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A GAL4-based yeast three-hybrid system for the identification of small molecule–target protein interactions

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Abstract

We report the development of a yeast strain designed for assaying compound–protein interactions through activation of reporter gene expression. Activation of *lacZ* expression, driven by the GAL4 promoter, has been demonstrated for precedented compound–protein interactions between FK506 and FK506 binding protein 12 (FKBP12) and also between methotrexate (MTX) and dihydrofolate reductase (DHFR). Reporter gene expression was completely abrogated in a competitive manner by the presence of excess FK506 or MTX, respectively. In addition, a strain expressing a mutated DHFR clone with decreased binding affinity for MTX was not capable of activating reporter gene expression. While strain sensitivity is compound-dependent, the minimum compound concentration necessary to drive reporter gene expression was 20 nM for the FK506–FKBP12 interaction. The utility of this strain as a tool for identifying unknown compound-binding proteins has been demonstrated by screening a mouse cDNA library for clones that encode proteins capable of binding MTX. Four library clones of mouse DHFR were identified after screening 5×10^6 clones. The screen background was low and false positives were easily identified, making this yeast system particularly amenable for use in a screening context for novel compound–protein interactions. © 2002 Published by Elsevier Science Inc.

Keywords: Yeast three-hybrid; GAL4; Dexamethasone; FK506; Methotrexate; Glucocorticoid receptor

1. Introduction

Interactions between small molecules and their receptor proteins modulate many fundamental cellular processes. Synthetic small molecules are used widely in basic biological research and form the basis for disease-modification through pharmacological agents. Despite their widespread use, the efficient identification of target proteins for small molecular weight ligands is a time consuming and technically challenging process. Current biochemical strategies involve sequencing of the interacting protein only after it has been purified from a highly complex cell extract via radiolabelled ligand-binding, photocrosslinking and affinity chromatography [1,2].

An alternative route for identifying proteins that interact with a small molecule of interest uses an approach that exploits the genetic and molecular tools developed in the yeast two-hybrid system for studying protein–protein interactions [3,4]. The two-hybrid system uses a transcription factor that can be separated into two domains, a DNA binding domain (BD) and an activation domain (AD). When co-expressed within the same cell, transcriptional activation can only occur if these composite domains are brought into close proximity. In the yeast two-hybrid system, functional assembly of the transcription factor can be made to occur in the nucleus of living yeast cells by fusing the domains with two proteins that interact. A yeast 'three-hybrid' approach has been developed through a modification of the two-hybrid system to incorporate a small molecule heterodimer [5]. Functional assembly of the transcription factor is driven by two independent small molecule–protein interactions (Fig. 1). As a result, interaction of the test compound with its binding protein drives activation of measurable reporter gene expression.

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Abbreviations: FKBP12, FK506 binding protein 12; MTX, methotrexate; DHFR, dihydrofolate reductase; GRhbd, glucocorticoid receptor hormone binding domain; Dex, dexamethasone; BD, binding domain; AD, activation domain; WT, wild type.

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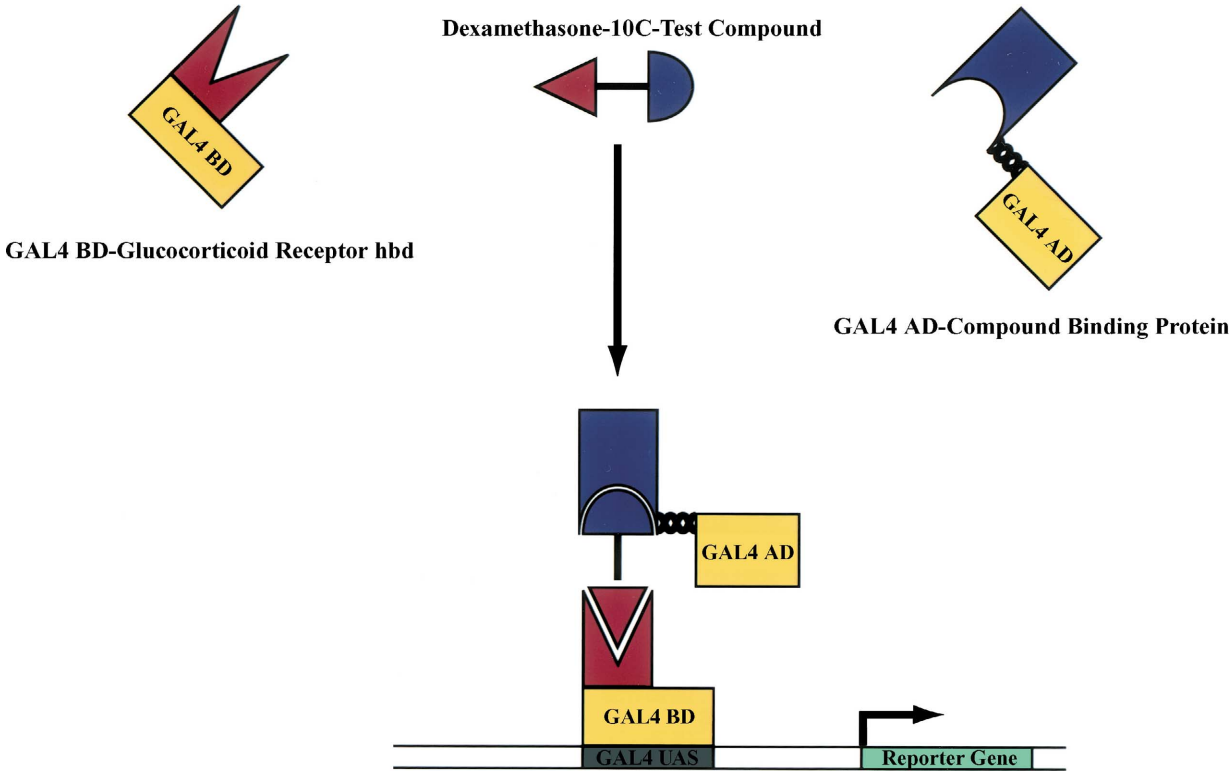

Fig. 1. The yeast three-hybrid screening strain. The GAL4 DNA binding domain (BD) and activation domain (AD) are brought into close proximity by two compound–protein interactions. Interaction of dexamethasone with the glucocorticoid receptor hormone binding domain is constant, whilst the compound–protein interaction to be tested is variable.

A yeast three-hybrid system has been described in which a glucocorticoid receptor hormone binding domain (GRhbd)-fusion and an FKBP12-fusion can be dimerised by a Dex–FK506 heterodimer [5]. The heterodimeric molecule was chosen because Dex and FK506 possess the well characterised binding partners GRhbd and FKBP12, respectively, they are both permeable to yeast cells and they both possess chemical functionality that can be modified without disrupting receptor binding. By similar reasoning, another yeast three-hybrid system has been reported more recently, in which a GRhbd-fusion and a DHFR-fusion can be dimerised by a Dex–MTX heterodimer [6]. The success of the two-hybrid system in identifying novel protein interactions with a protein of interest suggests that the three-hybrid system may have similar potential for identifying novel protein interactions with a compound of interest. Establishing the true potential of this approach requires the construction of an appropriate yeast strain and an assessment of practicalities such as signal to noise ratio, sensitivity, ease of eliminating false positives and breadth of application.

We present modifications of the published system that for the first time use the GAL4 transcription factor as the basis for the yeast three-hybrid strain. The large number of GAL4 activation domain libraries available suggests screens should be feasible for proteins from many different organisms or specific mammalian tissues. We demonstrate the sensitivity of this system, its efficacy for multiple target compounds and its utility for cDNA library screening. In addition to identifying proteins that interact with a compound of interest, we also highlight the ease with which false positives can be eliminated and describe the nature of these false positives. Our results demonstrate that the identification of novel proteins that interact with small molecules is now technically feasible and comparatively easy for any laboratory with rudimentary molecular biology capability. The subsequent identification of such proteins has potential implications relating to understanding both compound mode of action and side effect profiles.

2. Materials and methods

2.1. Dexamethasone–methotrexate synthesis

The Dex–MTX heterodimer was synthesised as shown in Fig. 2. The two molecules were covalently linked with a 10-carbon ($n = 8$) tether through positions that when modified did not abrogate target protein binding. The Dex–FK506 heterodimer was obtained from Liu and coworker [5].

2.2. Construction of expression vectors

All oligonucleotides used in this study are listed in Table 1. Full-length rat glucocorticoid receptor was amplified by

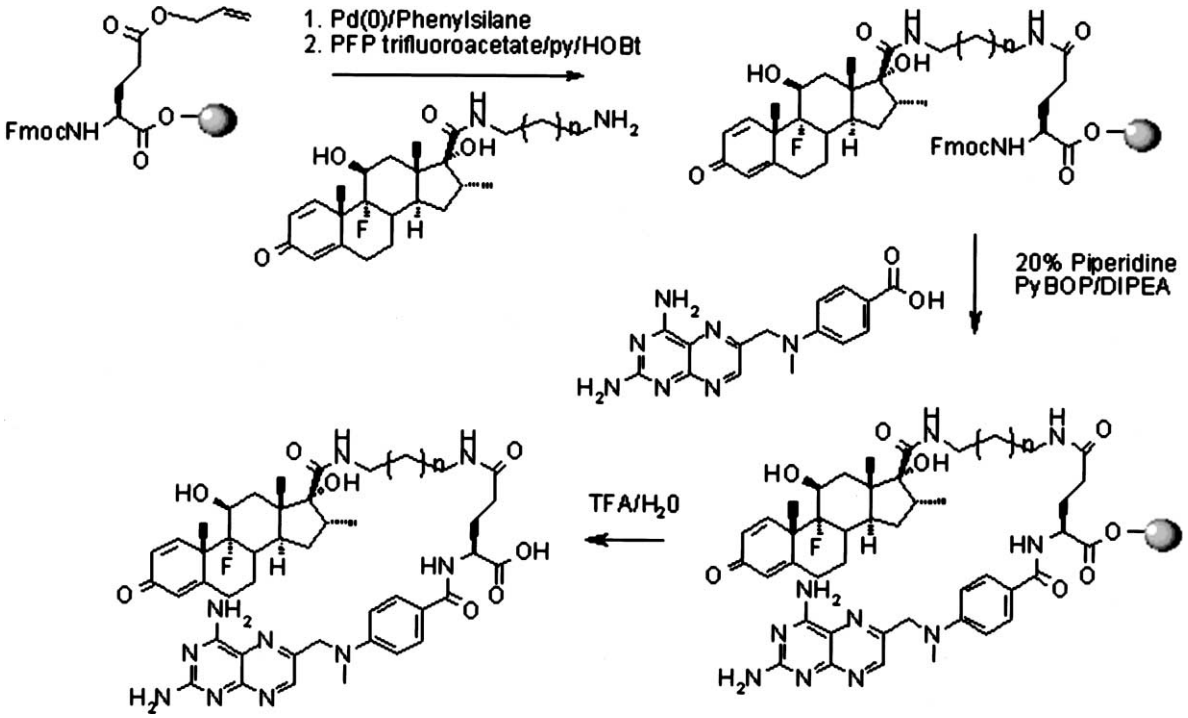

Fig. 2. Synthesis and structure of the dexamethasone–methotrexate heterodimer.

PCR using p6RGR [5] as a template and the primer pair 5′RGRdch/3′RGRii. This was subcloned into pYX032 (R&D systems). A two-step mutagenic PCR [7] was used to introduce F620S and C656G substitution mutations [5] into the sequence using primer pairs F620Sf/r and C656Gf/r, respectively. These mutations increase the stability of the rat glucocorticoid receptor in yeast and increase the affinity of the receptor for Dex. The product was used as a PCR template to amplify the rat GRhbd using primer pair 5′RGRhbd/3′RGRii. This was subcloned into pAS1-CHY2, a derivative of pAS1 [8], such that the GRhbd was in frame with the GAL4 DNA BD.

Human FKBP12 and mouse DHFR were amplified by PCR using pJGFKBP [5] and pQE40 (Qiagen) templates with primer pairs FKBPf/r and DHFRf/r, respectively. The resulting products were subcloned into pGADGH [9] to ensure frame with the GAL4 AD. DNA inserts in all plasmids were confirmed by sequencing.

2.3. Generation of the yeast three-hybrid strains

Yeast strains used in this study are represented diagrammatically in Fig. 3 and are listed in Table 2. Yeast were grown in synthetic dextrose (SD) media with appropriate

Table 1
Oligonucleotides used in this study

Primer	Sequence	Restriction site
5′RGRdch	AAAAAAGGATCCATGGACTCCAAAGAATCCTTA	*Bam*H1
3′RGRii	GGACGCGGATCCTCATTTTTGATGAAACAG	*Bam*H1
5′RGRhbd	AAAAGGATCCTAGCAGGAGTCTCACAAGACACTTCG	*Bam*H1
F620Sf	GTACTCATGGATGTCTCTCATGGCATTTGC	
F620Sr	GCAAATGCCATGAGAGACATCCATGAGTAC	
C656Gf	AGAATGTCTCTACCCGGCATGTATGACCAA	
C656Gr	TTGGTCATACATGCCGGGTAGAGACATTCT	
FKBPf	AAAAAGAATTCCATGGGAGTGCAGGTGGAAACCATC	*Eco*R1
FKBPr	AAACTCGAGTCATTCCAGTTTTAGAAGCTCCAC	*Xho*1
DHFRf	AAAAAGAATTCCATGGTTCGACCATTGAACTGCATC	*Eco*R1
DHFRr	AAACTCGAGTTAGTCTTTCTTCTCGTAGACTTC	*Xho*1
Arg22f	GGCAAGAACGGAGACCGGCCCTGGCCTCCGCTC	
Arg22r	GAGCGGAGGCCAGGGCCGGTCTCCGTTCTTGCC	
Trp31f	CCTCCGCTCAGGAACGAGTGGAAGTACTTCCAAAGAATG	
Trp31r	CATTCTTTGGAAGTACTTCCACTCGTTCCTGAGCGGAGG	
pACT2for	ATTACGCTAGCTTGGGTGGTC	
pACT2rev	CGGGGTTTTTCAGTATCTACG	

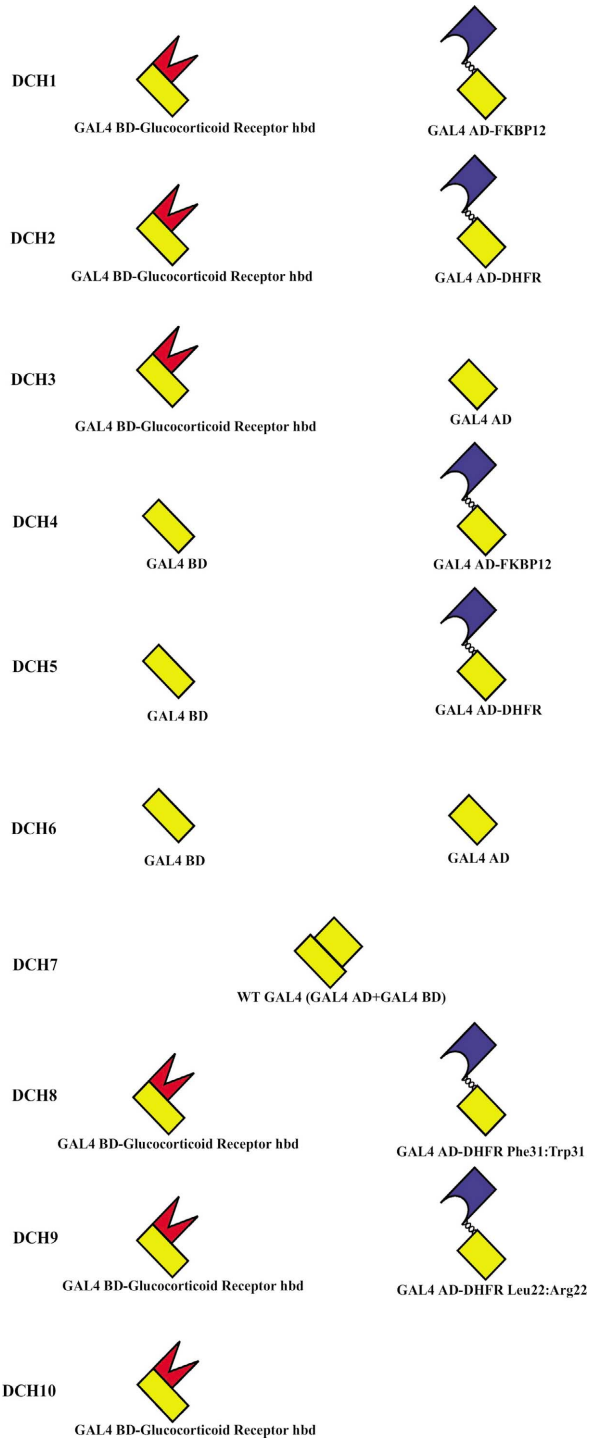

Fig. 3. Yeast three-hybrid strains and control strains. Yeast three-hybrid strain DCH1 expresses fusion proteins of GAL4BD with the rat GRhbd and of GAL4AD with FKBP12. Yeast three-hybrid strain DCH2 expresses fusion proteins of GAL4BD with the rat GRhbd and of GAL4AD with DHFR. Control strains DCH3–5 contain one of the fusion protein clones and the relevant non-fused GAL4 domain. A negative control strain DCH6 contains both the non-fused GAL4 domains, whilst a positive control strain DCH7 contains wild type GAL4 protein for constitutive activation of *lacZ* expression. Strains DCH8 and DCH9 express fusion proteins of GAL4BD with the rat GRhbd and of GAL4AD with either DHFR Phe:Trp31 or DHFR Leu:Arg22. Strain DCH10 only contains the fusion protein of GAL4BD with the rat GRhbd; this strain is transformed with a cDNA library fused to the GAL4AD to screen for novel binding proteins of a compound of interest.

auxotrophic supplements [10] and standard genetic methods were followed. To generate the three-hybrid strains, the pAS1-CHY2:GRhbd clone was co-transformed [11] with either pGADGH:FKBP12 or pGADGH:DHFR into yeast Y190 [4]. Transformants were grown on SD medium lacking leucine and tryptophan to generate strains DCH1 and DCH2. Control strains, DCH3–5 were generated by co-transforming only one of the fusion protein clones with the relevant empty vector. A negative control strain, DCH6 was produced by transforming Y190 with empty pAS1-CHY2 and empty pGADGH whilst a positive control strain, DCH7 was produced by co-transforming Y190 with pCL-1 [3].

Strains DCH1–7 were plated onto SD medium lacking leucine, tryptophan and histidine in the presence of 25 mM 3-aminotriazole (Sigma) and different concentrations of Dex–FK506 (in ethanol) ± FK506 (in ethanol) or Dex–MTX (in DMSO) ± MTX (in DMSO). After incubation at 30° for 3 days, colonies were transferred onto Whatmann 3 mm filter paper [12] and assayed for *lacZ* activation using the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

2.4. Generation of a MTX-resistant yeast three-hybrid strain

The Quikchange mutagenesis kit (Stratagene) was used according to manufacturer's instructions to produce either a Phe31:Trp31 or Leu22:Arg22 single amino acid substitution change in the pGADGH:DHFR clone using primer pairs Trp31f/r or Arg22f/r, respectively. The pAS1-CHY2:GRhbd clone was co-transformed into yeast Y190 with either pGADGH:DHFR Trp31 or pGADGH:DHFR Arg22. Transformants were grown upon SD medium lacking leucine and tryptophan to generate strains DCH8 and DCH9. Strains DCH2, DCH8 and DCH9 were plated onto SD medium lacking leucine, tryptophan and histidine in the presence of 25 mM 3-AT and either 1, 10 or 50 μM Dex–MTX. After incubation at 30° for 3 days, colonies were assayed for *lacZ* activation.

2.5. Yeast three-hybrid screening for MTX-interacting proteins

A yeast strain DCH10 was generated for three-hybrid screening by transforming pAS1-CHY2:GRhbd into Y190. The resulting strain was transformed according to manufacturer's instructions with 50 μg mouse 11-day embryo cDNA library containing pACT2-library insert clones (Clontech Matchmaker™, #ML4012AH). The number of yeast colonies screened was calculated as 5×10^6 by plating small aliquots of transformation mix onto SD medium lacking leucine and tryptophan. The remaining transformation mix was plated onto SD medium lacking leucine, tryptophan and histidine in the presence of 25 mM 3-AT and 10 μM Dex–MTX. After incubation at 30° for

Table 2
Yeast strains used in this study

Strain	Genotype	Source
Y190	*MATa*, *ura3-52*, *his3-200*, *lys2-801*, *ade2-101*, *trp1-901*, *leu2-3,112*, *gal4Δ*, *gal80Δ*, *cyh*^{r}*2*, *LYS2::GAL1*$_{UAS}$*-HIS3*$_{TATA}$*-HIS3*, *URA3::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ*	[4]
DCH1	Y190 + pAS1-CHY2:GRhbd + pGADGH:FKBP12	This study
DCH2	Y190 + pAS1-CHY2:GRhbd + pGADGH:DHFR	This study
DCH3	Y190 + pAS1-CHY2:GRhbd + pGADGH	This study
DCH4	Y190 + pAS1-CHY2 + pGADGH:FKBP12	This study
DCH5	Y190 + pAS1-CHY2 + pGADGH:DHFR	This study
DCH6	Y190 + pAS1-CHY2 + pGADGH	This study
DCH7	Y190 + pCL-1	This study
DCH8	Y190 + pAS1-CHY2:GRhbd + pGADGH:DHFR Phe31:Trp31	This study
DCH9	Y190 + pAS1-CHY2:GRhbd + pGADGH:DHFR Leu22:Arg22	This study
DCH10	Y190 + pAS1-CHY2:GRhbd	This study

10 days, the 56 resulting colonies showing histidine activation were assayed for *lacZ* activation. True positive hits were identified as those whose *lacZ* expression was competed out by 50 μM MTX and which did not show *lacZ* expression in the presence of 10 μM Dex. Putative interacting clones were back-extracted from 5 mL yeast cultures grown to saturation in SD medium lacking leucine. Cells were harvested by centrifugation (3,217 *g*, 5 min). Cell pellets were resuspended in 300 μL zymolyase solution (1.2 M sorbitol, 0.1 M KPO_4 (pH 7.5), 400 μg/mL zymolyase) and incubated at 37° for 2 hr. Each preparation had 300 μL buffer P2 (200 mM NaOH, 1% SDS; Qiagen) added prior to incubation at room temperature for 5 min and subsequent addition of 300 μL buffer P3 (3 M KAc, pH 5.5; Qiagen). The lysate was centrifuged (13,136 *g*, 1 min), the supernatant retained and 5 μL Strataclean resin (Stratagene) added. After vigorous mixing at room temperature for 5 min, resin was removed by centrifugation (13,136 *g*, 1 min). Plasmid DNA was precipitated using 0.7 mL of isopropanol and centrifugation (13,136 *g*, 15 min). The resulting DNA was resuspended in 20 μL H_2O. After pelleting undissolved material, 10 μL was used to transform DH5α™ competent cells according to manufacturer's instructions (Life Technologies). Plasmid DNA was prepared from *E. coli* transformants according to the standard mini-prep protocol (Qiagen). Rescued plasmids were re-transformed into DCH10 and re-screened for *lacZ* expression. The 5′ and 3′ ends of library insert cDNAs were sequenced using primers pACT2for and pACT2rev. Resulting sequences were searched against sequence databases to confirm the identities of the inserts.

2.6. Identification of glucocorticoid receptor interacting proteins

Library plasmids were back-extracted from strains whose *lacZ* expression was not competed out by MTX. These were re-transformed into DCH10 and re-screened for their ability to activate *lacZ* expression in the presence and absence of 10 μM Dex. The 5′ and 3′ ends of library insert cDNAs were sequenced using primers pACT2for and pACT2rev. Resulting sequences were searched against sequence databases to confirm the identities of the inserts.

3. Results

3.1. Generation of functional yeast three-hybrid strains

To determine whether a GAL4-based three-hybrid system could be developed to test for compound–protein interactions, a number of yeast strains were constructed (Fig. 3, Table 2). Yeast strain DCH1 expresses fusion proteins of the hormone binding domain of the rat glucocorticoid receptor fused to the GAL4 DNA-binding domain and of FKBP12 fused to the GAL4 transcriptional activation domain. An efficient three-hybrid strain requires transcription to be stimulated only in the presence of compound heterodimer and both fusion proteins and should show a low background of transcriptional activity. As shown in Fig. 4, DCH1 transformants activate *lacZ* expression only on media containing Dex–FK506 heterodimer (Fig. 4a). The positive control strain DCH7 activates *lacZ* expression in the presence or absence of compound. The remaining control strains DCH3, DCH4 and DCH6 show no activation of *lacZ* expression in the presence or absence of compound showing the DCH1 strain activation requires two independent compound–protein interactions to occur in the same cell. Activation of *lacZ* expression by DCH1 in the presence of 1 μM Dex–FK506 is completely abrogated in a competitive manner by the presence of 1 μM FK506 (Fig. 4b).

Yeast strain DCH2 was generated to confirm that the reporter expression observed was not specific to the FK506–FKBP12 interaction. This strain expresses fusion proteins of the hormone binding domain of the rat glucocorticoid receptor fused to the GAL4 DNA-binding domain and of DHFR fused to the GAL4 transcriptional activation domain. As can be seen in Fig. 4, DCH2 transformants activate *lacZ* expression only on media containing the Dex–MTX heterodimer (Fig. 4c). The positive control strain DCH7 activates *lacZ* expression

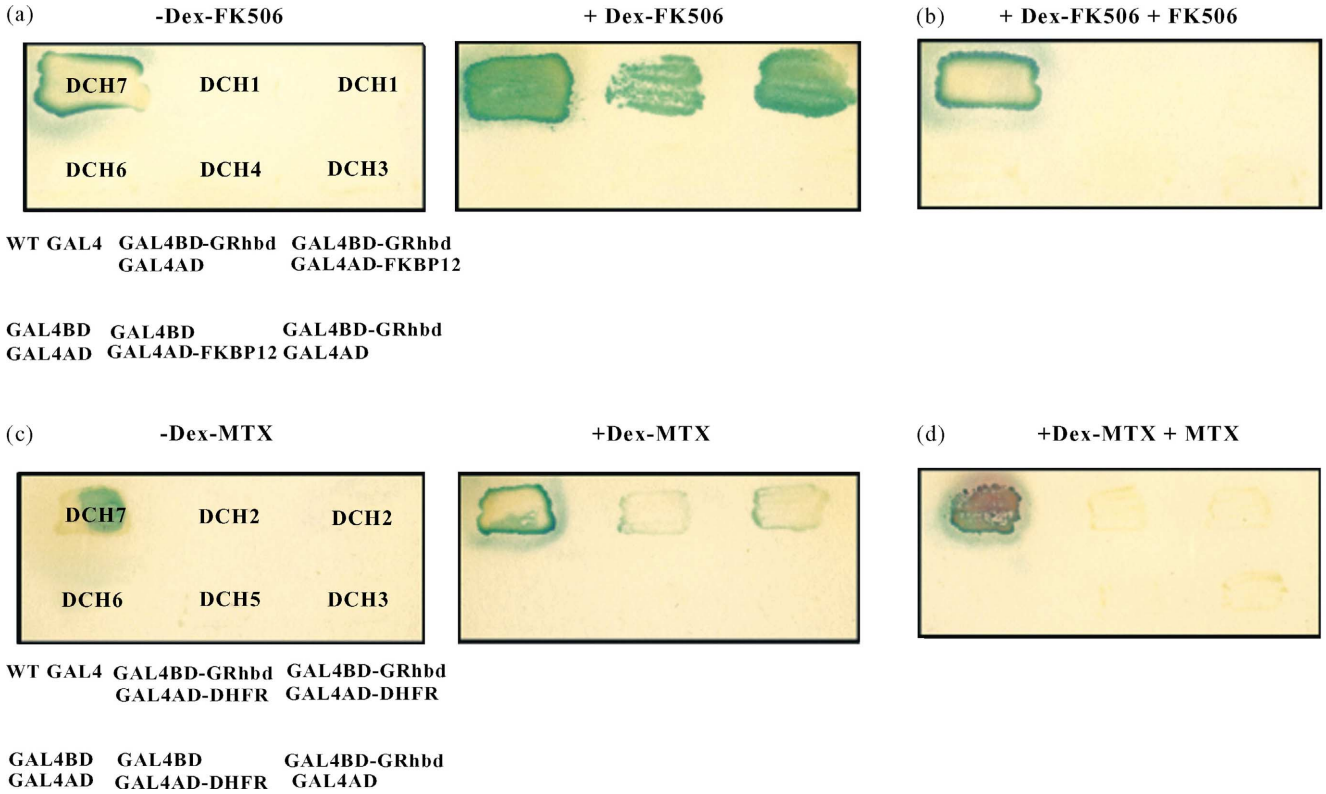

Fig. 4. Functionality of the yeast three-hybrid strains and control strains. Three-hybrid strains (DCH1, DCH2) and their corresponding control strains were tested for their ability to activate *lacZ* expression in the presence or absence of compound. (a) Activation of *lacZ* expression shows the FK506–FKBP12 interaction only occurs in the presence of Dex–FK506 and both fusion proteins. (b) Activation of *lacZ* expression in the presence of 1 μM Dex–FK506 can be competed by 1 μM FK506. (c) Activation of *lacZ* expression shows the MTX–DHFR interaction only occurs in the presence of Dex–MTX and both fusion proteins. (d) Activation of *lacZ* expression in the presence of 10 μM Dex–MTX can be competed by 50 μM MTX.

in the presence or absence of compound. The remaining control strains DCH3, DCH5 and DCH6 show no activation of *lacZ* expression in the presence or absence of compound showing the DCH2 strain activation requires two independent compound–protein interactions to occur in the same cell. Activation of *lacZ* expression by DCH2 in the presence of 10 μM Dex–MTX is completely abrogated in a competitive manner by 50 μM MTX (Fig. 4d).

Whilst it is possible to compete the observed interactions with free FK506 or free MTX, it is not possible to compete these interactions with free Dex. Transactivation of the strain occurs in the presence of 10 μM Dex; this transactivation occurs in the presence or absence of compound heterodimer, but is dependent on the presence of both fusion proteins (data not shown). To confirm that Dex transactivation was not responsible for the activation of *lacZ* expression in the three-hybrid strains, we asked whether Dex–MTX was capable of producing a positive readout in a strain expressing a fusion construct that did not bind MTX. To achieve this, the yeast three-hybrid strains DCH1 (GAL4AD–FKBP12) and DCH2 (GAL4AD–DHFR) were incubated on media containing Dex–MTX. Although *lacZ* expression was detected in DCH2 to represent the MTX–DHFR interaction, no *lacZ* expression was detected in DCH1, showing the activation of *lacZ* expression was dependent upon the presence of a specific compound–protein interaction and not Dex transactivation (data not shown).

3.2. Assessment of strain sensitivity

To explore the sensitivity of the reporter strains described above, titrations were performed to identify the minimal concentration of compound heterodimer capable of inducing a measurable three-hybrid interaction. For the FK506–FKBP12 interaction, the lowest concentration of Dex–FK506 that could activate *lacZ* expression was 20 nM (Fig. 5a). For the MTX–DHFR interaction, the lowest concentration of Dex–MTX that could activate *lacZ* expression was 1 μM (Fig. 5b).

3.3. Inhibition of the three-hybrid interaction by DHFR mutations

To confidently establish that reporter gene activation was specifically due to compound–protein interactions within the yeast strain, DHFR mutations reported to decrease MTX binding affinity were used. Showing that these mutated DHFR clones could no longer activate reporter gene expression would show the yeast three-

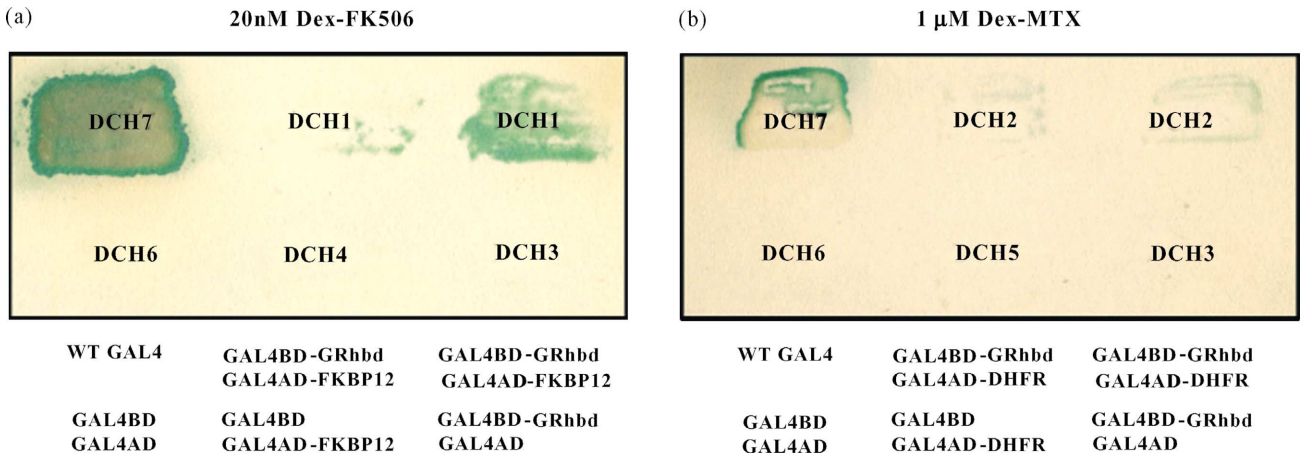

Fig. 5. Sensitivity of the yeast three-hybrid strains. Titrations of compound heterodimer identified the minimum concentration capable of inducing a three-hybrid interaction for strains DCH1 and DCH2. (a) Activation of *lacZ* expression shows the FK506–FKBP12 interaction is sensitive to 20 nM Dex–FK506. (b) Activation of *lacZ* expression shows the MTX–DHFR interaction is sensitive to 1 μM Dex–MTX.

hybrid strain activation to be dependent on compound–protein interactions. Site-directed mutagenesis was used to generate two murine DHFR clones that showed decreased binding affinity for MTX [13–15]. Yeast strains DCH8 and DCH9 were generated that express the DHFR Phe:Trp31 or DHFR Leu:Arg22 mutant clone, respectively.

As shown in Fig. 6, activation of *lacZ* gene expression was detected on 1 μM Dex–MTX for a wild type (WT) DHFR clone but was not detected for the DHFR Phe:Trp31 and DHFR Leu:Arg22 mutant clones. In the presence of 10 μM Dex–MTX, *lacZ* activation was detected for both the WT DHFR and DHFR Phe:Trp31 mutant, but even in the presence of 50 μM Dex–MTX, no *lacZ* activation was visible for the DHFR Leu:Arg22 mutant. The DHFR Phe:Trp31 and DHFR Leu:Arg22 mutations are predicted to increase the K_i value for MTX by 130-fold [15] and 7.5×10^5-fold [14],

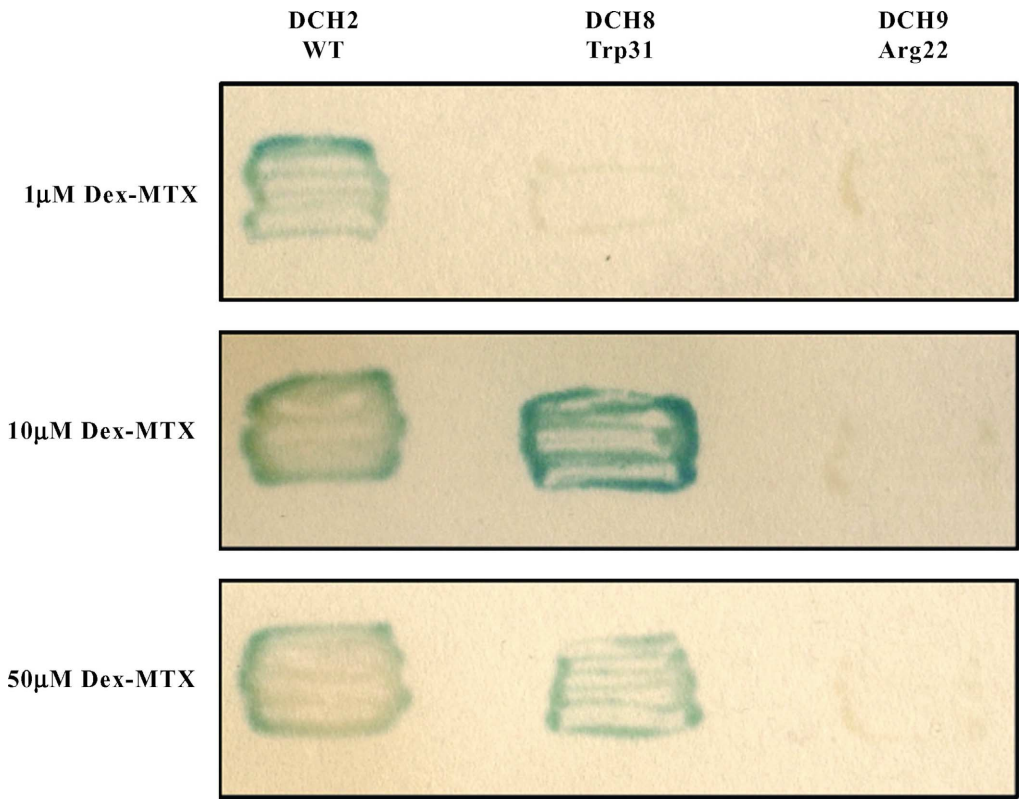

Fig. 6. Inhibition of the three-hybrid interaction by DHFR mutations. Strains containing wild-type DHFR (DCH2), DHFR Phe:Trp31 (DCH8) or DHFR Leu:Arg22 (DCH9) were tested for their ability to activate *lacZ* expression in the presence of different concentrations of Dex–MTX. WT DHFR can activate *lacZ* expression in the presence of 1 μM Dex–MTX, DHFR Phe:Trp31 requires 10 μM Dex–MTX to activate *lacZ* expression, but DHFR Leu:Arg22 cannot activate *lacZ* expression even in the presence of 50 μM Dex–MTX.

respectively. This correlates with our results and shows that induction of reporter gene expression in our three-hybrid strain is entirely dependent on interaction of a small molecule such as MTX with its target protein, DHFR.

3.4. Library screen to identify MTX-interacting proteins

While the results described above show the efficiency of the three-hybrid strain for visualising known compound–protein interactions, they do not demonstrate the practical utility of the strain as a screening tool for unknown protein binding partners. To explore the potential use of the strain in a screening context, MTX was used as a bait compound to screen a mouse cDNA library. By screening 5×10^6 colonies, 56 potential positives were identified through their ability to activate *lacZ* expression in the presence of 10 μM Dex–MTX.

A genuine hit was defined as a colony whose activation of *lacZ* expression in the presence of compound heterodimer could be competed by an excess of the uncoupled test compound and that in addition did not activate *lacZ* expression in the presence of uncoupled Dex. Four colonies out of 56 were defined as genuine hits based on the above criteria using 10 μM Dex–MTX, 50 μM MTX and a non-transactivating concentration of Dex. All four clones were identified as DHFR and sequencing of the rescued plasmids showed each DHFR sequence to be cloned in frame with the GAL4 AD to produce a functional fusion protein. One clone contains full length DHFR coding sequence, whilst the remaining three clones contain identical sequences that lack the initial six base pairs of coding sequence including the ATG, but are otherwise full length.

3.5. Identification of glucocorticoid receptor interacting proteins

After identification of genuine MTX-interacting proteins from the screen, the false positives were further analysed by scoring them based on their ability to activate *lacZ* expression in the presence or absence of 10 μM Dex. Five false positives were identified as being due to a genomic mutation in the yeast strain, shown by their ability to activate reporter gene expression in the absence of the GAL4AD–GRhbd fusion protein. The additional 45 hits were defined as GR-interacting proteins due to their ability to activate *lacZ* expression in a strain containing the GAL4BD–GRhbd fusion protein (DCH10) in the presence or absence of Dex. These protein–protein interactions are analogous to those identified in a yeast two-hybrid strain. The GR-interacting proteins fell into two categories. Twenty-eight of the library clones encoded proteins that could bind the GR in the presence or absence of Dex, whilst 17 of the library clones encoded proteins whose binding was Dex-dependent. In summary, two known and three novel GR-interacting proteins were identified from the screen. The known GR-interacting proteins showed Dex-dependent binding. Two of the novel GR-interacting proteins showed Dex-dependent binding, whilst one novel GR-interacting protein could bind in the presence or absence of Dex.

4. Discussion

In this report we describe a yeast three-hybrid strain that for the first time uses the functional reconstitution of a GAL4 transcription factor to activate reporter gene expression. We have shown the strain is functional using known compound–protein interactions between FK506–FKBP12 and MTX–DHFR to activate *lacZ* expression. These interactions can be competed by the presence of excess FK506 or MTX, respectively, resulting in loss of reporter gene expression. In addition, a strain expressing a mutated DHFR clone with decreased binding affinity for MTX is not capable of activating reporter gene expression. The amino acids chosen for mutation, Leu22 and Phe31, both reside within the active site of DHFR. The Leu22 residue is present in the hydrophobic pocket of the active site and functions to aid inhibitor and substrate binding in the pocket [14]. The Phe31 residue is present in a less critical position in the active site but functions to aid inhibitor and substrate stabilisation within the pocket [15]. Variants at the codon-22 position have been described generally as showing an increased resistance to a given level of MTX, whilst variants at codon-31 generally retain more catalytic activity at a given level of MTX [13]. This correlates with our data showing that WT DHFR requires 1 μM Dex–MTX to activate *lacZ* expression, DHFR Phe:Trp31 requires 10 μM Dex–MTX to activate *lacZ* expression, but DHFR Leu:Arg22 cannot activate *lacZ* expression even in the presence of 50 μM Dex–MTX. We have therefore demonstrated that the induction of reporter gene expression is entirely dependent on the interaction of a small molecule with its target protein.

Furthermore, we find that the strain shows a high degree of sensitivity, but that this varies to some extent depending on the compound–protein interaction being tested. The larger concentration of Dex–MTX required for reporter gene activation compared to Dex–FK506 (Fig. 5) could be due to the presence of endogenous yeast DHFR. One hypothesis is that yeast DHFR may be competing with the mouse DHFR for binding of MTX. This MTX sequestering may result in larger concentrations of Dex–MTX being required to achieve the critical threshold of compound necessary for strain activation. The difference in sensitivity between the two strains may alternatively result from FK506 and MTX being differentially permeable to yeast, being metabolised at different rates within the cell or possessing different binding affinities for their respective binding proteins, FKBP12 and DHFR.

We have also illustrated the utility of the strain as a screening tool for unknown compound-binding proteins by

1382 LLKTDADGNQQVQQVQVFADVQCTVNLVGGDP

YLNQPGPLGTQKPTSGPQTPQAQQKSL**LQQLL**TE* 1447

Fig. 7. SRC-1a amino acid sequence encoded by library cDNA insert. The amino acid sequence encoded by the library SRC-1a clone contains nuclear receptor binding motif four, LQQLL, highlighted in bold and underlined.

identifying four DHFR clones from a mouse library screen using the Dex–MTX heterodimer as a test compound. The screen background is low and genuine hits can easily be separated from false positives by quick, secondary tests for compound dependency and competition by the unconjugated bait compound, MTX. This analysis demonstrates that the yeast three-hybrid strain reported here can be used in a screening context to identify target proteins for a small molecule of interest from a representative library.

Further to its use for identifying proteins that interact with a compound of interest, we describe how this three-hybrid screen has identified two known and three novel GR-interacting proteins. Four of these proteins show a ligand-dependent interaction with the GR, whilst one protein can bind the GR in the presence or absence of Dex. The known GR-interacting proteins identified, steroid receptor coactivator 1a (SRC-1a) and receptor interacting protein 140 (RIP140) [16,17] provide confidence that the novel hits identified may be physiologically relevant. As reported in the literature, both SRC-1a and RIP140 only bind the GR in the presence of ligand, consistent with our observations. Many nuclear receptor coactivators, including SRC-1, bind the receptor in a ligand-dependent manner via LXXLL nuclear receptor binding motifs. Although SRC-1a contains four LXXLL motifs, published data suggests that it preferentially uses motif four for binding to the GR [18]. We have established that our isolated cDNA library clone of SRC-1a encodes this motif as shown in Fig. 7. It is interesting to note that using the same compound heterodimer to screen different cDNA libraries may result in the identification of different GR interacting proteins. In addition, using different compound heterodimers to screen the same cDNA library may also result in the identification of different GR-interacting proteins if the coupling of the test compound changes the binding affinity of the GR for different cofactors.

It should be noted that there are caveats to this screening technology since the limitations of the yeast two-hybrid system also apply to the three-hybrid system. A target protein for a small molecule of interest will not be identified if the protein requires complex folding, post-translational modification or insertion into a membrane for functional activity. Similarly, proteins that bind small molecules as part of a protein complex will not be identified. However, it is important to realise that these problems underlie all current techniques that are used to isolate compound-binding proteins and are not specific to the three-hybrid system. A further caveat is that although the test compound-binding partner interaction can be abrogated by the presence of free test compound, the Dex–GRhbd interaction cannot be competed due to transactivation of the strain by free Dex. We have established that Dex transactivation is not responsible for the activation of *lacZ* expression in the three-hybrid strains.

Points to consider when proposing a compound for three-hybrid screening are whether it is likely to be permeable to yeast, whether it is likely to retain functional activity after being coupled to Dex and whether there is a biological assay available for testing this heterodimer activity. Structure–activity relationship (SAR) studies should be used to identify optimal points for linker attachment that should have minimal effect upon the activity of the test compound. It may be necessary to generate a series of heterodimeric compounds using different linker attachment points for testing in a biological assay alongside the uncoupled compound. The carbon linker length is also a design consideration; although a 10-carbon linker has been shown to generate functional Dex–FK506 and Dex–MTX heterodimers, longer lengths may be required to attach larger molecules in order that both compounds remain biologically active.

Identification of novel target proteins for small molecular weight ligands has always been a time consuming and technically challenging process requiring complex biochemical procedures. We show that identification of such proteins for small molecules of interest is now technically feasible for any laboratory with classical molecular biology capability using the yeast three-hybrid system. We show that partial clones are often obtained from the screen, but using simple molecular techniques combined with information from genome sequencing programmes, the appropriate full-length clones should be easily obtainable.

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