Published in final edited form as:

Anal Biochem. 2009 March 1; 386(1): 129–131. doi:10.1016/j.ab.2008.11.042.

A Tetracycline Repressor-based Mammalian Two-hybrid System to Detect Protein-protein Interactions *in vivo*

Gabrielle Nina Thibodeaux^a, Roshani Cowmeadow^a, Aiko Umeda^b, and Zhiwen Zhang^{a,*}

^a Division of Medicinal Chemistry, College of Pharmacy, The University of Texas at Austin

^b Institute Cellular and Molecular Biology, College of Natural Sciences, The University of Texas at Austin

Protein-protein interactions are essential to many fundamental biological processes including signal transduction, cell cycle control, transcription, translation, DNA replication, and apoptosis [1]. A comprehensive understanding of protein-protein interactions within biological systems is needed to understand and regulate cellular functions. The yeast two-hybrid system has facilitated the study of protein-protein interactions tremendously since its introduction [2]. However, it is unclear whether mammalian proteins behave the same when expressed in yeast [3]. To better study protein-protein interactions in mammalian cells, a mammalian cell-based two-hybrid system should provide more biologically relevant information than the yeast two-hybrid system. The first mammalian two-hybrid system was based on the functional reconstitution of the transcription activator GAL4, and was essentially a “cut-and-paste” of the yeast two-hybrid system into mammalian cells [4], so do most (if not all) of commercial mammalian two-hybrid systems. One of the issues using the GAL4-based mammalian two-hybrid system is the high-level background from the genetic assays, especially when the dissociation constant (K_d) of the interacting proteins under study is above 1 μ M. A different protein-fragment complementation assay (PCA) has also been recently developed and used to detect protein-protein interactions in mammalian cells [5]. Since this assay is based on the stoichiometric association of two expressed fragments of a reporter protein to restore its function, the sensitivity of this assay is potentially limited by the expression level of the reporters in mammalian cells. Herein, we report a mammalian two-hybrid system (termed as trM2H) with high sensitivity and low-level background. The system can be used to detect protein-protein and peptide-peptide interactions in mammalian cells with dissociation constants (K_d) ranging from 0.99 nM to 55 μ M. The trM2H system can also be disrupted by the small molecule doxycycline (Dox).

The design of the trM2H system is based upon the reconstruction of the functional tetracycline repressor (TetR). TetR is a transcriptional regulator and its dimeric form binds specifically to the tet operator (*tetO*) to inhibit the downstream gene expression of tetracycline resistance genes [6]. The dimerization of TetR is controlled by the specific interaction of its C-terminal dimerization domain (DD) [7]. In a commercial Tet-Off[®] Advanced Inducible Gene Expression System (Clontech), TetR is connected to three nominal transcriptional activation domains (AD) taken from herpes simplex virus VP16 [8,9]. Dimerization of the TetR-VP16 fusion allows the N-terminal TetR to bind to the Tet-Responsive Element (TRE_{mod}) while the

*Division of Medicinal Chemistry, College of Pharmacy, The University of Texas at Austin, The University of Texas at Austin, PHARMED CHEM BME 6.202E, 1 University Station, C8050, Austin, TX 78712-0128 (512) 471-4551 office, (512) 232-2606 fax, zhang@mail.utexas.edu email.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

C-terminal VP16 is free to recruit the transcriptional machinery [10,11]. This system was designed to tightly regulate the expression of protein levels in mammalian cells with the ratio of signal to noise reaching 1000:1 in the absence of tetracycline [8,12]. Our approach is to replace the TetR C-terminal dimerization domain ($\alpha 8$ - $\alpha 10$) with “bait” and “prey” molecules respectively. When the bait and prey interact, the resulting TetR-VP16 will dimerize to reconstitute the functional transcriptional activator and initiate transcription followed by over-expression of a downstream reporter gene (GFP in this case). In contrast, absence of GFP signal indicates that there is no interaction between bait and prey molecules and the monomeric TetR-VP16 fails to initiate the transcription of GFP gene (Figure 1).

To locate the minimal region within TetR that is responsible for dimerization, the C-terminal α -helices ($\alpha 1$ to $\alpha 10$) of TetR-VP16 were sequentially deleted and replaced with homodimeric GCN4 leucine zipper (GCN4LZ), which has a K_d of 0.5 μ M [13,14]. The effectiveness of trM2H system was studied using fluorescence microscopy, based on the expression of GFP *in vivo*. In a typical experiment, human embryonic kidney (HEK) 293H cells were transfected with pTRE-Tight-AcGFP (Clontech) and pTetOff mutants at 70–80% confluency using FuGene6. Figure 2A and 2H show that native TetR dimerizes and initiates the expression of GFP. By replacing $\alpha 8$ - $\alpha 10$ with GCN4LZ dimerization of TetR-VP16, the expression of GFP was restored (Fig. 2B, left lane of 2I). To address the possibility of non-specific binding (background), a GCN4LZ mutant that cannot dimerize replaced $\alpha 8$ - $\alpha 10$ helices of TetR [15]. As expected, this mutant failed to dimerize TetR-VP16, and no GFP was expressed (Fig. 2C, right lane of 2I). Deletion of other helices either completely destroyed the functions of TetR-VP16 or were unable to dissociate the TetR dimer (data not shown). This set of experiments not only identified the multiple cloning site (MCS) that permits incorporation of “prey” and “bait” molecules to the C-terminus of TetR-VP16, but also demonstrated that interaction between “prey” and “bait” can reconstruct the functional TetR-VP16 transcription activator.

The trM2H system can detect the hetero-dimerization of proteins in mammalian cells. The hetero-dimeric pair of Fos and Jun peptides (K_d of 0.99 nM) [16] were cloned into the MCS of TetR-VP16 as bait and prey, respectively. Plasmids encoding TetR-Fos-VP16 (pWTFos) and TetR-Jun-VP16 (pWTJun) were co-transfected into HEK293H cells containing pTRE-Tight-AcGFP. Interaction of Fos-Jun dimerized TetR-VP16 and created a functional transcriptional regulator that promoted the expression of GFP in mammalian cells (Figure 2D, left lane of Fig. 2J). As a control, Fos was mutated at sites that abolish its binding to Jun [17]. This pair of mutant Fos and Jun peptides was unable to restore the functional TetR-VP16 dimer. However, it is known that Jun can form a relatively weaker homodimer with a K_d of 0.27 μ M [18], which results in the formation of homodimeric TetR-Jun-VP16 transcription activator (Figure 2E, right lane of Fig. 2J). It is interesting that the expression level of GFP in these cells is lower than that in cells containing stronger Fos-Jun interaction (Compare Figure 2D and 2E). Western blot experiment confirmed the genetic data (Fig. 2J).

The trM2H system is highly sensitive to detect weak protein interactions in mammalian cells. Sortase A (SrtA), a transpeptidase, has been recently shown to be a dimeric protein with a K_d of 55 μ M *in vitro* [19]. A single mutation (K137A) on its surface disrupts the dimerization of SrtA *in vitro* [20]. A truncated sortase A with the deletion of its N-terminal 59 amino acids (SrtA $_{\Delta 59}$) can be readily expressed in mammalian cells (data not shown). The gene encoding SrtA $_{\Delta 59}$ was cloned into the MCS of TetR-VP16 to afford the plasmid pWTSrtA $_{\Delta 59}$. As a negative control, the gene encoding a mutant SrtA $_{\Delta 59}$ K137A was cloned into the same position of TetR-VP16 to afford plasmid pMUSrtA $_{\Delta 59}$. HEK 293H cells co-transfected with plasmids pWTSrtA $_{\Delta 59}$ and pTre-Tight GFP showed strong GFP signals (Figure 2F, left lane of Fig. 2K), illustrating that association of SrtA $_{\Delta 59}$ introduces the dimerization of TetR-SrtA-VP16 and activates the transcription of GFP. In contrast, HEK 293H co-transfected with plasmids pMUSrtA $_{\Delta 59}$ and pTre-GFP showed no or little background GFP signal (Fig. 2G, right lane

of Fig. 2K). In this case, the SrtA_{Δ59} mutant K137A was unable to dimerize the TetR-K137A-VP16 to initiate the transcription of the GFP gene. Thus, the genetic data of sortase A from trM2H system matches and supports the biochemical *in vitro* results [19].

Small molecules such as tetracycline and doxycycline (Dox) can specifically disrupt trM2H system, since they are known to allosterically regulate the binding of TetR to the *tetO* transcriptional element [12]. HEK 293H cells were co-transfected with pWTGCN4 and pTre-Tight-AcGFP and showed the positive GFP signal after 72 hours (Supplement Fig. S1). In a separate experiment, doxycycline (1mM) was added to the culture 24 hours after co-transfection. The culture was incubated for an additional 48 hours and only about 1% HEK 293H cells were fluorescent (Supplement, Fig. S2). In this case, Dox disrupted the dimerization of TetR-GCN4-VP16 and the expression of GFP was turned off. In another experiment, doxycycline (1mM) was added immediately after the co-transfection. After 72 hours, less than 1% of the HEK 293H cells expressed GFP (Supplement, Fig. 3C).

In conclusion, a small molecule-regulated mammalian two-hybrid system (trM2H) has been developed to detect peptide-peptide and protein-protein interactions *in vivo*. Both homo- and hetero-dimerizations can be detected using this system. To our best knowledge, this system is the most sensitive genetic assay in mammalian cells capable of detecting weak interactions with dissociation constants in the high μ M range, with excellent background. The described trM2H system can be extended as an efficient genetic selection system in mammalian cells to identify library-derived ligands that specifically bind to the target protein (forward assay) or specifically disrupt the protein-protein interaction (reverse mammalian two-hybrid system).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Professor Shawn Bratton's lab (University of Texas at Austin) for technical assistance and helpful discussions and Tiffany Chen and Rachel Hartsell for technical assistance, and Christopher Thibodeaux for proof reading our manuscript. This work was funded in part by a grant from Welch Foundation (F1618) and a grant from the American Heart Association (0665198Y) and NIH R01CA120168.

Abbreviation used

trM2H	tetracycline repressor-based mammalian two-hybrid system
TetR	tetracycline repressor
<i>tetO</i>	tet operator
Dox	doxycycline
K_d	dissociation constants
GFP	green fluorescent protein
GCN4LZ	

GCN4 leucine zipper

SrtA

Staphylococcus aureus sortase A

MCS

multiple cloning site

References

1. Phizicky EM, Fields S. Protein-protein interactions: Methods for detection and analysis. *Microbio Rev* 1995;59:94–123.
2. Fields S, Song O-k. A novel genetic system to detect protein-protein interactions. *Nature* 1989;340:245–246. [PubMed: 2547163]
3. Aravind L, Dixit VM, Koonin EV. Apoptotic molecular machinery: Vastly increased complexity in vertebrates revealed by genome comparisons. *Science* 2001;291:1279–1284. [PubMed: 11181990]
4. Luo Y, Batalao A, Zhou H, Zhu L. Mammalian two-hybrid system: A complementary approach to the yeast two-hybrid system. *Biotechniques* 1997;22:350–352. [PubMed: 9043710]
5. Michnick SW, Ear PH, Manderson EN, Remy I, Stefan E. Universal strategies in research and drug discovery based on protein-fragment complementation assays. *Nat Rev Drug Discov* 2007;6:569–582. [PubMed: 17599086]
6. Hillen W, Berens C. Mechanisms underlying expression on tn10 encoded tetracycline resistance. *Ann Rev Microbio* 1994;48:345–369.
7. Schnappinger D, Schubert P, Berens C, Pfeleiderer K, Hillen W. Solvent-exposed residues in the tet repressor (tetR) four-helix bundle contribute to subunit recognition and dimer stability. *J Biol Chem* 1999;274:6405–6410. [PubMed: 10037732]
8. Baron U, Gossen M, Bujard H. Tetracycline-controlled transcription in eukaryotes: Novel transactivators with graded transactivation potential. *Nucl Acid Res* 1997;25:2723–2729.
9. Triezenberg SJ, Kingsbury RC, McKnight SL. Functional dissection of vp16, the transactivator of herpes simplex virus immediate early gene expression. *Gene Devel* 1988;2:718–729. [PubMed: 2843425]
10. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline- responsive promoters. *Proc Natl Acad Sci USA* 1992;89:5547–5551. [PubMed: 1319065]
11. Flint J, Shenk T. Viral transactivating proteins. *Ann Rev Genet* 1997;31:177–212. [PubMed: 9442894]
12. Henssler EM, Bertram R, Wisshak S, Hillen W. Tet repressor mutants with altered effector binding and allostery. *FEBS J* 2005;272:4487–4496. [PubMed: 16128817]
13. Wendt H, Baici A, Bosshard HR. Mechanism of assembly of a leucine zipper domain. *J Am Chem Soc* 1994;116:6973–6974.
14. Dragan AI, Frank L, Liu Y, Makeyeva EN, Crane-Robinson C, Privalov PL. Thermodynamic signature of gcn4-bzip binding to DNA indicates the role of water in discriminating between the ap-1 and atf/creb sites. *J Mol Biol* 2004;343:865–878. [PubMed: 15476806]
15. Hu JC, O'shea EK, Kim PS, Sauer RT. Sequence requirements for coiled-coils: Analysis with lambda repressor-gcn4 leucine zipper. *Science* 1990;250:1400–1403. [PubMed: 2147779]
16. Heuer KH, Mackay JP, Podzebenko P, Bains N, Weiss PSAS, King GF, Easterbrook-Smith SB. Development of a sensitive peptide-based immunoassay: Application to detection of the jun and fos oncoproteins. *Biochemistry* 1996;35:9069–9075. [PubMed: 8703910]
17. Schuermann M, Neuberger M, Hunter JB, Jenuwein T, Ryseck RP, Bravo R, Muller R. The leucine repeat motif in fos protein mediates complex formation with jun/ap-1 and is required for transformation. *Cell* 1989;56:507–516. [PubMed: 2492453]
18. Oyama R, Takashima H, Yonezawa M, Doi N, Miyamoto-Sato E, Kinjo M, Yanagawa H. Protein-protein interaction analysis by c-terminally specific fluorescence labeling and fluorescence cross-correlation spectroscopy. *Nucl Acid Res* 2006;34:e102.

19. Lu C, Zhu J, Wang Y, Umeda A, Cowmeadow RB, Lai E, Moreno GN, Person MD, Zhang Z. Staphylococcus aureus sortase a exists as a dimeric protein in vitro. *Biochemistry* 2007;42:9346–9354. [PubMed: 17658894]
20. Zhu J, Lu C, Standland M, Lai E, Moreno GN, Umeda A, Jia X, Zhang Z. Single mutation on the surface of staphylococcus aureus sortase a can disrupt its dimerization. *Biochemistry* 2008;47:1667–1674. [PubMed: 18193895]

Figure 1.

General scheme for the mammalian two-hybrid system (trM2H). Replacement of the dimerization domain (DD) of Tetracycline repressor (TetR) with interacting “bait” and “prey” molecules will yield a functional transcriptional regulator. A small molecule doxycycline can disrupt the TetR dimer and turn off the function of transcription activator and the expression of the downstream reporter protein.

Figure 2.

Expression of GFP signals in a mammalian two-hybrid system was monitored by fluorescence microscopy (A-G) and Western blot (H-K, probed with anti-GFP antibody). Dimerization of native TetR activated the expression of GFP in HEK 293H cells (A, H). Dimerization of yeast GCN4LZ (in place of the C-terminal dimerization domain (DD) of TetR) reconstructed the functional TetR dimer and activated the expression of GFP (B, left lane of I). The monomeric GCN4LZ mutant failed to activate the expression of GFP (C, right lane of I). Interaction between Fos and Jun as bait and prey reconstructed the functional TetR dimer and activated the expression of GFP (D, left lane of J). In the presence of a Fos mutant, weaker homodimerization of Jun-Jun restored the function of the TetR dimer and a low level of GFP expression was observed (E, right lane of J). Dimerization of sortase A reconstructed the TetR dimer and activated the GFP expression (F, left lane of K). Monomeric sortase A mutant K137A failed to dimerize TetR and no expression of GFP expression was observed (G, right lane of K).