction of S2 with RXR (48). These observations strongly support our finding that the Arg+6 residue of N2 is also generally required for NR binding in addition to the res-
idues involved in core hydrophobic interactions with NRs (Leu+1, Ile+5, and Leu+9).

We hope that these corepressor mutants showing differential binding specificities for various NRs will facilitate the elucidation of the physiological roles and the molecular basis of the repressive functions of specific NRs. In addition, identification of specific residues that mediate high affinity binding between NRs and corepressors will be helpful in dissecting the complex network of corepressor/NR interactions in the absence of the crystal structures for NR-corepressor complexes.

**One- plus Two-hybrid System**—The one- plus two-hybrid system is designed for the rapid and efficient selection of missense mutations that specifically disrupt known protein/protein interactions and has three major advantages over the existing methods. First, this system allows rapid and efficient genetic selection for missense mutations. This advantage originates from the simultaneous operation of dual reporter systems in the same cell and the use of the gap repair system for the one-step construction of a mutant cell library containing only missense mutations. For the reverse two-hybrid systems, several alternative strategies have been used to isolate full-length alleles by adding an easily detectable C-terminal fusion such as β-galactosidase (7), green fluorescent protein (9–11), or epitope tag (12). In these modified systems, truncation of the prey by an uninformative mutation is indicated by white color phenotype on X-gal plates, absence of green fluorescence, or defective epitope/antibody interactions in immuno-blot analysis, indicating that these methods cannot positively select for the non-interacting full-length alleles. To circumvent this technical obstacle, Gray et al. (13) developed a novel method specifically designed to generate full-length, high coverage allele libraries. In this system, mutagenized prey proteins are expressed as C-terminal fusions of kanamycin resistance gene, enabling the construction of full-length allele libraries in *E. coli* by the positive selection of antibiotic-resistant colonies. To operate, this system requires two in vitro recombinational cloning steps and the generation of a full-length allele library in *E. coli* prior to isolating the non-interacting mutants by the yeast reverse two-hybrid system. This indicates that this strategy has more methodological complexity and difficulties in the construction of a full-length allele library compared with our system using the modified one-hybrid system in yeast. Thus, our one- plus two-hybrid system is the first positive selection system for full-length alleles fortified with the one-step construction of full-length allele library in yeast.

Second, no or a low level of background of false positives was generated in the first positive selection of the full-length alleles as well as in the second screening of the non-interactors by the one- plus two-hybrid system. There was no truncation mutant among the 50 transformants with the His+ phenotype during the selection of the full-length allele (Fig. 3A), indicating that our modified one-hybrid system works well and is relatively stable. We recommend that the YOK400 strain be made freshly or carefully checked for the His- phenotype before initiating each round of screening to minimize the generation of false positives in the first selection step of the full-length clones. In a second screening step for interaction-defective prey mutants, we found that only six of 42 N1 and three of 17 N2 mutant candidates were false positives (less than 20%). This result is in contrast to reports indicating that reverse two-hybrid systems generally create a high background of false positive (more than 65%). This discrimination might be due to differences in the stabilities of two-hybrid reporter systems utilized by these methods.

Third, the one- plus two-hybrid system can be generally applied for the characterization of a wide spectrum of protein/protein interactions without knowledge of the specific system or mechanism involved in the interactions. For example, we have successfully identified residues that are important for NR-specific interactions within the NR interaction motifs of various coactivator proteins.² In addition, we have discovered novel motifs within RD3 of corepressors that mediate the interactions with class II histone deacetylases³ and have mapped the amino acid residues required for the direct interaction between the two subunits of the activating signal cointegrator 2-containing complex (45).⁴ These (unpublished) examples using the one- plus two-hybrid system prove that our system is suitable for general applications as well as for systematic applications on a larger scale. Notably our system can also be used to analyze the interaction interfaces of DNA-binding proteins using dual one-hybrid reporter systems: one for the generation of full-length allele library and the other for the isolation of interaction-defective mutants.

However, our system may have two potential limitations. First, because the prey protein is expressed as a triple fusion between B42 and GBD, a complex protein whose proper three-dimensional structure is impaired by B42 and GBD fusions may not be a suitable target for mutant screening. Second, another potential limitation for the general use of our system is a restriction in the length of the prey protein because of the difficulty to maintain the optimal mutation rate with increasing size of the prey. Thus far, we have successfully analyzed prey proteins of up to 200 amino acids.

Finally our “one- plus two-hybrid method” could be improved by introducing a positive selection system for non-interactors as has already been tried in the reverse or split hybrid systems. For example, instead of using the episomal *LacZ* reporter, a yeast counterselectable marker, such as *URA3* or *CYH2*, could be used in the two-hybrid screen for simultaneous double positive selection of missense mutations and non-interacting mutants.

In conclusion, the one- plus two-hybrid system rapidly and efficiently generates missense mutations that can specifically

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² Y. L. Son and Y. C. Lee, unpublished data.
³ M. J. Park and Y. C. Lee, unpublished data.
⁴ M. J. Kang, Y. C. Lee, and J. W. Lee, unpublished data.
disrupt a known protein/protein interaction without knowledge of the specific system or mechanism involved in the interaction. The use of loss-of-interaction mutants will be very helpful in determining the functional significance of their interactions in the relevant biological system and understanding the underlying molecular mechanism for their interactions. More importantly, information regarding the residues involved in the interactions and the effects of various mutations on these residues will be critical for the molecular modeling of the protein/protein interactions.

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REFERENCES

1. Fields, S., and Song, O. (1989) A novel genetic system to detect protein-protein interactions. Nature 340, 245–246
2. Chien, C. T., Bartel, P. L., Stemmler, R., and Fields, S. (1991) The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. U. S. A. 88, 9578–9582
3. Vidal, M., and Legrain, P. (1999) Yeast forward and reverse ‘n’-hybrid systems. Nucleic Acids Res. 27, 919–929
4. Legrain, P., and Hanhinni, M. (1996) The reverse two-hybrid system: a genetic scheme for selection against specific protein/protein interactions. Nucleic Acids Res. 24, 3341–3347
5. Vidal, M., Brachmann, R. K., Fattaey, A., Harlow, E., and Boeke, J. D. (1996) Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein and DNA-protein interactions. Proc. Natl. Acad. Sci. U. S. A. 93, 10315–10320
6. Vidal, M., Braun, P., Chen, E., Boeke, J. D., and Harlow, E. (1996) Genetic characterization of a mammalian protein-protein interaction domain by using a yeast reverse two-hybrid system. Proc. Natl. Acad. Sci. U. S. A. 93, 10321–10326
7. Shih, H. M., Goldman, P. S., DeMaggio, A. J., Hollenberg, S. M., Goodman, R. H., and Hoeckstra, M. F. (1996) A positive genetic selection for disrupting protein-protein interactions: identification of CREB mutations that prevent association with the coactivator CBP. Proc. Natl. Acad. Sci. U. S. A. 93, 13896–13901
8. White, M. A. (1996) The yeast two-hybrid system: forward and reverse. Proc. Natl. Acad. Sci. U. S. A. 93, 10001–10003
9. Endoh, H., Walthou, A. J., and Vidal, M. (2000) A green fluorescent protein-based reverse two-hybrid system: application to the characterization of large numbers of potential protein-protein interactions. Methods Enzymol. 328, 74–88
10. Puthalakath, H., Strasser, A., and Huang, D. C. (2001) Rapid selection against truncation mutants in yeast reverse two-hybrid screens. BioTechniques 30, 984–988
11. Mendelsohn, A. R., Hamner, J. D., Wang, Z. B., and Brent, R. (2002) Cyclin D3 activates Caspase 2, connecting cell proliferation with cell death. Proc. Natl. Acad. Sci. U. S. A. 99, 6871–6876
12. Barr, R. K., Hopkins, R. M., Watt, P. M., and Bogoyevitch, M. A. (2004) Reverse two-hybrid screening identifies residues of JNK required for interaction with the kinase interaction motif of JNK-interacting protein-1. J. Biol. Chem. 279, 43178–43189
13. Gray, P. N., Busser, J. K., and Chappell, T. G. (2007) A novel approach for generating full-length, high coverage allele libraries for the analysis of protein interactions. Mol. Cell. Proteomics 6, 514–526
14. Beato, M., Herrlich, P., and Schutz, G. (1995) Steroid hormone receptors: many actors in search of a plot. Cell 83, 851–857
15. McKenna, N. J., and O’Malley, B. W. (2002) Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 106, 465–474
16. Mangelsdorff, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) The nuclear receptor superfamily: the second decade. Cell 83, 839–839
17. Chen, J. D., and Evans, R. M. (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377, 454–457
18. Horlein, A. J., Naar, A. M., Heintz, T., Torchia, J., Gless, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K., and Rosenfeld, M. G. (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear co-repressor. Nature 377, 397–404
19. Jepsen, K., and Rosenfeld, M. G. (2002) Biological roles and mechanistic actions of co-repressor complexes. J. Cell Sci. 115, 689–698
20. Hu, X., and Lazar, M. A. (2000) Transcriptional repression by nuclear hormone receptors. Trends Endocrinol. Metab. 11, 6–10
21. Cohen, R. N., Wondisford, F. E., and Hollenberg, A. N. (1998) Two separate NCOR (nuclear receptor corepressor) interaction domains mediate corepressor action on thyroid hormone response elements. Mol. Endocrinol. 12, 1567–1581
22. Dotzlaw, H., Moehren, U., Mink, S., Cato, A. C., Iniguez Lluhi, J. A., and Banaihmad, A. (2002) The amino terminus of the human AR is target for corepressor action and androgen agonism. Mol. Endocrinol. 16, 661–673
23. Liao, G., Chen, L. Y., Zhang, A., Godavarthy, A., Xia, F., Ghosh, J. C., Li, H., and Chen, J. D. (2003) Regulation of androgen receptor activity by the nuclear receptor corepressor SMRT. J. Biol. Chem. 278, 5052–5061
24. Seol, W., Mahon, M. J., Lee, Y. K., and Moore, D. D. (1996) Two receptor interacting domains in the nuclear hormone receptor corepressor RIP13/N-CoR. Mol. Endocrinol. 10, 1646–1655
25. Wagner, B. L., Valledor, A. F., Shao, G., Daige, C. L., Bischoff, E. D., Petrovski, M., Jepsen, K., Baek, S. H., Heyman, R. A., Rosenfeld, M. G., Schulman, I. G., and Glass, C. K. (2003) Promoter-specific roles for liver X receptor/corepressor complexes in the regulation of ABCA1 and SREBP1 gene expression. Mol. Cell. Biol. 23, 5780–5789
26. Hu, X., and Lazar, M. A. (1999) The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. Nature 402, 93–96
27. Nagy, L., Kao, H. Y., Love, J. D., Li, C., Banayo, E., Gooch, J. T., Krishna, V., Chatterjee, K., Evans, R. M., and Schwabe, J. W. (1999) Mechanism of receptor binding and release from nuclear hormone receptors. Genes Dev. 13, 3209–3216
28. Perissi, V., Staszewski, L. M., Mclnerney, E. M., Kurokawa, R., Krones, A., Rose, D. W., Lambert, M. H., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1999) Molecular determinants of nuclear receptor-corepressor interaction. Genes Dev. 13, 3198–3208
29. Cohen, R. N., Putney, A., Wondisford, F. E., and Hollenberg, A. N. (2000) The nuclear corepressors recognize distinct nuclear receptor complexes. Mol. Endocrinol. 14, 911–924
30. Hu, X., Li, Y., and Lazar, M. A. (2001) Determinants of CoRNR-dependent repression complex assembly on nuclear hormone receptors. Mol. Cell. Biol. 21, 1747–1758
31. Makowski, A., Brzostek, S., Cohen, R. N., and Hollenberg, A. N. (2003) Determination of nuclear receptor corepressor interactions with the thyroid hormone receptor. Mol. Endocrinol. 17, 273–286
32. Cohen, R. N., Brzostek, S., Kim, B., Chourev, M., Wondisford, F. E., and Hollenberg, A. N. (2001) The specificity of interactions between nuclear hormone receptors and corepressors is mediated by distinct amino acid sequences within the interacting domains. Mol. Endocrinol. 15, 1049–1061
33. Hu, X., Li, S., Wu, J., Xia, C., and Laia, D. S. (2003) Liver X receptors interact with corepressors to regulate gene expression. Mol. Endocrinol. 17,
1019–1026
34. Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993) Cdc1, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell 75, 791–803
35. Na, S. Y., Choi, J. E., Kim, H. J., Jhun, B. H., Lee, Y. C., and Lee, J. W. (1999) Bcl3, an IkB protein, stimulates activating protein-1 transactivation and cellular proliferation. J. Biol. Chem. 274, 29491–29496
36. Lee, S. K., Anzick, S. L., Choi, J. E., Babendorf, L., Guan, X. Y., Jung, Y. K., Kallioniemi, O. P., Kononen, J., Trent, J. M., Azorsa, D., Jhun, B. H., Cheong, J. H., Lee, Y. C., Meltzer, P. S., and Lee, J. W. (1999) A nuclear factor, ASC-2, as a cancer-amplified transcriptional coactivator essential for ligand-dependent transactivation by nuclear receptors in vivo. J. Biol. Chem. 274, 34283–34293
37. Lee, S. K., Jung, S. Y., Kim, Y. S., Na, S. Y., Lee, Y. C., and Lee, J. W. (2001) Two distinct nuclear receptor-interaction domains and CREB-binding protein-dependent transactivation function of activating signal cointegrator-2. Mol. Endocrinol. 15, 241–254
38. Forman, B. M., Umesono, K., Chen, J., and Evans, R. M. (1995) Unique response pathways are established by allosteric interactions among nuclear hormone receptors. Cell 81, 541–550
39. Guarente, L., Yocum, R. R., and Gifford, P. (1982) A GAL10-CYC1 hybrid yeast promoter identifies the GAL4 regulatory region as an upstream site. Proc. Natl. Acad. Sci. U. S. A. 79, 7410–7414
40. Estojak, J., Brent, R., and Golemis, E. A. (1995) Correlation of two-hybrid affinity data with in vitro measurements. Mol. Cell. Biol. 15, 5820–5829
41. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153, 163–168
42. Cadwell, R. C., and Joyce, G. F. (1992) Randomization of genes by PCR mutagenesis. PCR Methods Appl. 2, 28–33
43. Muhlrad, D., Hunter, R., and Parker, R. (1992) A rapid method for localized mutagenesis of yeast genes. Yeast 8, 79–82
44. Lee, Y. C., and Kim, Y. J. (1998) Requirement for a functional interaction between mediator components Med6 and Srb4 in RNA polymerase II transcription. Mol. Cell. Biol. 18, 5364–5370
45. Goo, Y. H., Sohn, Y. C., Kim, D. H., Kim, S. W., Kang, M. J., Jung, D. J., Kwak, E., Barlev, N. A., Berger, S. L., Chow, V. T., Roeder, R. G., Azorsa, D. O., Meltzer, P. S., Suh, P. G., Song, E. J., Lee, K. J., Lee, Y. C., and Lee, J. W. (2003) Activating signal cointegrator 2 belongs to a novel steady-state complex that contains a subset of trithorax group proteins. Mol. Cell. Biol. 23, 140–149
46. Zhang, J., Hu, X., and Lazar, M. A. (1999) A novel role for helix 12 of retinoid X receptor in regulating repression. Mol. Cell. Biol. 19, 6448–6457
47. Xu, H. E., Stanley, T. B., Montana, V. G., Lambert, M. H., Shearer, B. G., Cobb, J. E., McKee, D. D., Galardi, C. M., Plunket, K. D., Nohe, R. T., Parks, D. J., Moore, J. T., Kliwer, S. A., Willson, T. M., and Stimmel, J. B. (2002) Structural basis for antagonist-mediated recruitment of nuclear co-repressors by PPARα. Nature 415, 813–817
48. Ghosh, J. C., Yang, X., Zhang, A., Lambert, M. H., Li, H., Xu, H. E., and Chen, J. D. (2002) Interactions that determine the assembly of a retinoid X receptor/corepressor complex. Proc. Natl. Acad. Sci. U. S. A. 99, 5842–5847