Interactions between *Drosophila* USP and ECR-A was Identified in Yeast Two-hybrid system

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ABSTRACT

Ultraspiracle gene product(USP) is one of several orphan receptors in *Drosophila*, sharing significant homology with the mammalian retinoid X receptor. In *Drosophila* the response to the hormone is mediate in part by USP and ecdysone receptors (ECR), which are members of the nuclear receptor superfamily. Heterodimers of these proteins bind to ecdysone response elements(EcRE) and ecdysone to modulate transcription. We used the yeast two-hybrid assay for detection of protein-protein interactions *in vivo* to screen for novel partners of USP. The GAL4 DNA-binding domain fused to USP was used as bait to screen a *Drosophila* embryonic cDNA library in which the cDNA was fused to the GAL4 activation domain. Several cDNA clones encoding proteins that interact with USP were isolated, one of which corresponded to the ecdysone receptor A isoform (ECR-A). Domain analysis on USP revealed that the ligand binding domain is required for heterodimerization with ECR-A. Given the ability of USP to dimerize preferentially with ECR-A, this strategy should be useful for cloning novel partners for USP from a variety of cell types.

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INTRODUCTION

Steroid hormones and other nuclear hormone receptors are a family of ligand-modulated transcription factors that regulate cell differentiation and development as well as homeostasis and reproduction (Segraves, 1991 ; Thummel, 1995). In *Drosophila melanogaster*, the steroid hormone ecdysone plays a key role in molting and metamorphosis by triggering a cascade of gene expression that is thought to be modulated by changing hormone titers and regulated expression of receptors. Ecdysone titers show complex dynamics that are related to the developmental program (Raddiford, 1993). The actions of the ecdysone are mediated by the ecdysone receptor (ECR), a member of the nuclear hormone receptor superfamily (Koelle *et al.*, 1991). The ability of ECR to bind to hormone and to interact with ecdysone response element (EcRE) in the genome depends on the heterodimerization with USP, the product of the *ultraspiracle*(usp) gene locus (Yao *et al.*, 1992 ; Thomas *et al.*, 1993 ; Yao *et al.*, 1993). USP is one of several orphan receptors in *Drosophila*, sharing significant homology with the mammalian retinoid X receptor (RXR) (Henrich *et al.*, 1990 ; Oro *et al.*, 1990 ; Shea *et al.*, 1990). Both USP and RXRs can form heterodimers with several vertebrate nuclear receptors to modulate their affinity for genomic response elements (Kliewer *et al.*, 1992 ; Bugge *et al.*, 1992 ; Leid *et al.*, 1992 ; Zhang *et al.*, 1992 ; Yao *et al.*, 1992, 1993).

The *usp* gene was originally identified based on its lethal mutant phenotypes, which suggested a role in mediating ecdysone response during development (Perrimon *et al.*, 1985 ; Oro *et al.*, 1992). USP functions in abdominal cuticle synthesis during midembryogenesis and larval cuticle molting. Clonal analysis has also revealed a function for *usp* in the proper selection of the R7 photoreceptor cell fate in the adult eye, suggesting that it may interact with *seven-up*(*svp*) in this developmental pathway. However, adult thoracic and abdominal metamorphosis can occur in the absence of USP, indicating that these responses are either not regulated by ecdysone or are dependent on the activity of another nuclear receptor(Oro *et al.*, 1992). USP is expressed in many tissues throughout development with fluctuations in mRNA and protein levels (Henrich *et al.*, 1994 ; Kim *et al.*, 1995).

The behaviors of USP in heterodimerization formation with other receptors *in vitro*, together with the pleiotropy of *usp* gene function necessitate the identifying novel partners of USP. As a first step toward screening for novel partners of USP,

we used the yeast two-hybrid assay which is a genetic assay to detect protein-protein interaction *in vivo* (Fields and Song, 1989). In this screening, we have isolated a cDNA clone encoding ECR-A isoform and performed the domain analysis of USP.

MATERIALS AND METHODS

Strains and Vectors

Yeast HF7c strain (*Saccharomyces cerevisiae* : *MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS2* : : *GAL1-HIS3, URA3* : : $(GAL4\ 17\text{-}mer)_3$-*CYC1-lacZ*) was used as host cells for the yeast two-hybrid assay. The host cell carries *HIS3* and *lacZ* as reporters and *trp1* and *leu2* as transformation markers. Plasmid pGBT9 (Clonetech) was used to generate fusions of the USP proteins with the GAL4 DNA-binding domain (DB). This plasmid has a GAL4 DB sequence (amino acid 1-147) and TRP1 transformation selective marker. *Drosophila* embryomic MATCHMAKER cDNA library (Clontech) constructed in pGAD10 activation domain (AD) vector was used to screen the interacting proteins with USP. This plasmid contains GAL4 AD sequence and *LEU2* transformation selective marker.

Yeast Culture

Yeast was cultured in a complete YPD medium (20 g peptone, 10 g yeast extract and 20 g dextrose/liter) at 30℃. The transformed yeast cell was cultured in an appropriate selective SD synthetic media (1.7 g yeast nitrogen base, 5 g ammonium sulfate, 20 g dextrose and 100 ml of 10×Dropout solution/liter) lacking Trp, Leu, and His.

Constructions of GAL4 Fusion Plasmids

The GAL4 DB (amino acids 1 to 147) fusion of USP were constructed by insertion of the appropriate PCR fragments produced from usp cDNA clone (Henrich *et al.*, 1990) into a pGBT9 yeast expression vector. Three GAL4-USP fusions were contructed as shown in Figure 1. Reading frame and protein molecular weight were confirmed by DNA sequencing and Western blot analysis of yeast extracts, respectively.

Transformation

Yeast was transformed by the lithium acetate method (Ito *et al.*, 1983). For transformation of *E. coli* cells with GAL4 AD fusion plasmids isolated from yeast, we used electroporation (Sambrook *et al.*, 1989). *E. coli* HB101 (carrying a *leuB* mutation) was used as host so transformants receiving the GAL4 AD hybrid plasmid can be selected by complementation by the yeast *leu2* gene.

β-Galactosidase Activity Assay

For 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyr-anoside(X-Gal) filter assays, cell colonies were transferred onto Whatman filters and frozen in liquid nitrogen for 10 sec before incubating on top of a filter socked in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, and 1 mM $MgSO_4$, 0.27% 2-mercaptoethanol, pH 7.0) containing 0.1% X-Gal. The quantitative β-Gal activity assays were performed as described (Bartel *et al.*, 1993). The transformants were grown in SD medium to an OD_{600} of 1.0. The 1.5 ml of yeast cells were removed into a microcentrifuge tube, centrifuge at 14,000 rpm for 30 sec. The cell pellets were suspended in 100 μℓ of Z buffer. Cells were lysed by placing the tubes in liquid nitrogen until frozen. β-galactosidase activity was measured by using o-nitrophenyl-β-galactosidase as the substrate (Miller, 1972).

DNA Sequencing

Double strand DNA sequencing was performed by the dideoxy-chain termination method (Sanger *et al.*, 1977) using a sequenase kit (United Stated Biochemicals), [α-^{35}S]dATP, commercial primer, subcloned cDNA template into *E. coli*. DNA sequence comparision was performed by using the GenBank databases.

RESULTS

Screening of cDNA library with GAL4-USP (ES) "Bait"

The yeast two-hybrid assay for detection of protein-protein interactions *in vivo* was performed to screen for novel partners of USP. The GAL4 DB fused to USP[pGBT-USP(ES), Fig. 1] was used as bait to screen for *Drosophila* embryonic cDNA expression library in which the cDNA was fused to the GAL4 AD. The yeast strain used for these experiment also contains the *HIS3* gene linked to GAL4 UAS. Screening of 500,000 transformants by cotransformation with GAL DB-USP for cDNAs that

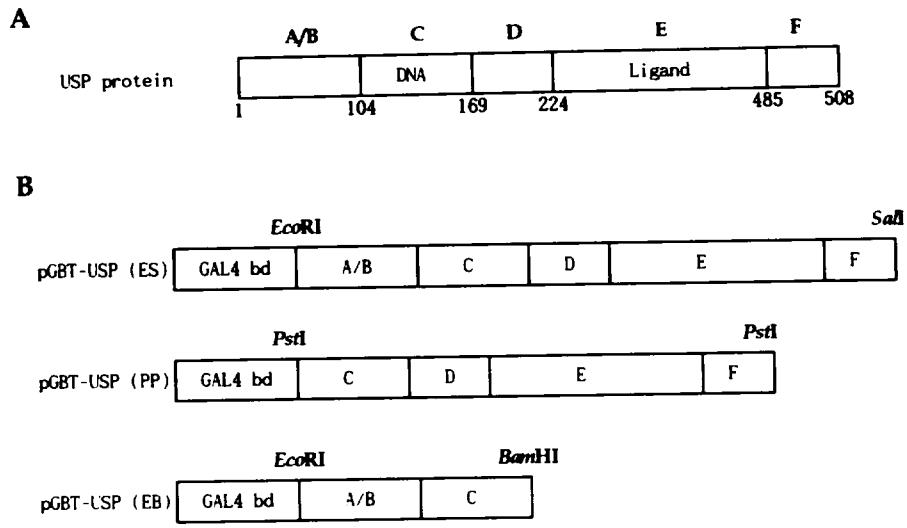

Figure 1. Structure of *Drosophila* USP protein and subcloning of *usp* cDNA fragments into pGBT9. (A) Schematic representation of USP protein. USP contains a variable N-terminal region (A/B), conserved DNA binding domain (C), variable hinge region (D), a conserved ligand binding domain (E), and a variable C-terminal region (F). (B) Construction of USP fusions into pGBT9 expression vector. *usp* gene fragments was amplified by PCR using *usp* cDNA clone as template, and then three constructions were produced by ligation into the filled in *Eco*RI/*Sal*I [pGBT-USP (ES)], *Pst*I [pGBT-USP (PP)], *Eco*RI/*Bam*HI [pGBT-USP (EB)] sites of pGBT9 vector, respectively.

could rescue activation of the *lacZ* and HIS marker linked to the GAL4 UAS yielded three clonies. When stained for *lacZ* expression, one clone (U74) showed expression after incubation for 12 hr and the additional two clonies showed expression after incubation for 24 hr.

We chose to focus our attention on the U74 clone and the complete nucleotide sequences of the cDNAs were determined by the dideoxy sequencing method. Comparision of the nucleotide sequence of the cDNA with the GenBank data base showed that U74 corresponded to the ecdysone receptor A isoform (Talbot *et al.*, 1993) and contained the sequence beginning in the exon 2 of ECR-A-specific nucleotide sequence and extending to poly A region fused in-frame with GAL4 activation domain. The ECR-A-specific nucleotide sequence isolated here spliced to the common region of ECR isoforms, which begins at nucleotide 1748 of the ECR-B1 cDNA sequence reported by Koelle *et al.* (1991). This region of ECR-A isoform contains 42 amino acids of ECR-A specific N-terminal and the ECR common region among three ECR isoforms (Fig. 2). The ECR common region contains the DNA- and ligand-binding domains, as defined the homology of these regions to other members of the steroid superfamily.

Domain Analysis of USP

To determine which domains of USP is required for heterodimerization with ECR-A, we created bait expression vectors in which the domains of USP were fused to the GAL4 DB (Fig. 1). And we used as the trap vector the ECR-A isoform cDNA in which the ECR-A protein was fused to the GAL4 transcription activation domain. Potential interaction between the two hybrid proteins was then tested by cotransformation into a yeast strain (HF7c) harboring *lacZ* and *HIS3* genes under control of the GAL4 UAS. The β-galactosidase activities were determined from the liquid culture using ONPG as a substrate for the comparision of the levels of the lacZ gene expression (Fig. 3). The pairwise combination of pGBT-USP(ES) and pGAD-ECR fusions led to moderate β-galactosidase expression, but a fusion containing N-teminal deletion(missing A/B domain) of USP showed most strong β-galactosidase expression. Whereas the pGBT-USP (EB) containing conserved C-terminal deletion failed to interact with ECR, consistent with its known dimerization function.

DISCUSSION

The fundamental question in insect endocrinology is how

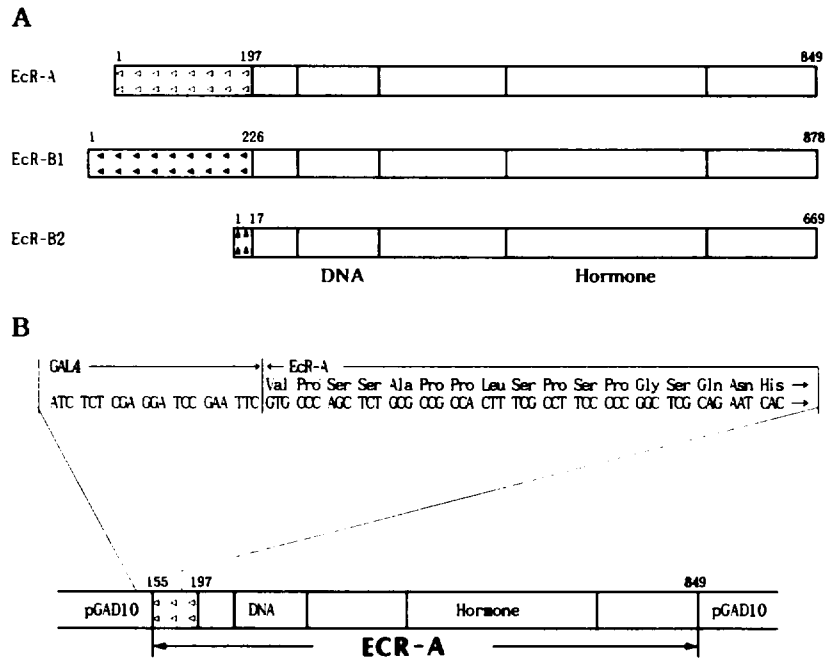

Figure 2. Three ECR isoforms with distinct N-terminal sequence 197, 226, and 17 residues in length, respectively. But they share a 652 residue C-terminal common region that contains the DNA- and hormone-binding domains characteristic of members of the steroid receptor superfamily. (A) Schemetic representations of ECR isoforms. The ECR common region contains the DNA- and hormone-binding domains. The common region extends from Gly-227 to Ala-878 of ECR-B1 (Koelle *et al*, 1991). The predicted sequence of the ECR-A-specific region from Met-1 to Thr-197. The predicted sequence of the ECR-B2-specific region is MDTCGLVAELAHYIDAY (Talbot *et al*, 1993). (B) Derived amino acid sequence of the junction between GAL (AD) and ECR-A. The amino acid sequence of the cDNA insert in clone U74 was determined by DNA sequencing using a primer upstream of the junction of GAL (AD) and the cDNA insert. Nucleotide sequence and deduced open reading frame for a portion of the sequence are shown, and the regions corresponding to GAL (AD) and ECR-A are indicated.

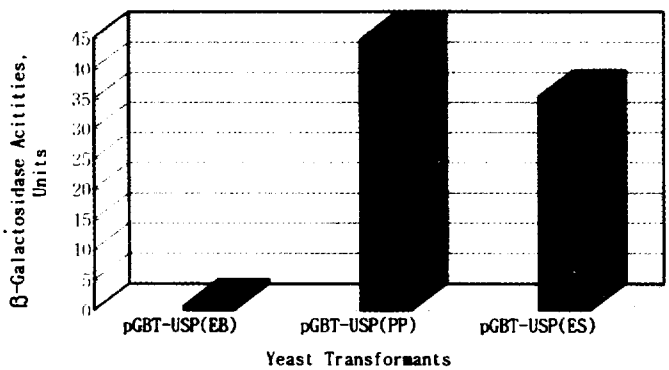

Figure 3. Functional analysis of the domains of USP fused to the GAL4 DNA binding domain in yeast cells. Combinations of GAL4-USP and GAL4-ECR-A fusions were cotransformed into yeast strain HF7c containing the GAL1 UAS (upstream activating sequence) and the TATA portion of the *GAL1* promoter fused to *HIS3*. *In sddition, in HF7c strain, three copies of the GAL4* 17-mer consensus sequence and the TATA portion of the *CYC1* promoter are fused to *lacZ*. β-galactosidase activities were determined by using the substrate ONPG. The values represents the average of at least three independent assays.

can a single hormone induce a variety of tissue response. Ecdysone manifests its effect on development via its interaction with the ecdysone receptor-the only ligand-dependent nuclear receptor identified to date in *Drosophila*. The functional ecdysone receptor consists of a heterodimer between ECR and USP. The identification of USP as a RXR homolog raised the possibility that this protein could heterodimerize with *Drosophila* receptors other than ECR, thus increasingly the spectrum of potential regulatory interactions. Similarly, heterodimer combinations among orphan receptors or between orphan receptors and ECR could generate an array of distinct receptors with different target gene specificities (Richard, 1992).

To search novel partner of USP, we used the yeast two-hybrid assay which is a genetic assay to detect protein-protein interaction *in vivo*. Through screening embryonic cDNA library, only one known heterodimer have been discovered. We showed that USP interact with ECR-A in yeast. This result is consistent with experiments *in vitro*. Three ecdysone receptors (ECRs) is encoded by the *EcR* gene (Koelle *et al.*, 1991), designate ECR-A, ECR-B1, and ECR-B2 (Talbot *et al.*, 1993). These proteins differ in their amino-terminal sequence but contain identical DNA binding domain and ligand binding domain (Fig. 3A). All three isoforms acquired the ability to bind DNA upon heterodimerization with USP (Yao *et al.*, 1992 ; Thomas *et al.*, 1993).

Two alternative heterodimers of USP have been discovered by others. Assays for dimerization using the yeast two-hybrid system revealed a strong and specific interaction between USP and DHR38 (Sutherland *et al.*, 1995). In transfected tissue culture cells, addition of DHR38 can disrupt ECR-USP binding to an *hsp27* EcRE and suppress the ability of ECR-USP to induce transcription through this sequence. These observation suggest a model for negative regulation of ecdysone response in which DHR38 heterodimerizes with USP, thus blocking its ability to form a functional receptor complex with ECR (Sutherland *et al.*, 1995). Recent evidence also suggest that negative regulation of ecdysone signaling may be achieved through heterodimerization with ECR. Studies using the yeast two-hybrid system have demonstrated that *seven up* protein (SVP) can interact with ECR (Zelhof *et al.*, 1995). SVP can also reduce the ability of ECR and USP to transactivate transcription through the *hsp27* EcRE, although SVP is unable to bind this sequence. Taken together, these observations suggest that SVP may form an inactive heterodimer with ECR, reducing the levels of functional ecdysone receptor in the cell. Thus, both direct DNA binding and the formation of distinct heterodimer combination provide different levels at which ecdysone responses can be negatively controlled.

The nuclear receptors are characterized by a central DNA binding domain (DBD), which targets the receptor to specific DNA sequence known as hormone response elements. The DBD is composed of two highly conserved zinc fingers that set the nuclear receptors apart from other DNA-binding proteins. The C-terminal half of the receptor encompasses the ligand-binding domain (LBD), which possesses the essential property of hormone recognition and ensures both specificity and selectivity of the physiologic response (Mangelsdorf *et al.*, 1995). Domain analysis on USP revealed that the deletion of C-terminal of USP disrupts the ability of USP to interact with ECR. This result is consistent with its known dimerization function of RXR, mammalian homolog of USP. Previously studies have revealed the LBD to comprise a carboxy-terminal region of approximately 225 amino acids that is capable of autonomous ligand binding (Evans, 1988). In addition to functioning as a LBD, this region also included homo-and heterodimerization interfaces, hormone-dependent transcriptional activation functions, and (in some cases) hormone reversible transcriptional repression. It is interesting that the interaction of USP with ECR was more enhanced in the absence of A/B domain (a variable N-terminal region) than full-length USP. However, we don't know why the deletion of A/B domain makes the heterodimer complex more stronger. Given the ability of USP to dimerize preferentially with ECR-A, this strategy should be useful for cloning novel partners for USP from a variety of cell types.

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