

Cloning of JNK activation domain and Fas-binding domain of human apoptotic Daxx gene by homologous recombination in yeast

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ABSTRACT

The death domain-associated protein (Daxx) was originally cloned as a Fas-interacting protein. It is ubiquitously expressed and highly conserved in mammals. Currently, the role of Daxx is controversy between apoptotic or anti-apoptotic molecules. To understand the precise function of human Daxx, yeast two hybrid screening will be used as a tool to identify interactions between human Daxx and other human proteins. The first step in a yeast two hybrid method is to express a bait plasmid in the recombinant form in yeast. We utilized the *in vivo* homologous recombination in yeast to insert a JNK activation domain and a Fas-binding domain of human Daxx, into the bait plasmid, pEG-NRT. Furthermore, the expression of a JNK activation domain and a Fas-binding domain of human Daxx was demonstrated by Western blot analysis. Yeast expressing a JNK activation domain and a Fas-binding domain of human Daxx will be used as bait strains to screen for human proteins interacting with Daxx, which may provide us the clue to uncover the mysterious function of human Daxx.

Keywords: cloning, human Daxx, JNK activation domain, Fas-binding domain, yeast

INTRODUCTION

Daxx is a 740 amino acid protein that contains amino-terminal amphipathic helices (PAH1, PAH2), a coiled-coiled domain (CC), an acidic domain (D/E), and a carboxyl-terminal serine/proline/threonine rich domain (S/P/T). The Daxx carboxyl-terminal region (amino acids 625-739) interacted strongly with Fas receptor in a yeast two-hybrid system. This region was sufficient to bind a GST-Fas fusion protein in a GST pull down assay. Daxx mutants missing this region had very

modest cell death activity (Yang *et al.*, 1997). This region is also required for interactions with several apoptotic proteins; for example, Pml, ETS1, and DJ-1 (Li *et al.*, 2000; Zhong *et al.*, 2000; Junn *et al.*, 2005). Daxx and Pml participate in a nuclear pathway of apoptosis (Ishov *et al.*, 1999; Zhong *et al.*, 2000). Daxx itself represses transcriptional activation of ETS1 target genes including Bcl-2 (Li *et al.*, 2000). Finally, interaction of Daxx with DJ-1 inhibits ASK1 activity and cell death activity (Junn *et al.*, 2005). However, Daxx mutants containing amino acids 501-625, which lies

immediately amino-terminal to the Fas-binding domain of Daxx (a JNK activation domain) contained most of the cell death activity (Yang *et al.*, 1997; Chang *et al.*, 1998).

Yeast two hybrid is one of the tools to identify interactions among proteins. It has three critical components. First, a plasmid expressing a protein of interest fused to DNA binding domain refers to bait. When a bait plasmid is transformed into yeast with a reporter gene, the bait protein binds to a reporter gene promoter, but it cannot activate transcription. Second, a cDNA prey plasmid expressing cDNA encoded protein fused to activation domain refers to prey. When a prey plasmid is placed into yeast containing a reporter gene, the prey protein cannot activate transcription because it does not have DNA binding domain to bind to a reporter gene promoter. Third, reporter genes contain binding sites that are upstream activation sequence for DNA binding domain bindings. When the bait and prey plasmids are expressed into yeast, the interaction will bring DNA binding domain and activation domain into close contact. Then, the transcription factor is constitutively activated and reporter genes are expressed.

The first step in yeast two hybrid screening is to express a protein of interest fused to DNA binding protein such as LexA. We utilized the *in vivo* homologous recombination in yeast to insert a JNK activation domain and a Fas-binding domain,

into the bait plasmid, namely pEG-NRT (Limjindaporn *et al.*, 2007). The expression of a JNK activation domain and a Fas-binding domain in yeast was demonstrated by Western blot analysis.

MATERIALS AND METHODS

PCR amplification

A Fas-binding domain of human Daxx (303 bps), a JNK activation domain (339 bps), and a region between the a JNK activation domain and a Fas-binding domain of human Daxx (282 bps) flanked with recombination tags (RT) at the 5' and 3' ends, were amplified by PCR using pCDNA3.1 Hygro-Daxx (Limjindaporn and Netsawang, 2008) as a DNA template. The primers used are listed in Table 1. The PCR reactions were carried out in a GeneAmp PCR System 9700 (Applied Biosystem) started with 94 °C for 5 minutes and followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 60 °C for 60 seconds, extension at 72 °C for 1 minute and 20 seconds, and one cycle of final extension at 72 °C for 7 minutes.

To screen the correct yeast clones containing truncated Daxx genes by colony PCR, yeast colonies were used as templates. LexA NRTf and NRT ADHT_r, whose sequences are located within the yeast vector at the 5' and 3' ends of inserts, were used as primers (Table 1). The PCR reaction was carried out in a GeneAmp PCR System 9700 (Applied Biosystem) started with 96 °C for

Table 1 Primers used in this study.

Names	Sequences
Jnkf	5' TTG ACT GTA TCG CCG CGC ATA GTG TCA CC 3'
Jnkr	5' CCG GAA TTA GCT TGG CTG CAG TTG CTT CTT CTC CTT C 3'
Fasf	5' TTG ACT GTA TCG CCG CAA ACA GGA TCA GGG 3'
Fasr	5' CCG GAA TTA GCT TGG CTG CAG ATC AGA GTC TGA GAG 3'
Jnk-fasf	5' TTG ACT GTA TCG CCG GAG ATT GAA GCT TTG 3'
Jnk-fasr	5' CCG GAA TTA GCT TGG CTG CAG TAT CTT TTT CCC ATT 3'
Lex A NRTf	5' TCG TTT TAA AAC CTA AGA GTC 3'
NRT ADHT _r	5' AGC TTC ACC ATT GAA GGG CTG 3'

5 minutes and followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 48 °C for 30 seconds, extension at 72 °C for 1 minutes, and one cycle of final extension at 72 °C for 10 minutes.

In vivo DNA cloning and DNA sequencing

Yeast strain RFY231 (*MAT α his3, trp1 Δ ::hisG, ura3, 3LexAop-LEU2::leu2 MAL+*) (Finley and Brent, 1994) was transformed with 300 ng of linearized pEG-NRT and 100 ng of the PCR product using the LiOAc method (Gyuris *et al.*, 1993). The negative control was transformed with the same method but no PCR product was added. After the heat shock step, yeast cells were resuspended in 100 μ L of sterile distilled water and plated onto a Glu / –his plate. The plates were incubated at 30°C for 4 days, and colonies were transferred to the new plates. Colony PCR was conducted using the primers located in the pEG-NRT to check whether the clones had the correct inserts. The DNA from the clones, which had the correct inserts, was then subjected to DNA sequencing.

Verification of protein expression by Western blot analysis

Western blot analysis was performed by growing the yeast strain RFY231, which contained bait plasmid, in Glu/CM / –his broth to express a LexA fusion protein. The crude protein was extracted from yeast culture by adding 20% SDS and physically breaking with glass bead. The lysates were subjected to 12% SDS-PAGE gel and subsequently blotted onto nitrocellulose membrane using a semi-dry blotting apparatus. Western blot analysis was performed by using rabbit anti-LexA monoclonal antibody as a primary antibody followed by horseradish peroxidase (HRP) conjugated swine anti-rabbit IgG secondary antibody. The signal of proteins was visualized by ECL detection according to PIERCE manufacturer's protocol.

RESULTS

Homologous recombination in yeast was performed to insert domains of human Daxx, for example, a JNK activation domain and a Fas-binding domain, into the bait plasmid, pEG-NRT, which contains LexA, 5'recombination tag (RT) and 3'RT (Limjindaporn *et al.*, 2007). The domains of Daxx flanking with 5'RT and 3'RT (Fig. 1) were easily cloned into pEG-NRT, which also has 5'RT and 3'RT by homologous recombination in yeast. Colony PCRs were conducted to select clones containing the correct inserts from a Glu/–his plate. Three clones in lane 2, 3 and 4 of Figure 2 contained the expected inserts of PCR products, which are a JNK activation domain, a region between a JNK activation domain and a Fas-binding domain and a Fas-binding domain, respectively. Finally, to demonstrate that the yeast clones actually expressed the expected fusion proteins, Western blot analysis was performed by using antibody to LexA. Undoubtedly, the expected sizes of the truncated Daxx fusion proteins were expressed (Fig. 3). Therefore, cloning of JNK activation domain and Fas-binding domain of Daxx by homologous recombination in yeast was successful.

DISCUSSION

In vivo homologous recombination functions of a host strain can insert a gene of interest into plasmids without *in vitro* steps. This approach is well described in the yeast *Saccharomyces cerevisiae*. A new DNA fragment can be integrated into a plasmid in the cell as long as both regions (20-40 amino acids) flanking the gap are homologous to regions flanking a DNA fragment targeted for integration. Crossing-overs occur at the two homologous regions, resulting in the insertion of the DNA fragment (Uetz *et al.*, 2000).

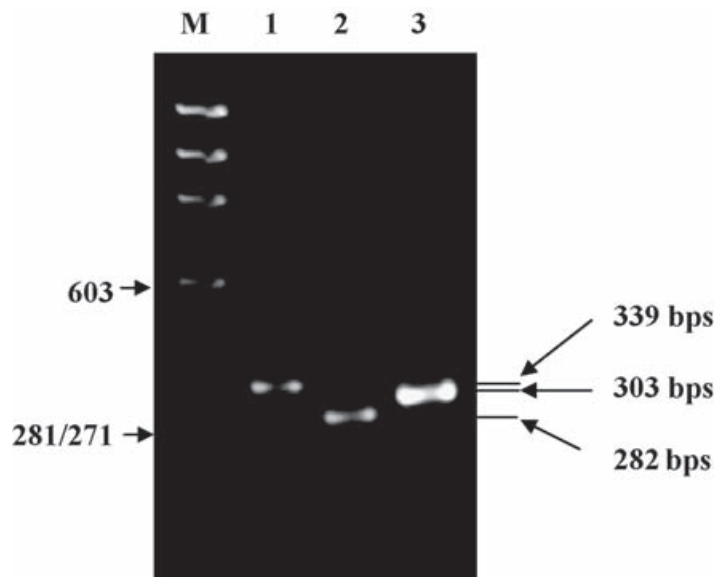


Figure 1 PCR amplification of JNK activation domain and Fas-binding domain of human Daxx. Lanes 1, 2, and 3 are a JNK activation domain (339 bps), a region between a JNK activation domain (282 bps) and a Fas-binding domain (303 bps), respectively. Sizes of the PCR fragments were compared to that of *Hae*III-digested ϕ X174 DNA fragments (lane M) which served as a DNA marker.

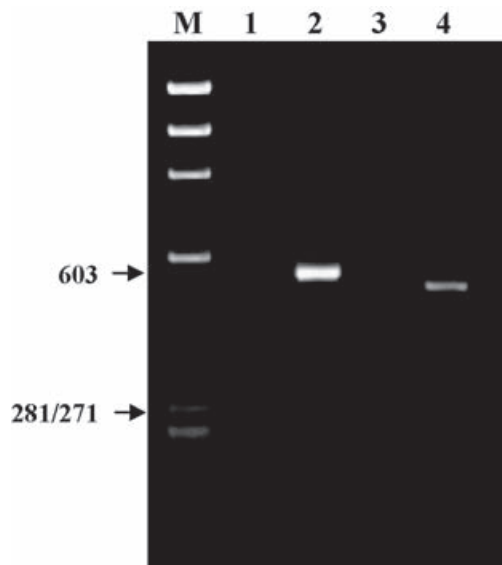


Figure 2 Verification of positive yeast clones containing JNK activation domain and Fas-binding domain of human Daxx by colony PCR. Lane 1, PCR product was from a yeast clone containing a control vector that contained only LexA gene. Three yeast clones in lane 2, 3 and 4 contained the expected inserts of PCR products. Sizes of the PCR fragments were compared to that of *Hae*III-digested ϕ X174 DNA fragments (lane M) which served as a DNA marker.

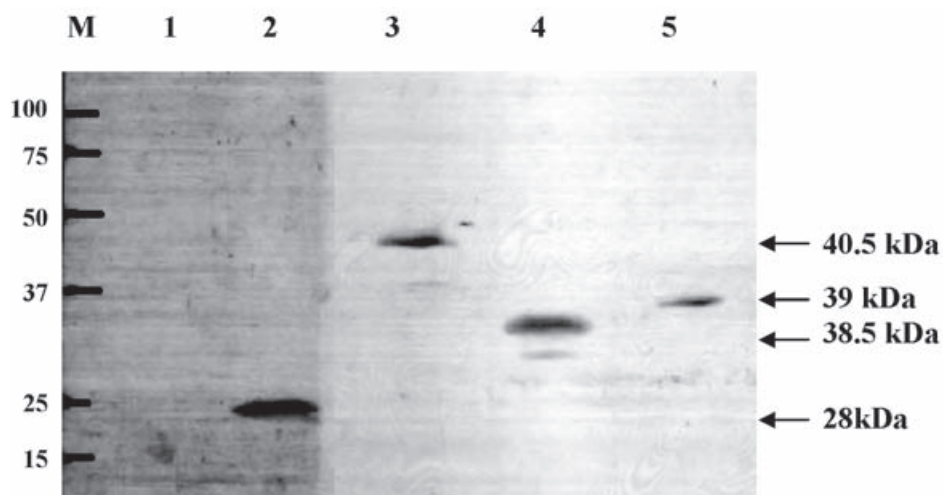


Figure 3 Protein expression of JNK activation domain and Fas-binding domain of human Daxx in yeast. Western blot analysis using anti-LexA antibody showed the LexA- a JNK activation domain (lane 3), the LexA-a region between a JNK activation domain and a Fas-binding domain (lane 4), and the LexA-Fas binding domain (lane 5), respectively. Control in lane 1 is the lysates of yeast strain RFY 231 and lane 2 is the lysates of yeast strain RFY 231, which contained only the LexA protein.

We utilized the *in vivo* homologous recombination in yeast to insert a JNK activation domain and a Fas-binding domain of human Daxx, into the bait plasmid, pEG-NRT, which is a bait plasmid in a yeast two-hybrid system. The expression of a JNK activation domain and a Fas-binding domain of human Daxx was further demonstrated by Western blot analysis. These clones will be used as bait strains to screen human proteins interacting with Daxx, which may uncover the unclear function of human Daxx via protein-protein interaction between Daxx and known human proteins.

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