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Structural and functional characterization of a novel type of ligand-independent RXR-USP receptor

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Retinoid X receptor (RXR) and Ultraspiracle (USP) play a central role as ubiquitous heterodimerization partners of many nuclear receptors. While it has long been accepted that a wide range of ligands can activate vertebrate/mollusc RXRs, the existence and necessity of specific endogenous ligands activating RXR-USP *in vivo* is still matter of intense debate. Here we report the existence of a novel type of RXR-USP with a ligand-independent functional conformation. Our studies involved *Tribolium* USP (TcUSP) as representative of most arthropod RXR-USPs, with high sequence homology to vertebrate/mollusc RXRs. The crystal structure of the ligand-binding domain of TcUSP was solved in the context of the functional heterodimer with the ecdysone receptor (EcR). While EcR exhibits a canonical ligand-bound conformation, USP adopts an original apo structure. Our functional data demonstrate that TcUSP is a constitutively silent partner of EcR, and that none of the RXR ligands can bind and activate TcUSP. These findings together with a phylogenetic analysis suggest that RXR-USPs have undergone remarkable functional shifts during evolution and give insight into receptor–ligand binding evolution and dynamics.

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Introduction

Arthropods represent an enormous variety of animals comprising insects, crustaceans, myriapods and chelicerates. Since their segmented body is covered by a rigid exoskeleton, successive molts allow increase in their body size, while metamorphosis and the imaginal molt for certain species represent the last transformation to the adult stage. Ecdysteroids trigger these developmental events by binding to a transcription factor, the heterodimer of the ecdysone receptor (EcR, NRH1) and Ultraspiracle (USP, NR2B4), two members of the nuclear receptor (NR) superfamily (Koelle *et al*, 1991; Yao *et al*, 1992, 1993; Thomas *et al*, 1993). By binding to specific response elements in the promoters of target genes, EcR/USP controls the cascades of early and late gene expression leading to arthropod development and reproduction (Henrich, 2005).

The USP protein is the arthropod homologue of the vertebrate retinoid X receptor (RXRα, β, γ; NR2B1, 2, 3) of vertebrates, molluscs and cnidarians (reviewed in Laudet and Gronemeyer, 2002). RXR and USP are ubiquitous heterodimerization partners for class II NRs (Brelivet *et al*, 2004). USP was unambiguously identified as the partner of EcR and DHR38 (Sutherland *et al*, 1995; Baker *et al*, 2003). In addition, *in vitro* binding studies and transient transfection assays indicated that USP can functionally replace RXR for dimerization with RAR, TR and VDR (Christianson *et al*, 1992; Hatzivassiliou *et al*, 1997). Compared to RXR, USP sequences feature a conserved DNA-binding domain (DBD) and a more divergent ligand-binding domain (LBD). However, for Mecopterida, a group of insects that includes Diptera (flies and mosquitos) and Lepidoptera (moths and butterflies), a phylogenetic analysis indicated a strong evolutionary divergence of the USP and EcR sequences as compared to sequences of other arthropod species (Bonneton *et al*, 2003, 2006). The functional reasons behind this increased evolutionary divergence in Mecopterida are still unknown.

Consistent with the sequence divergence, the crystal structures of the USP LBDs of the lepidopteran *Heliothis virescens* (HvUSP) (Billas *et al*, 2001) and of the dipteran *Drosophila melanogaster* (DmUSP) (Clayton *et al*, 2001) revealed significant structural differences compared to human RXR LBD structures (Egea *et al*, 2000). For these two similar USP structures, the activation helix H12 is locked in a so-called antagonist conformation by intra-protein interactions with a structural motif conserved in Mecopterida USPs. However, the biological significance of these structures is unclear, since

they feature a large hydrophobic ligand-binding pocket (LBP) filled by a fortuitous phospholipid. For the other arthropods, the USP sequences are very similar to RXR sequences, notably with a high degree of conservation of the residues, which, in the structures of HsRXR, were shown to interact with RXR ligands (Egea *et al*, 2000). It would therefore be expected that these USP receptors could bind and become activated by RXR ligands in a similar manner.

RXRs and USPs are present in all metazoan organisms from cnidarians and molluscs, to insects and mammals. Several molecules were described as being potential natural ligands for RXR, 9-*cis* retinoic acid (9cRA) (Heyman *et al*, 1992; Levin *et al*, 1992), phytanic acid (Kitareewan *et al*, 1996) and docosahexaenoic acid (DHA) (de Urquiza *et al*, 2000). However, none of these have been proved to be the bona fide endogenous ligand so far (Calleja *et al*, 2006). In addition to these ligands, RXR can be activated *in vitro* by polyunsaturated fatty acids (PUFAs) (de Urquiza *et al*, 2000; Lengqvist *et al*, 2004) and by various synthetic compounds (Szanto *et al*, 2004). On the other hand, no ligands have yet been unambiguously identified for USP. In insects, besides ecdysteroids, other lipophilic hormones, juvenile hormones (JH) act as to moderate the actions of ecdysteroids necessary for molting and metamorphosis (Dubrovsky, 2005). These molecules belong to the same family of sesquiterpenoids as retinoids. It was therefore tempting to suggest that JHs could be endogenous ligands of USP, thus integrating the ecdysone and juvenile hormone signaling pathways through the same EcR/USP system. Some evidence was presented that *Drosophila* USP could be a JH receptor (Fang *et al*, 2005), but the *in vivo* relevance of these data remained speculative. Regarding other species for which USP sequences are highly similar to RXR sequences, information on the binding of potential ligands to USP is very limited (Guo *et al*, 1998; Hayward *et al*, 2003). As a general consensus, USP is commonly thought to be an orphan receptor, although this is still a contentious issue.

Since Mecopterida USPs exhibit a strong sequence divergence as compared with other arthropod USPs, the general relevance of the observations made on these model species to other arthropods is questionable. Therefore, we have focused our studies on the beetle *Tribolium castaneum* USP (TcUSP), a representative non-Mecopterida USP. In this paper, we show that despite the high sequence homology to RXR, TcUSP does not bind and is not activated by RXR ligands, as demonstrated by *in vitro* and *in vivo* experiments. To gain insight into the mechanism of action of this new type of USP, we solved the crystal structure of TcUSP in the context of its functional heterodimer with EcR. The structure of TcUSP is markedly different from vertebrate and mollusc RXR structures by lacking a conventional LBP. It also strongly differs from the structures of the Mecopterida *Drosophila* and *Heliothis* USP, but resembles the partial structure of the Hemiptera *Bemisia tabaci* USP (BtUSP), that completely lacks the N-terminal region (Carmichael *et al*, 2005). These functional and structural results, supported by a phylogenetic analysis of evolutionary constraints, suggest that USPs of non-Mecopterida insects define a third class of RXR-USP NRs. This suggests that several important functional shifts occurred during the evolution of RXR-USP, highlighting the evolutionary flexibility of NRs.

Results

In order to better understand the structural and functional differences existing between USP of Mecopterida (including *Drosophila*) and those of other arthropods, we cloned *usp* and *EcR* orthologs of the beetle *Tribolium castaneum*. The cloning and characterization of TcEcR and TcUSP, as described in the Supplementary data, show that these sequences are *bona fide* coleopteran sequences that conform to the evolutionary trend described previously (Bonneton *et al*, 2003, 2006).

TcUSP is not activated by RXR ligands in cultured cells

Since TcUSP shares a high degree of sequence similarity with vertebrate RXRs, we first hypothesized that TcUSP, as a representative member of non-Mecopterida USPs, could bind and be activated by RXR ligands. Therefore, transcriptional activation of TcUSP was assayed by using the potent agonist 9cRA, the JH analogue methoprene acid (Harmon *et al*, 1995) and the PUFA phytanic acid (Kitareewan *et al*, 1996). The detection system consisted of TcUSP D/E domains fused to the GAL4 DBD, along with a luciferase (LUC) reporter plasmid containing a UAS binding site in front of either the thymidine kinase (TK) or TATA promoter. For comparison, we performed assays using the GAL4-HsRXRα or GAL4-DmUSP fusion constructs. By using chimeric proteins, possible formation of a functional dimer between TcUSP and endogenous RXR of the mammalian cells can be minimized (Guo *et al*, 1998). Transient transfection assays were performed using human HEK293 EBNA or *Drosophila* Schneider 2 (S2) cells.

To show that GAL4-TcUSP is fully functional, we performed assays in HEK293 cells with *Tribolium* and *Drosophila* full-length EcR and GAL4-USP proteins, in the presence of ponasterone A (ponA), a potent ecdysone agonist. As shown in Figure 1A, no activation is observed upon ponA induction in the absence of EcR, whereas in its presence, TcEcR/GAL4-TcUSP and DmEcR-B1/GAL4-DmUSP could be activated to about 10-fold. In S2 cells, endogenous DmEcR can heterodimerize with either GAL4-TcUSP, GAL4-HsRXRα or GAL4-DmUSP, resulting in the transcriptional activation upon induction with ponA (Figure 1B). In contrast, the transactivation properties of TcUSP and HsRXRα upon induction with RXR ligands, are markedly different. Whereas for HsRXRα, a significant transcriptional activation is observed upon induction with 9cRA or methoprene acid, no transcriptional activity was detected for TcUSP in HEK293 (Figure 1A) and in S2 cells (Figure 1B), as well as for DmUSP (shown for S2 cells in Figure 1B). Notice that the low-level transcriptional activation of GAL4-TcUSP observed upon induction with 9cRA in HEK293 cells is due to dimerization of the transfected construct with endogenous HsRXR. In addition, the transactivation properties of TcUSP (and DmUSP) are independent of the presence of the dimerization partner. As shown in S2 cells (Figure 1B), in presence of 9cRA and ponA, the activation levels of GAL4-TcUSP and GAL4-DmUSP are identical to those measured for ponA alone, whereas the activity of GAL4-HsRXRα is markedly increased (50-fold). For the transient transfection assays using PUFAs, we determined the dose dependency of transactivation in HEK293 and S2 cell lines using phytanic acid (Figure 1C and D). In HEK293 cells, a significant activation of HsRXRα was observed at 30 μM. In S2 cells, the activation fold of HsRXRα is lower, but the

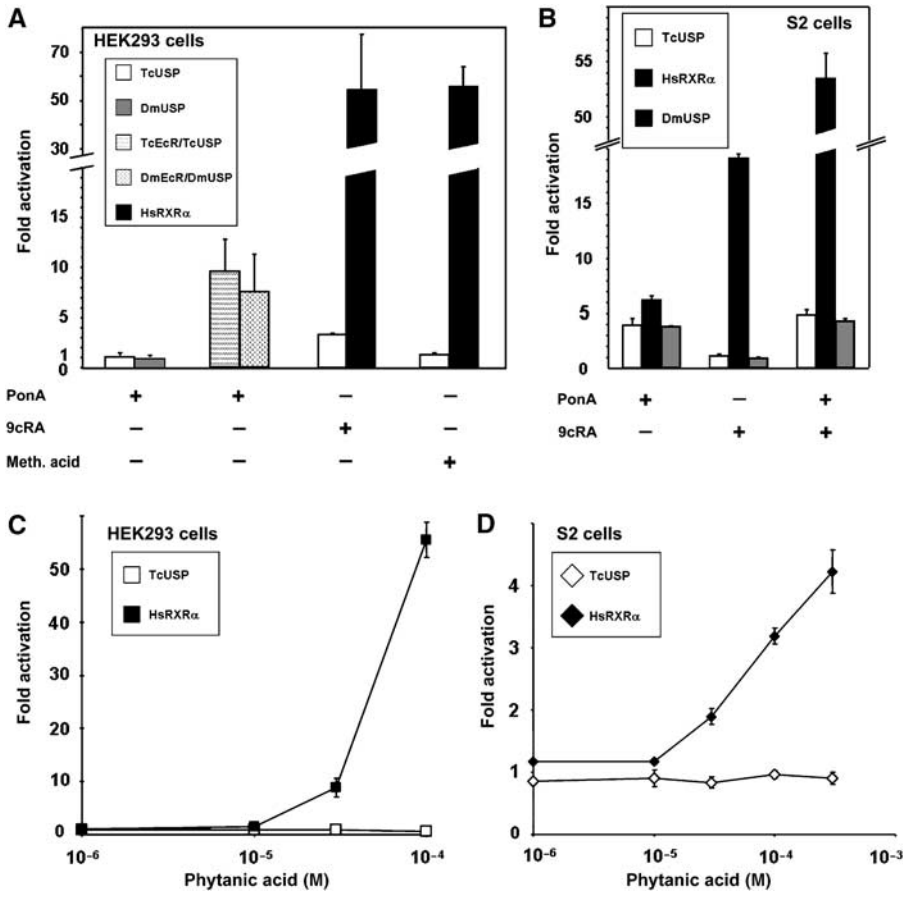

Figure 1 GAL4-USP confers responsiveness to EcR, but cannot activate reporter gene transcription upon RXR ligands induction. (**A**) HEK293 cells were transfected with GAL4-TcUSP (white), GAL4-DmUSP (gray) or cotransfected with GAL4-TcUSP/TcEcR (diagonal slashes) or GAL4-DmUSP/DmEcR-B1 (vertical slashes), along with a UAS-TK-LUC reporter plasmid and induced with 10^{-6} M ponA. For assays using 9cRA and methoprene acid, HEK293 cells were transfected with GAL4-TcUSP (white) and GAL4-HsRXRα (black), along with a UAS-TATA-LUC reporter plasmid and induced with 10^{-6} M 9cRA or 10^{-4} M methoprene acid. (**B**) S2 cells were transfected with GAL4-TcUSP (white) and GAL4-HsRXRα (black), GAL4-DmUSP (gray), along with a UAS-TATA-LUC reporter plasmid and induced with 10^{-6} M ponA, 10^{-6} M 9cRA or with ponA/9cRA, both at 10^{-6} M. (**C, D**) Dose–response curves for phytanic acid activating GAL4-TcUSP (white) and GAL4-HsRXRα (black) in (C) HEK293 and (D) S2 cells.

general trend is comparable. In contrast, for TcUSP, no transcriptional activation could be detected even at the highest concentration tested (300 μM). In conclusion, both in mammalian and insect cultured cells, TcUSP acts as a functional dimerization partner that confers proper transactivation properties to EcR upon ecdysteroid activation. However, it cannot transactivate reporter gene transcription in presence of RXR ligands.

TcUSP is not activated by RXR ligands in organ culture

Since the lack of transactivation ability of TcUSP in the presence of various RXR ligands could be due to the artificial system of cultured cells, we decided to address the problem using an *in vivo* approach. We used the GAL4-UAS approach (Duffy, 2002) to study the putative TcUSP LBD activity in *Drosophila* transgenic flies. We introduced into the *Drosophila* genome a transgene coding for a protein constituted by GAL4-DBD fused with TcUSP D/E domains under the control of a heat shock promoter. In the same way, we constructed transgenic flies expressing the GAL4-DBD fused with HsRXRα D/E domains. We used animals carrying one copy of one of these transgenes and one copy of a reporter transgene under the control of an UAS promoter and coding for the enhanced green fluorescent protein (GFP). We conducted our study by using late third instar larvae, just before the ecdysone peak of pupariation that characterizes the onset of metamorphosis (Kozlova and Thummel, 2002, 2003). Immediately after heat-shock treatment, we dissected the salivary glands and separated the two lobes. One lobe was incubated in a medium containing no hormone, whereas the sister lobe was incubated in a medium supplemented with 9cRA (Figure 2B and J), various JH analogs, methoprene acid (Figure 2C and K), methoprene (Figure 2D and L), kinoprene (Figure 2E and M) or PUFAs, DPA (Figure 2F and N), arachidonic acid (Figure 2G and O) and DHA (Figure 2H and P). An important GFP induction was observed in the salivary glands expressing GAL4-HsRXRα and incubated with ligands, whereas no GFP expression was detected in salivary glands cultivated in absence of ligands (Figure 2A–H). On the other hand, for salivary glands expressing the GAL4-TcUSP transgene, no induction of the reporter gene was observed independently of the presence of ligands (Figure 2I–P). The same result was obtained with flies carrying two copies of the GAL4-TcUSP transgene and two copies of the GFP reporter transgene (data not shown). This lack of induction is not due to a lack of expression of the transgene, as shown by RT–PCR

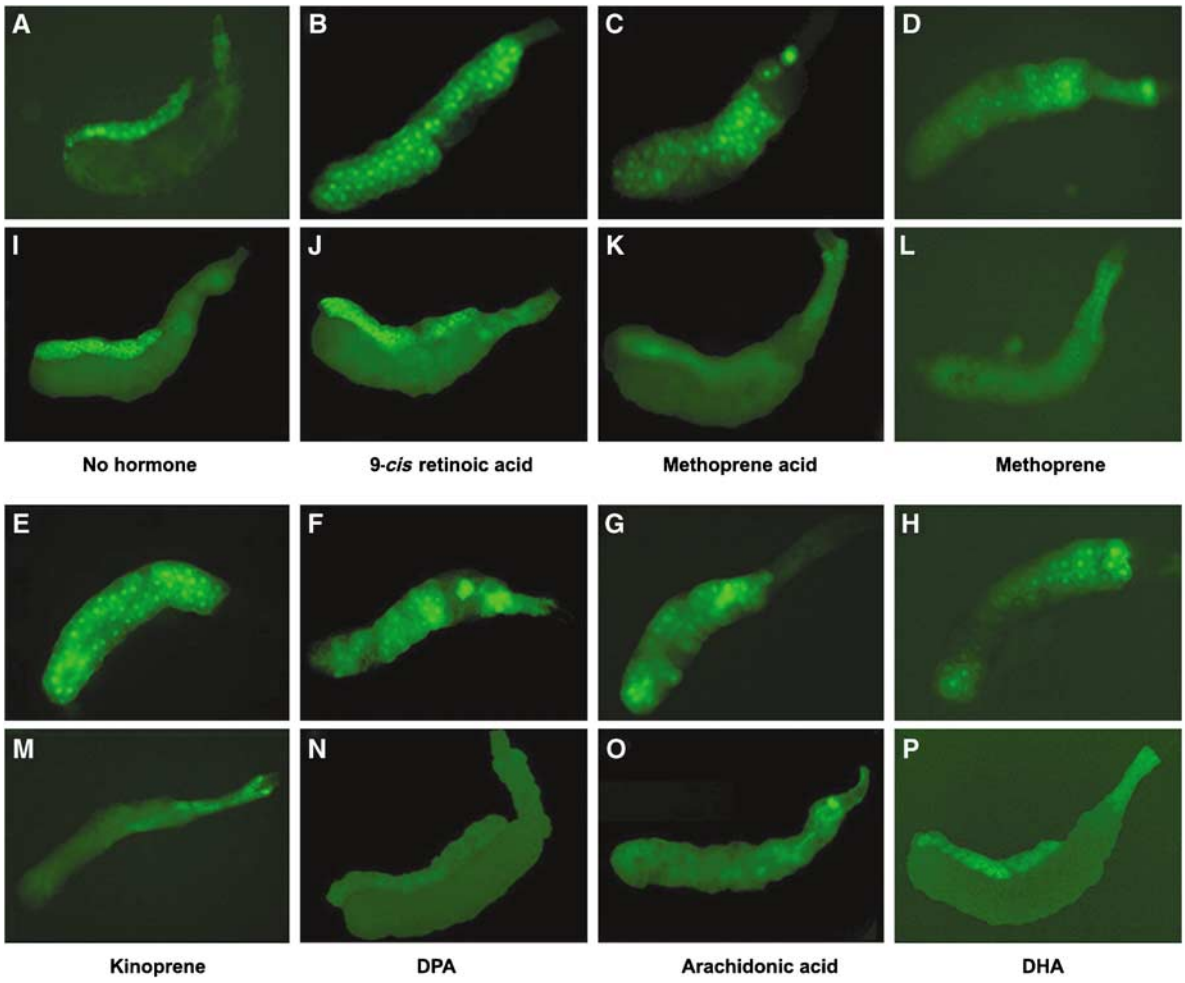

Figure 2 GAL4-TcUSP is not activated by RXR Ligands in *Drosophila* larval organ culture. GFP induction from salivary glands expressing the GAL4-HsRXRα (upper pictures) or the GAL4-TcUSP fusion protein (lower pictures), incubated without hormone (**A, I**) or with 1.6 μM 9cisRA (**B, J**), 1.6 μM methoprene acid (**C, K**), 100 μM methoprene (**D, L**), 16 μM kinoprene (**E, M**), 200 μM DPA (**F, N**) or 100 μM arachidonic acid (**G, O**) and 100 μM DHA (**H, P**). Addition of the ligands in the culture medium induced the GFP expression in salivary glands expressing GAL4-HsRXRα, whereas these ligands had no visible effect on the GFP induction in salivary glands expressing GAL4-TcUSP.

and by immunochemistry using anti-GAL4 antibodies (data not shown). Interestingly, the absence of activation of GAL4-DmUSP by JH and JH analogs was also recently reported in organ explants from transgenic flies expressing the GAL4-DmUSP transgene, except for fenoxycarb which weakly activates GAL4-USP most likely due to xenobiotic response (Palanker *et al*, 2006). Finally, our observations are consistent with the cell transfection assays and strongly suggest that RXR ligands do not activate TcUSP *in vivo*.

TcUSP does not bind RXR ligands in vitro

In order to decipher whether the inability of TcUSP to be activated by RXR ligands *in vitro* and *in vivo* was due to a lack in ligand binding, we performed a comparative electrospray ionization mass spectrometric (ESI-MS) analysis of TcUSP and HsRXRα LBDs, as described in the Supplementary data. In the absence of any added ligand, ESI mass spectra of non-denatured protein samples displayed three major peaks corresponding to the 12+, 11+ and 10+ charge states of the apo monomeric form of HsRXR (Figure 3A) and TcUSP (Figure 3B), and a second minor ion series corresponding to various charge states of HsRXRα or TcUSP homodimers. These results demonstrate that TcUSP homodimerizes in a way similar to RXR and is found in an apo state, in contrast to HvUSP and DmUSP that bind a phospholipid in solution (Potier *et al*, 2003).

ESI mass spectra were then recorded in the presence of RXR ligands (Figure 3E–H) and compared to unliganded protein mass spectra (Figure 3C and D). Specific binding of 9cRA to HsRXRα was observed, as indicated by a new series of peaks corresponding to the 12+, 11+ and 10+ charge states of HsRXRα/9cRA non-covalent complex (Figure 3E). For TcUSP LBD, the mass spectrum indicates the attachment of one and two 9cRA molecules to the LBD (Figure 3F). Since the experiments were performed with a large molar excess of 9cRA as compared with TcUSP, these observations are characteristic of a nonspecific binding of 9cRA to TcUSP. The lack of specific binding of 9cRA to TcUSP was furthermore checked by surface plasmon resonance (SPR) experiments as described below and shown in Supplementary Figure S3. Similarly, incubating TcUSP LBD with methoprene acid also resulted in nonspecific ligand binding (data not shown). In addition, we examined the binding of a representative set of PUFAs (DHA, DPA, phytanic, and arachidonic, linoleic,

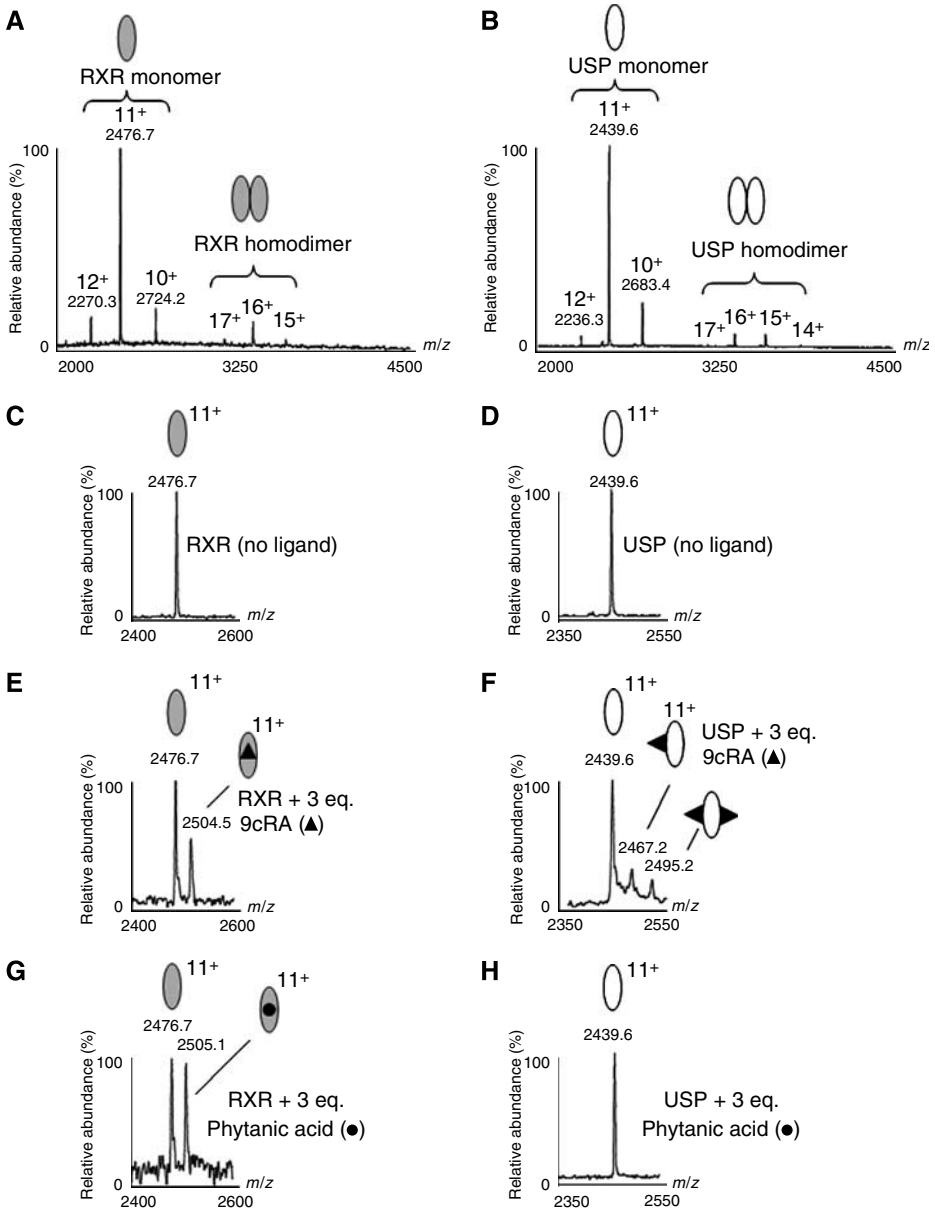

Figure 3 TcUSP LBD does not bind RXR ligands *in vitro*. (**A, B**) ESI mass spectra of native HsRXRα (A) and TcUSP (B) LBD protein samples, showing the different charge states of monomer and homodimer populations. (**C, D**) Close-up views of the region of the 11+ charge state of (C) HsRXRα and (D) TcUSP in the absence of added ligand. (**E, F**) Close-up views of the region of the 11+ charge state of HsRXRα (E) or TcUSP (F) after incubation with a three-fold molar excess of 9cRA. Under these experimental conditions, specific binding of 9cRA to HsRXRα, but not to TcUSP, is demonstrated. (**G, H**) Close-up views of the region of the 11+ charge state of (G) HsRXRα or (H) TcUSP after incubation with a three-fold molar excess of phytanic acid, also revealing specific binding to HsRXRα, but not to TcUSP.

linolenic acids) to TcUSP and HsRXRα. For HsRXRα, the resulting ESI mass spectra indicated the formation of specific non-covalent receptor–ligand complexes. The highest binding rate was observed with phytanic acid, where about 40% of receptor–ligand complex is present in solution (Figure 3G). In contrast, for TcUSP, no binding could be observed (Figure 3H). These results are consistent with limited proteolysis assays, for which no RXR ligand was found to protect the coleopteran *Tenebrio molitor* USP (95.2% similarity to TcUSP) from proteolytic digestion, in contrast to observations made on mice RXRα (data not shown). Altogether, these data demonstrate that ligands, which bind to RXR do not bind to TcUSP, explaining the lack of activation observed *in vitro* and *in vivo*.

Structure of the heterodimer TcEcR/TcUSP

Surprisingly, despite high sequence similarity, our results suggest that TcUSP and HsRXR function differently. In order to gain insight into these functional differences, the crystal structure of TcUSP LBD was solved in the context of the functional heterodimer TcEcR/TcUSP. The LBDs of TcEcR/TcUSP were coexpressed in *Escherichia coli*, purified in the presence of ponA and crystallized in $P2_1$ space group. The structure was solved at 2.75 Å resolution by molecular replacement (Navaza, 1994). The final model encompasses four heterodimers in the asymmetric unit. Three of the four dimers superimposed very well to each other, while the fourth one exhibited a significant shift of EcR with respect

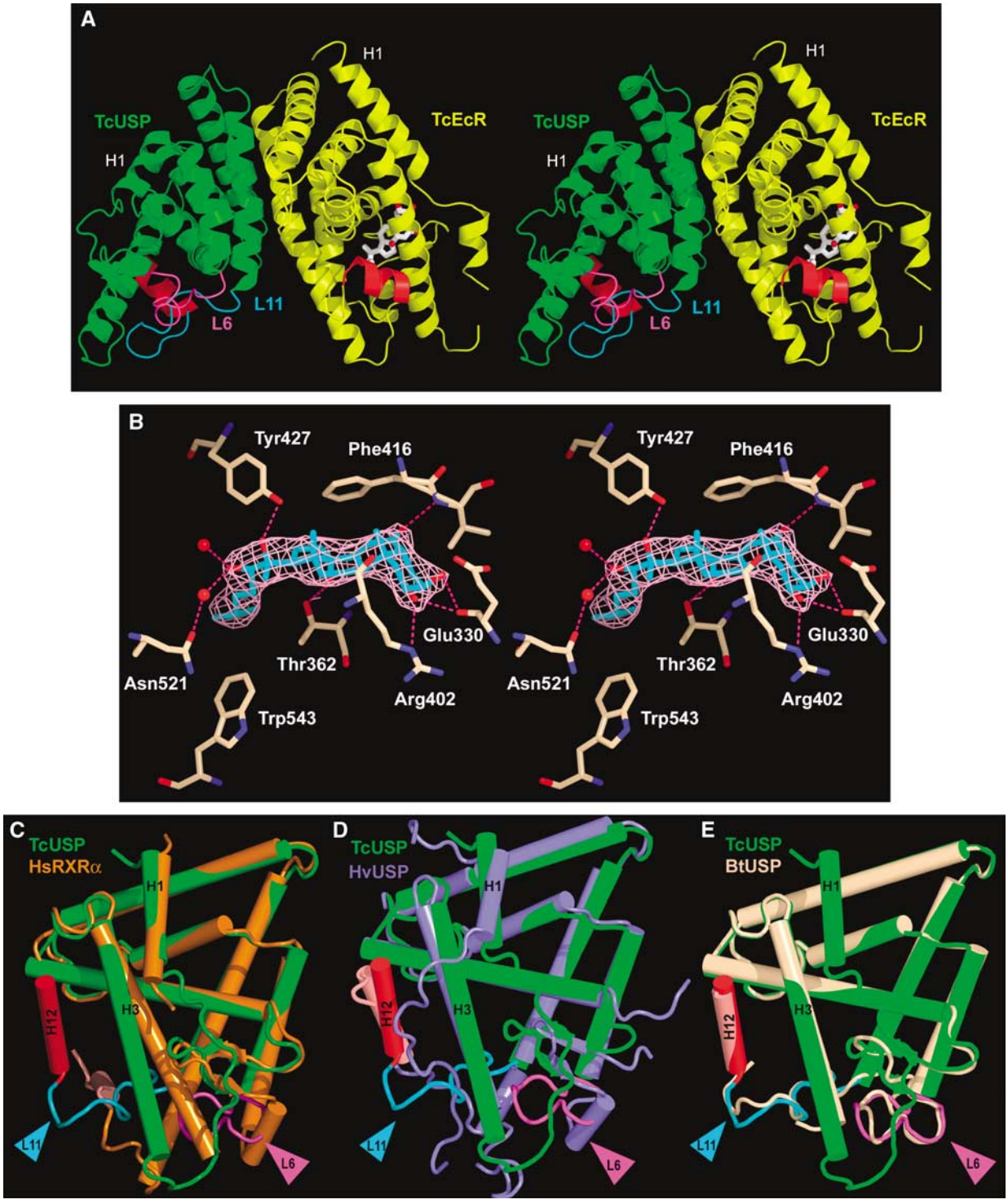

Figure 4 Structure of the LBDs of TcEcR/TcUSP. (**A**) A stereoview of the overall heterodimer structure in complex with the steroid hormone ponA, with TcEcR in yellow and TcUSP in green. Helices H12 are shown in red, the loops L6 and L11 of TcUSP are shown in pink and light blue, respectively. PonA is shown in stick representation colored by atom type (blue for carbon and red for oxygen). (**B**) Stereoview of the sigma-A-weighted 2Fo–Fc electron density map for ponA bound inside the LBP of TcEcR. The map is contoured at 1.4 σ and overlaid on the final refined model. Hydrogen bonds between ligand and residues are indicated by pink dotted lines. (**C**–**E**) Superimpositions of the LBDs of TcUSP (in green) with (C) HsRXRα (in orange), (D) HvUSP (in blue) and (E) BtUSP (in salmon). Helices H12 are shown in red for TcUSP and pink for HsRXRα, HvUSP or BtUSP. TcUSP loops L6 and L11, indicated by arrows, are shown in pink and light blue, respectively.

to USP, probably due to crystal packing. Data and refinement statistics are summarized in Supplementary Table S3.

The overall structure of *Tribolium* EcR/USP is similar to the structures of *Heliothis* and *Bemisia* EcR/USP (Figure 4A). The subunit TcEcR exhibits a structure that very closely resembles the structures of HvEcR and BtEcR in complex with ponA. Its activation helix H12 packs against the core of the receptor in the canonical agonist conformation. The LBP

and the main interactions of the residues with ponA are the same as those observed for HvEcR and BtEcR (Figure 4B).

The superimposition of TcUSP with HsRXRα and HvUSP are shown in Figure 4C and D, respectively. The latter, as well as DmUSP, exhibited a phospholipid in their LBP and were characterized by a peculiar conformation of the loop connecting H1 and H3 (L1–3) that locked H12 in a so-called antagonist conformation. In contrast, no ligand is bound to TcUSP and L1–3 adopts a path similar to that observed for human RXR and mollusc RXR (de Groot *et al*, 2005) (Figure 4C). Interestingly, the structure of BtUSP that completely lacks the N-terminal region is very similar to TcUSP (Figure 4E), indicating that H1 is dispensable for the stabilization of apo USP. The core of TcUSP composed of H4, H5, H7, H8, H9, H10 and the β-sheet superimposes very well to BtUSP. However, the helices H6 and H11 observed in the various structures of RXR, as well in HvUSP and DmUSP, are missing in TcUSP and replaced by loops (L6 and L11, respectively). The same structural features are seen in BtUSP, where 'folding in' of the loops connecting the β-sheet to H7 and H10 to H12 is observed. These two structural elements are positioned in such a way as to fill the interior of the receptor, leaving no room for a potential LBP.

TcUSP is in an apo state and lacks a conventional ligand-binding pocket

The LBP of TcUSP is filled by residues that stabilize an apo conformation. Two major structural elements play a key role in this mechanism: the loop connecting H10–H12 (L11) and the one that links the β-sheet to H7 (L6). The side chains of the hydrophobic residues V286, V288, I291 in L6 and L382 and F383 in L11 fill the space that is otherwise occupied by RXR ligands, as shown in Figure 5A. Residues of L6 superimpose with the β-ionone ring of 9cRA, while residues of L11 are located at the place of the 9cRA aliphatic side chain. For HsRXRα, the transition from the apo to the holo state upon ligand binding was shown to be accompanied by the concerted motion of H11, exposing one face toward the solvent in the apo state and the other face in the holo state. For TcUSP, two of the three conserved Phe residues of H11 (F383, F384) play an important role in the structural stabilization of the apo conformation, while the third one (F385) is oriented toward the solvent. F383 is buried inside the LBD, while F384 makes van der Waals contacts with H12 (in particular with F396). The stabilization of the TcUSP apo structure also involves an unusual interaction pattern around W251 (H5) (Figure 5B). The Trp residue of H5, conserved in all RXR (HsRXRα-W305), was shown to be one of the major actors in the stabilization of the agonist H12 conformation of HsRXRα holo-LBD. In all RXR structures determined so far, and in the *Heliothis* and *Drosophila* USP structures, this residue adopts an identical rotamer in a similar environment, whereas in TcUSP and BtUSP, the conformation of this Trp residue is radically different, involving the interaction network shown in Figure 5B.

TcUSP AF-2 helix is stabilized in a so-called antagonist conformation

The residues of H12 and the two residues (E399 in H12 and K230 in H3) that clamp the LXXLL motif of coactivators in the groove formed by H3, H4, H5 and H12 (Darimont *et al*, 1998; Xu *et al*, 1999) are conserved in TcUSP, suggesting that TcUSP could interact with cofactors homologous to those identified in vertebrates. Since in the crystal structure of TcUSP, H12 is positioned in a so-called antagonist conformation, the question arises whether H12 is mobile in solution and can adopt the canonical agonist conformation. Therefore, interactions between TcUSP LBD and coactivator peptides containing the LXXLL motif of SRC-1 and TIF2/GRIP1 were investigated by using SPR technique. These peptides are good representatives of the canonical binding motif that would normally bind to the USP coactivator binding cleft on the basis of residue conservation with HsRXR. For comparison purpose and internal control, SPR experiments were also performed on apo HsRXR LBD under identical experimental conditions. As demonstrated in Figure 5C and D, no interaction is observed between TcUSP and the coactivator peptides, whereas even in the absence of ligand, HsRXR can recruit both the SRC-1 and TIF2/GRIP1 derived peptides. Incubation of HsRXR with agonist ligands, including 9cRA, resulted in the increase of the binding affinity of the peptides for the LBD. For TcUSP incubated with 9cRA, no change in the lack of recruitment of SRC-1 and TIF2 derived peptides as well as peptides derived from the insect cofactor Taiman (Bai *et al*, 2000) to the LBD was observed (Supplementary Figure S3). The SPR analysis therefore suggests that the so-called antagonist conformation of TcUSP is stable in solution.

The analysis of evolutionary rates supports the existence of three types of RXR-USP receptors

Residues that play a key functional role are likely to show the weakest evolutionary rates because of a strong evolutionary pressure. It is thus possible to detect variations in the functional constraint acting on a given amino acid by measuring its substitution rate when a large dataset of sequences is available, which is the case for RXRs and USPs (Yang and Wang, 1995; Yang, 1996). In this context, we performed a phylogenetic analysis to identify changes in the substitution rates in RXR and USP LBD sequences. Trees obtained after alignment of available USP and RXR LBD sequences indicated three distinct receptor groups: RXR (chordates and molluscs), USP (non Mecopterida) and USP (Mecopterida) (Bonneton *et al*, 2006). Remarkably, as shown above and in the literature, each group is characterized by a specific LBD structure and specific ligand binding properties. Using maximum likelihood methods (Yang, 1996), we estimated the relative residue substitution rates within each group (Figure 6A–C). These values were then projected onto the crystal structures of one representative of each group (Figure 6D–F). This analysis indicates that the dimerization surface formed by residues of H7 and H10 is highly constrained in the three groups. In contrast, the pattern of relative substitution rate in the LBP differs significantly, with a low evolutionary rate in the RXR group and to a lesser extent also in the group of Mecopterida USPs. In contrast, for non-Mecopterida USPs, residues that would belong to a potential RXR-like LBP exhibit high evolutionary rates, suggesting a relaxation of the evolutionary pressure in this region that may be related to a loss in ligand binding capability. Taken together, these data suggest that the role of USPs and RXRs as heterodimerization partner has been retained throughout evolution, but that their ability to interact with ligands has been specifically modified in two different ways. Whereas the analysis of the evolution rates strongly supports the existence of endogenous ligands

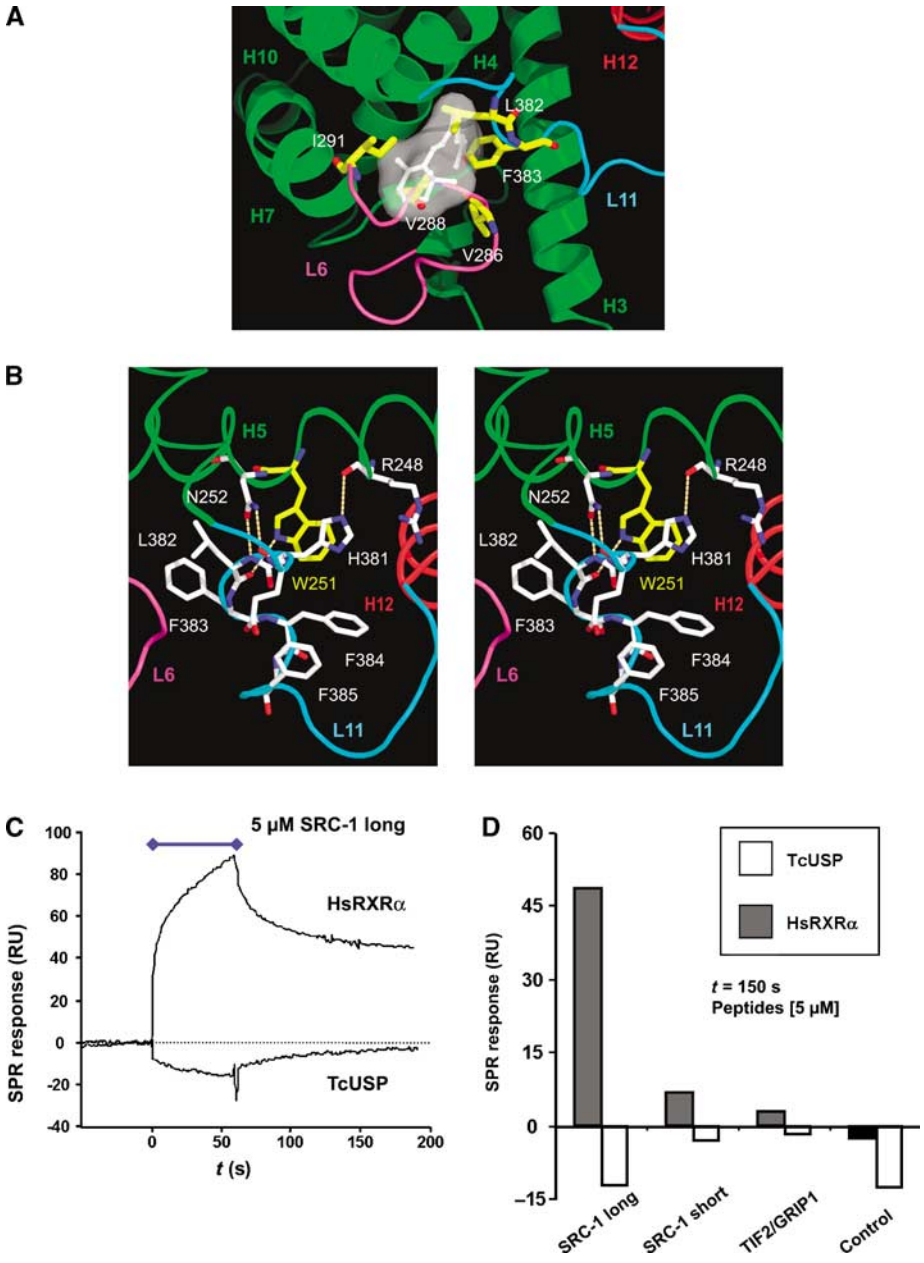

Figure 5 TcUSP is in an apo state with a stable so-called antagonist receptor conformation. (**A**) Superimposition of TcUSP with 9cRA-bound HsRXRα. TcUSP is depicted as green ribbons, with loops L6 and L11 in magenta and cyan, respectively. HsRXRα is not shown for the sake of clarity. The view is restricted to the region of 9cRA, which is shown in a stick representation with carbon atoms colored in white and oxygen atoms in red, and surrounded by a transparent light gray surface. Residues of TcUSP that fill the 9cRA LBP are show by sticks with carbon atoms colored in yellow, oxygen atoms in red and nitrogen atoms in blue. (**B**) Stereoview of the interaction network that stabilize the position of L11 and H12 in TcUSP. Helix H12 is shown in red, L6 and L11 in magenta and cyan, respectively and the rest of the protein in green. Residues that participate to the stabilization of L11 are represented by sticks colored by atom types (red for oxygen, blue for nitrogen, white for carbon, except for carbon atoms of W251 which are depicted in yellow). Hydrogen bonds are indicated by light orange dotted lines. (**C, D**) SPR analysis of the interactions between peptides derived from NR coactivators and receptor LBDs, demonstrating the lack of interactions between TcUSP and coactivator peptides. (C) Typical binding profiles (sensorgrams) are shown here for a 60 s injection (indicated by a blue line) of 5 μM SRC-1 long peptide over surfaces captured with 1500–1800 response units (RU) of apo HsRXRα (upper curve) and TcUSP (lower curve). (D) Histogram representations of the coactivator peptide binding to TcUSP (white) and HsRXRα (dark gray), showing the SPR response at $t = 150$ s, that is, 90 s after the end of the injection of 5 μM peptide. The peptides are derived from SRC-1 NR-box 2 (SRC-1 long and SRC-1 short) and from TIF2/GRIP1 NR-box 2 (TIF2/GRIP1).

for RXRs, it suggests that non-Mecopterida USPs have lost the ligand binding capability, while Mecopterida USPs have retained the capacity of binding a ligand, as shown in the crystal structures of the phospholipid-bound *Drosophila* and *Heliothis* USP.

Discussion

Tribolium USP is not responsive to RXR ligands

In the present work, we provide compelling functional, structural and evolutionary evidence for a novel type of RXR-USP

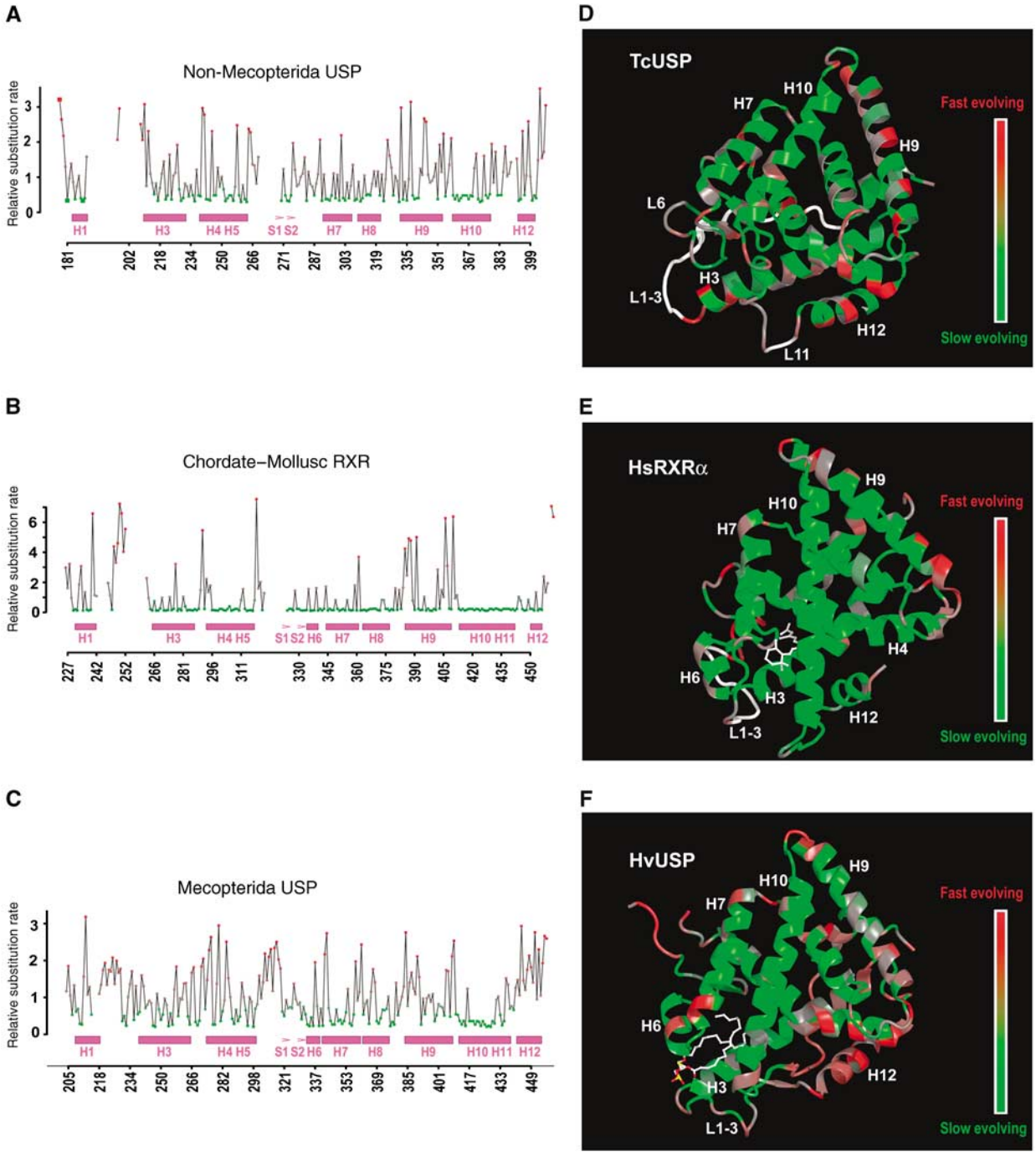

Figure 6 Mapping of evolutionary rates on the LBD sequences and structures. The phylogenetic analysis by maximum likelihood gives an estimation of site-specific substitution rates for (**A**) non-Mecopterida USPs, (**B**) chordate-mollusc RXRs and (**C**) Mecopterida USPs. The relative substitution rate (with a mean was fixed to 1) is given as a function of the sequence residues. For each group, values of substitution rates are mapped onto one representative LBD sequence and projected onto the corresponding crystal structure: (**D**) TcUSP, (**E**) HsRXRα and (**F**) HvUSP. Secondary structures are indicated in pink. 9cisRA and the phospholipid are shown by sticks, with carbon atoms in white, oxygen atoms in red and nitrogen atoms in blue, and phosphorous atoms in yellow. The evolutionary conserved residues are shown in green, fast evolving residues in red, and gray corresponds to an average rate. Not aligned residues are in white.

NR. Despite the high sequence similarity with human RXR, TcUSP does not bind RXR ligands. Independent experimental evidence supports our findings. *In vitro* transient transfection assays, in mammalian and insect cells, demonstrated that RXR ligands do not transactivate TcUSP. This can be explained by the lack of ligand binding to the receptor, as shown by MS analysis, SPR experiments and limited proteolysis assays. Furthermore, *in vivo* studies on transgenic flies provided a neutral functional test. Organ explants from flies bearing the GAL4-TcUSP transgene were not responsive to RXR ligands, whereas distinct response to various RXR ligands was observed in similar experiments with the GAL4-HsRXRα transgenic flies. In addition, the study of selection pressure acting on the receptor showed that TcUSP and its orthologs in non-Mecopterida arthropods are not subject to the same evolutionary constraints as vertebrate RXRs or Mecopterida USPs.

Structural studies reveal an original stabilization mode of the apo receptor state

In order to interpret the functional data, the crystal structure of the TcUSP LBD was solved in the context of the functional heterodimeric complex TcEcR/TcUSP. While the structure of ponA-bound TcEcR is very similar to those of HvEcR and BtEcR, TcUSP exhibits an apo structure, in contrast to HvUSP and DmUSP, but in line with BtUSP. For the latter, the lack of an LBP in the structure was difficult to interpret due to a truncated N-terminal form of the crystallized LBD. Remarkably, the TcUSP and BtUSP structures are otherwise very similar. Therefore, the structural work presented here indicates that the stable apo state is likely to be relevant to most if not all non-Mecopterida USPs. The stabilization of the apo state relies on the compact filling of the interior of the receptor by hydrophobic side chains of residues belonging to loops L6 and L11. These two structural elements are normally α-helices (H6 and H11) for RXR and for most other NRs. The peculiar conformation of L11 is stabilized by a characteristic interaction network around the conserved Trp residue in H5 (W251), a residue shown to be specific of class I NRs (Brelivet *et al*, 2004). Given the strong structural constraints on the LBD to maintain this apo conformation, the existence of some yet unknown ligands that would bind to USP and induce allosteric changes to the agonist receptor conformation is rather unlikely. However, we cannot exclude the possibility that some unknown transcriptional cofactor appears transiently at some developmental stages that would activate TcUSP and render it ligand responsive. Post-translational modifications could also be another possibility for this process.

Due to the peculiar conformation of loops L6 and L11, H12 is forced to adopt a so-called antagonist position that is stable enough to prevent the binding of coactivator peptides, as shown by SPR experiments. Interestingly, this antagonist conformation is a common feature of all the USP structures solved so far, irrespective of the insect order. With USP in a stable antagonist conformation, EcR would represent the sole anchoring point of cofactor proteins to the heterodimer. This would imply that the insect hormonal regulation is essentially dependent on ecdysteroids, merely suggesting a role of regulator for USP. Since the development of all arthropods is regulated hormonally in a similar manner by ecdysteroids and JHs or their precursors, our observations seriously call into question the possibility that USP could be the juvenile hormone receptor.

Lack of RXR ligand binding capability of USP and evolutionary implications

The phylogenetic analysis of EcR and USP revealed a marked divergence between sequences of Mecopterida and those of non-Mecopterida arthropods, such as *Tribolium* (Bonneton *et al*, 2003, 2006). For the latter, the USP sequences are highly similar to RXRs, with a high level of residue conservation for the LBP, as well as for other regions of the LBD. This sequence similarity first suggested that the properties of non-Mecopterida USPs could be similar to those of RXRs from Bilateria (Szanto *et al*, 2004; Bouton *et al*, 2005; de Groot *et al*, 2005) and Cnidaria (Kostrouch *et al*, 1998), for which ligand binding capability is well established. However, our results on *Tribolium* USP do not support this hypothesis and demonstrate the existence of a subgroup of RXR-USP receptors that can function in a ligand-independent manner. Earlier studies on other non-Mecopterida USPs already suggested that these receptors function differently when compared to RXRs. Transactivation assays on the tick *Ambylomma americanum* USP indicated that this receptor was unlikely to bind retinoic acids (Guo *et al*, 1998). Similarly, no binding of JHIII to the locust *L. migratoria* USP was detected (Hayward *et al*, 2003). For decapod (such as the crab *Uca pugilator*) (Durica *et al*, 2002) and branchiopod (such as *Daphnia magna*) crustaceans (Wang *et al*, 2007), the JHIII precursor, methyl farnesoate, has been hypothesized as being a physiological ligand for USP based on its known biological activities, but the binding of this terpenoid compound or any other ligand to USP has not been formally demonstrated. Together with these data, our work suggests that the capability of ligand binding to RXR, apparently acquired by a common ancestor of Cnidaria and Bilateria and conserved throughout evolution, was specifically altered in arthropods. With all the available data placed in a phylogenetic context, it appears that for USP, two main functional shifts took place in the arthropod lineage (Figure 7): a loss of ligand binding capability for non-Mecopterida USP, giving rise to a stable apo LBD structure, and a divergence in ligand binding specificity for Mecopterida, resulting in phospholipid-bound LBD structure. For the latter, the chemical nature of the endogenous ligand and its function still need to be clarified. Note that both types of USP are not capable of binding RXR ligands. Despite differences seen at the structural level for both groups of USP, the functional outcome is identical. In fact, both exhibit an activation helix H12 in a stable antagonist conformation, suggesting a constitutively silent role for USP in general. The phylogenetic scenario is interesting to consider in the light of the discussion arising on the evolution of ligand binding

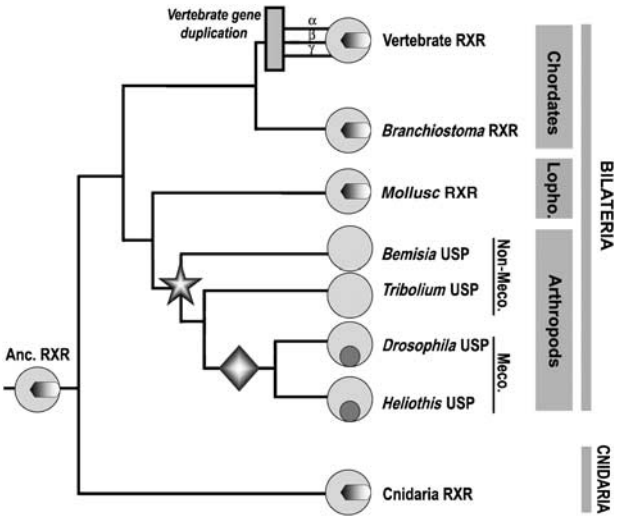

Figure 7 Functional evolutionary flexibility of arthropod USPs. Schematic representation of the RXR-USP phylogeny, suggesting a scenario where functional shifts took place in the arthropod lineage (indicated by a star and a diamond), resulting in a loss of ligand binding capability for non-Mecopterida USPs (light gray circle) and a divergence in ligand binding specificity for Mecopterida USPs (light gray circle with smaller dark gray circle). On the other hand, the capability of ligand binding to chordates, mollusc and cnidarian RXRs was apparently acquired by a common ancestor of Cnidaria and Bilateria (Anc. RXR), and conserved throughout evolution.

capability of NRs. It has been proposed by one of us that, starting from orphan receptors, ligand binding was gained several times independently by NRs during evolution (Escriva *et al*, 1997, 2000). In contrast, others have proposed that ligand binding was ancestral and that orphan receptors lost their ligand binding abilities during evolution (Thornton *et al*, 2003; Krylova *et al*, 2005; Keay *et al*, 2006). The case of USP discussed in this paper shows in fact that NRs are much more evolutionarily plastic than previously anticipated and that both scenarios can be reconciled.

Perspectives

Our observations that non-Mecopterida USPs are constitutively silent NRs that exhibit an original apo conformation of remarkable stability emphasize the importance of the dynamics of the receptor LBD in the ligand binding process, where ligand capture is only possible when the apo state is in an equilibrium with other receptor conformers. This is a meaningful lesson demonstrating that sequence identity is a necessary, but not sufficient condition to predict binding of similar ligands to similar receptors. Our work illustrates the remarkable evolutionary plasticity of this NR LBD that can accommodate variable selective pressures and specific functional shifts, such as changes in the ligand binding abilities, together with the conservation of the dimerization interface. This evolutionary plasticity, which has also been illustrated by studies of steroid and retinoid receptors as well as nematode NRs (Robinson-Rechavi *et al*, 2005; Van Gilst *et al*, 2005; Bridgham *et al*, 2006; Escriva *et al*, 2006) suggests that NRs may represent outstanding models for studying how evolution changes the biological function of the molecules themselves, a feature that has been relatively neglected up to now.

Furthermore, the results of our studies are of relevance for the entire field of RXR research, where controversial issues are constantly raised as concerning the existence of endogenous ligands activating RXR *in vivo*. By showing the existence and properties of USPs that function in a ligand-independent manner, our findings conversely support the existence of endogenous molecules that bind to RXR *in vivo*, either as activating ligands or as sensor molecules and should motivate further prospecting work in this direction. Remarkably, studies of Mecopterida and non-Mecopterida USPs and the search for cofactors or other activation mechanisms should give more insight into the roles and functions of RXR-USP receptors in general.

Materials and methods

Chemicals

PonA was purchased from SciTech (Prague, Czech Republic); 9cRA and methoprene and methoprene acid from MP Biomedicals Europe (Illkirch, France); Kinoprene from Chem Service Inc. (West Chester, USA); PUFAs from Sigma Chimie (Saint-Quentin Fallavier, France). All the ligands were dissolved in ethanol.

Transactivation assays

HEK293 EBNA cells transient transfection assays were carried out in 24-well plates (10^5 cells per well) using a standard calcium phosphate co-precipitation technique described by Greiner *et al* (1996). Cells were cotransfected with 25 ng pSG5-GAL4-HsRXR, pSG5-GAL4-TcUSP or pSG5-GAL4-DmUSP expression plasmid, 250 ng UAS-TK-LUC reporter and 75 ng pCH110 β-galactosidase expression plasmid, and when necessary, with 25 ng pSG5-DmEcR or pSG5-TcEcR expression plasmids. S2 cells were transiently transfected in 24-well plates (4×10^5 cells per well) using Effectene (Qiagen), following the manufacturer's protocol. Cells were cotransfected with 25 ng pPAC5C-GAL4-HsRXR or pPAC5C-GAL4-TcUSP expression plasmid, 250 ng UAS-TK-LUC reporter and 75 ng pPAC5C-β-galactosidase expression plasmids. HEK293 EBNA and S2 cells were treated with the appropriate ligand for 20 h, and cell extracts were assayed for luciferase and β-galactosidase activity. Luciferase values were normalized to β-galactosidase activity. Data points represent the value of assays performed in duplicate. All experiments were confirmed by at least two independent experiments.

Transgenic fly generation and analysis

TcUSP-GAL4 DBD and HsRXRα-GAL4-DBD were excised from pG4M plasmids by digestion with *Eco*RI and Bgl2, and inserted in pCaSpeR-hs. Each P element was inserted into the w^{1118} germ line according to standard transformation procedure. Seven independent p{hs-GAL4-TcUSP} and nine independent p{hs-GAL4-HsRXRα} transgenic fly lines were obtained. Two homozygous viable p{hs-GAL4-TcUSP} insertions localized on the third chromosome were used that showed the same induction level after a heat-shock treatment and a homozygous viable p{hs-GAL4-HsRXRα} insertion also located on the third chromosome. w;+;p{hs-GAL4-TcUSP},p{w^{+mc} = UAS-eGFP} and w;+;p{hs-GAL4HsRXRα}, p{w^{+mc} = UAS-eGFP} larvae were obtained by crossing the selected w;+;p{hs-GAL4-TcUSP} or p{hs-GAL4-HsRXRα} transgenic males with w;+;p{w^{+mc} = UAS-eGFP} females. Third instar larvae were harvested when they left the food medium, just at the beginning of the wandering stage. Then, animals were put into a tube immersed in a 37°C water bath for 20 min. Following this heat-shock treatment, the salivary glands were dissected and incubated in 25 μl of culture medium (50:9:1-Grace's medium (Gibco):distilled water:ethanol). 9cRA, methoprene acid or PUFAs were added in the alcohol fraction. Salivary glands were incubated for 6–7 h at 25°C, then mounted in the culture medium and observed immediately with an epifluorescent microscope.

Data collection, structure determination and refinement

Cloning of the expression plasmids as well as the purification and crystallization procedures are described in the Supplementary data. Diffraction data were collected at beamline ID14-EH1 at the European Synchrotron Radiation Facility (Grenoble, France). Two data sets from two different locations of a single crystal were processed using the HKL package (Otwinowski and Minor, 1997). Crystals belonged to $P2_1$ space group. The crystal structure was solved by molecular replacement with AMORE (Navaza, 1994), using two models: a chimeric model composed of ponA/HvEcR and ΔH12-HsRXRα (no H12) and the crystal structure of BtEcR/ΔH12-BtUSP. The two models led to a different MR solution, encompassing three heterodimers in the asymmetric unit (a.u.), two being shared by both models. Combination of these two solutions resulted in the final solution with four heterodimers in the a.u. Refinement was performed with CNS using torsion angle dynamics with a maximum likelihood function target, including restrained anisotropic refinement of individual atomic temperature factors and bulk solvent correction (Brünger *et al*, 1998). Manual adjustments and rebuilding of the models were performed using the program O and sigma-A-weighted electron density maps (Jones *et al*, 1991). The missing regions correspond to flexible loops between H1 and H3 in EcR and to the chain termini. Structures exhibit good geometry with no Ramachandran outliers. Molecular graphics figures were created using PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA, USA; http://www.pymol.org).

Coactivator binding

SPR analyses were performed at 25°C with a BIAcore 2000 optical sensor, as described in the Supplementary data. The sequences of the peptides were the following:

SRC-1 NR-box 2: NH-CPSSHSSLTERHKILHRLLQEGSPS-COOH (SRC-1 long)
SRC-1 NR-box 2: NH-RHKILHRLLQEGSPS-COOH (SRC-1 short)
GRIP1 NR-box 2: NH-KHKILHRLLQDSS-COOH (GRIP1)
Control peptide: NH-CADYKLQESKKEEPEKQ-COOH (control)

Phylogenetic analysis and evolutionary rates estimation

We built a set of 51 protein-coding sequences of RXRs-USPs (Supplementary Table S1). Sequences were aligned using SEAVIEW (Galtier *et al*, 1996). All positions with gaps were excluded from analysis, resulting in 227 complete aligned sites for chordates-molluscs

RXR LBDs, 215 complete sites for non-Mecopterida USP LBDs and 247 complete sites for Mecopterida USP LBDs. In order to estimate site-specific substitution rates within each group of sequences, we predefined the taxonomic relationships within each group of species following proposed consensual trees (Supplementary Figure S2). The branch lengths of the trees were independently estimated by Maximum Likelihood using the PAML program (Yang, 1997) under the JTT amino-acid substitution model (Jones *et al*, 1992) plus rate heterogeneity between sites, estimated by a gamma law with 20 categories (Yang, 1996). We selected a high number of categories for the gamma law approximation in order to use the Bayesian approach (Yang and Wang, 1995) implemented in the PAML program, to estimate the relative substitution rate of each LBD site. These substitution rates, mapped along the LBD sequence for the three groups, were projected onto the corresponding 3D structure.

Accession number

The coordinates and structure factors have been deposited in the RCSB Protein Data Bank (http://www.rcsb.org/pdb/) under accession number 2NXX.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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