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Identification of interacting proteins of retinoid-related orphan nuclear receptor gamma in HepG2 cells

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The retinoid-related orphan nuclear receptor gamma (RORγ) plays critical roles in regulation of development, immunity and metabolism. As transcription factor usually forms a protein complex to function, thus capturing and dissecting of the RORγ protein complex will be helpful for exploring the mechanisms underlying those functions. After construction of the recombinant tandem affinity purification (TAP) plasmid, pMSCVpuro RORγ-CTAP(SG), the nuclear localization of RORγ-CTAP(SG) fusion protein was verified. Following isolation of RORγ protein complex by TAP strategy, seven candidate interacting proteins were identified. Finally, the heat shock protein 90 (HSP90) and receptor-interacting protein 140 (RIP140) were confirmed to interplay with RORγ by co-immunoprecipitation. Interference of HSP90 or/and RIP140 genes resulted in dramatically decreased expression of CYP2C8 gene, the RORγ target gene. Data from this study demonstrate that HSP90 and RIP140 proteins interact with RORγ protein in a complex format and function as co-activators in the RORγ-mediated regulatory processes of HepG2 cells. [BMB Reports 2012; 45(6): 331-336]

INTRODUCTION

Following the cloning of several nuclear hormone receptors (NHR) in the late 1980s, many laboratories began to search for novel members of this superfamily. One of the results was the identification of the retinoid-related orphan nuclear receptor (ROR) subfamily. RORα was the first member of the ROR subfamily to be identified, based upon its similar sequence to the retinoic X receptor and the acid retinoid receptor (1), followed by the identification of RORβ (2). The third member of RORs, RORγ, was identified and cloned by anchor PCR, according to its similarity to the most highly-conserved sequence of the DNA-binding domain of RORα and RORβ (3). Both the mouse and human RORγ gene generate two isoforms: γ1 and γ2 (4). RORγ1 (usually referred to as RORγ) expression is restricted to liver, adipose tissue, heart and skeletal muscle, while RORγ2 (most commonly referred to as RORγt) is exclusively detected in a few distinct cell types of the immune system (3, 5).

RORγ has been characterized for its critical functions in development, immunity and metabolism (6, 7). Besides playing an important role in the regulation of several metabolic pathways, particularly lipid and steroid metabolism (8), RORγ stimulates transcription of the CYP2C8 gene in human liver by binding to the canonical ROR element (RORE) within the CYP2C8 promoter, ultimately modulating the metabolism of many clinical drugs in current use (9).

Cellular functions are nearly always the result of the coordinated actions of several proteins in macromolecular assemblies and pathways (10). Protein complexes are intricately-ordered, dynamic structures that translate biological information into function (11). While some endogenous/synthetic ligands of RORγ were found such as 7-oxygenated sterol, SR1078, T0901317, and 24S-OHC, which have been demonstrated to regulate the transcriptional activity of RORγ (12-15), only protein Mi-2β has been identified to interact with RORγ to function as a corepressor by using the yeast two hybrid strategy (16). Considering RORγ probably function via forming protein-protein complex, it will be very beneficial to exactly explore the RORγ-interacting proteins by directly capturing and dissecting such complex by using a more suitable strategy, in contrast to the yeast two hybrid method.

To this end, in this study, the tandem affinity purification (TAP) method was designed to capture the endogenous interacting protein complex of RORγ in stably-transfected HepG2 cells. TAP is a generic, two-step affinity purification protocol originally designed to capture protein complexes in yeast, but was modified with a tag based on protein G and the streptavidin-binding peptide (GS-TAP) to facilitate a marked increase in protein-complex yield and higher specificity of the procedure (17). By using TAP and RNA interference strategies, we found

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that HSP90 and RIP140 exist in the protein complex with RORγ and silencing of HSP90 or/and RIP140 genes dramatically decreased CYP2C8 gene expression in HepG2 cells, indicating that both proteins may be the important co-activators in the RORγ-regulated biological processes.

RESULTS

Construct of pMSCVpuro RORγ-CTAP(SG)

We cloned the RORγ cDNA from total mRNA of HepG2 cells using a high fidelity DNA polymerase and constructed RORγ-CTAP(SG) fusion genes by cleavage and ligation of the RORγ gene into the pCeMM CTAP(SG) vector. The schematic structures of the fusion gene RORγ-CTAP(SG) and its control CTAP(SG) are shown in Fig. 1A. The vector pCeMM RORγ-CTAP(SG) had no selectable antibiotics gene to facilitate a stable transfected cell line, so we subcloned RORγ-CTAP(SG) or CTAP(SG) fragments into the pMSCVpuro vector to obtain the puromycin-resistant recombinant plasmid pMSCVpuro RORγ-CTAP(SG) or control pMSCVpuro CTAP(SG).

After the successful construction of pMSCVpuro RORγ-CTAP(SG), the plasmids pMSCVpuro RORγ-CTAP(SG) and pMSCVpuro CTAP(SG) were transiently transfected into HepG2 cells and analyzed by Western Blot to determine whether RORγ were expressed correctly in HepG2 cells. Our results demonstrated that the RORγ-CTAP(SG) fusion gene was able to be expressed in the pMSCVpuro RORγ-CTAP(SG)-transfected cells with the expected molecular size (79 kDa), as detected by anti-RORγ antibody. However, no such band was observed in HepG2 pMSCVpuro CTAP(SG)-transfected HepG2 cells. The endogenous RORγ protein expression was detected in all experimental cells (58 kDa) (Fig. 1B). When using anti-PortG, RORγ-CTAP(SG) fusion protein and CTAP(SG) (21 kDa) was able to be detected in the corresponding plasmids-transfected cells, and not in HepG2 cells (Fig. 1C). Similar results were observed when using SBP tag as the detecting ligand (Fig. 1D). The tubulin protein was detected as an internal control to reflect nuclear protein expression, and was found to be expressed constitutively in all experimental cells (Fig. 1E).

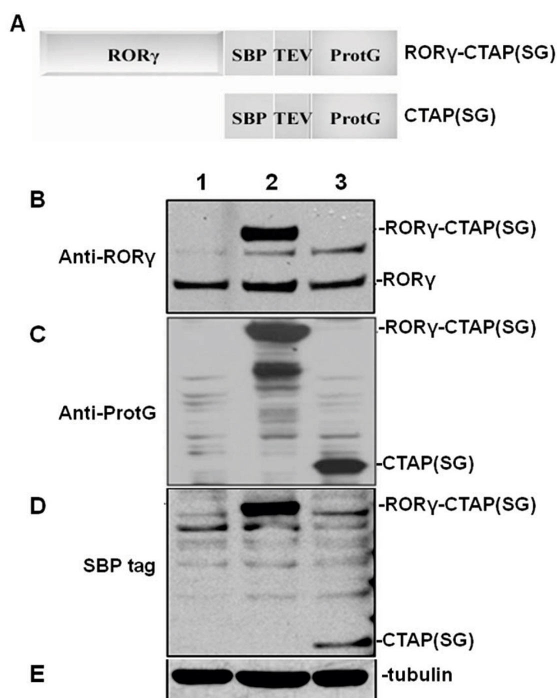

Fig. 1. Cloning of RORγ-CTAP(SG) fusion gene. (A) Schematics of the RORγ-CTAP(SG) fusion gene. The CTAP(SG) tags are located at the C-terminus of the RORγ gene, which is comprised of DNA sequences encoding protein G and the streptavidin-binding peptide (GS-TAP), separated by a TEV protease cleavage site. (B-D) Western blot analyses detecting the expressions of RORγ-CTAP(SG) and CTAP(SG) in the transfected HepG2 cells by using anti-RORγ (B), anti-ProtG (C), or SBP tag (D). Tubulin was detected as the internal nuclear protein control (E) and non-transfected HepG2 cells was detected as the cell control. Lanes: 1, non-transfected HepG2 cells; 2, pMSCVpuro RORγ-CTAP(SG)-transfected HepG2 cells; and 3, pMSCVpuro CTAP(SG)-transfected HepG2 cells.

Nuclear localization of RORγ-CTAP(SG) gene in stably-transfected HepG2 cells

RORγ exhibits a typical nuclear receptor domain structure by which it specifically binds to ROREs in the nucleus to regulate gene transcription. Fusion of CTAP(SG) tags into the C-terminus of the RORγ gene might have affected the nuclear localization of RORγ in HepG2 cells. Therefore, we checked whether the RORγ-CTAP(SG) fusion protein was still localized intranuclearly in the transfected HepG2 cells by detecting the SBP tag in immunofluorescence assays. As expected, the

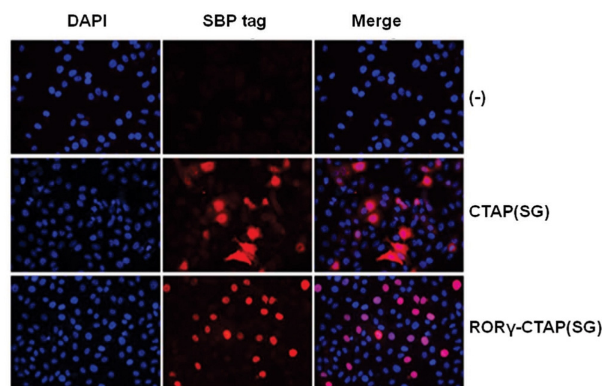

Fig. 2. Cellular localization of RORγ-CTAP(SG) and CTAP(SG) in transfected HepG2 cells. HepG2 cells were stably-transfected with RORγ-CTAP(SG) or CTAP(SG) recombinant plasmids and subjected to immunofluorescence analysis with an anti-SBP tag antibody. The non-transfected HepG2 cells were used as the control.

RORγ-CTAP(SG) fusion protein showed exclusive nuclear localization that completely overlapped with DAPI staining; whereas, the non-fused CTAP(SG) tags were present in both nucleus and cytoplasm (Fig. 2). The above results further support the expected ability of this system to capture endogenous RORγ protein complexes in HepG2 cells.

Isolation and identification of RORγ protein complexes by TAP/MS

The acquisition of protein complexes by tandem affinity purification followed by MS analysis of the captured samples is an effective and efficient method to identify biologically relevant macromolecular complexes (17). In our work, TAP was performed after induction of expression of RORγ-CTAP(SG) or its control CTAP(SG) tags in stably-transfected HepG2 cell lines. The purification yields from each step of the TAP procedure were evaluated by Western blot analysis (Fig. 3A). Results showed that the initial total input contained RORγ-CTAP(SG) fusion protein. After being treated with TEV (tobacco etch virus) protease, the RORγ-CTAP(SG) fusion protein was effectively cleaved into RORγ-SBP, as observed in the Western blot assay of the final eluate (Fig. 3A). The final eluates from pMSCVpuro RORγ-CTAP(SG)- or pMSCVpuro CTAP(SG)-transfected HepG2 cells were separated by SDS-PAGE and stained with silver regents. Results demonstrated a total of eight bands that differed from the control (Fig. 3B).

These eight bands were excised and digested with trypsin, peptides were extracted, separated by nanoflow LC, and introduced into an ion-trap mass spectrometer. MS/MS analysis and database searching of the sequenced peptides resulted in the identification of 8 proteins (Supplementary Table S1). Two representative MS/MS spectrum of the tryptic digest from band 1 and band 3 are shown in Supplementary Fig. S1. The peptide sequence (K)GMSSHLNGQAR(T) and (R)TLTLVDTGIGMTK(A) matched separately to RIP140 and HSP90 by database searching.

Confirmation of the RORγ complex

There is no doubt that any interacting protein detected by the TAP method must be confirmed by different assays. In this study, confirmation of the interaction was conducted by immunoprecipitating RORγ from total extracts, followed by Western blot assay to determine whether those newly-identified proteins were interacting with endogenous RORγ protein. Results showed that RIP140 and HSP90 proteins, rather than EMG1, ZNF21, DOC2A, Sp5 and RFXDC1 proteins, could be

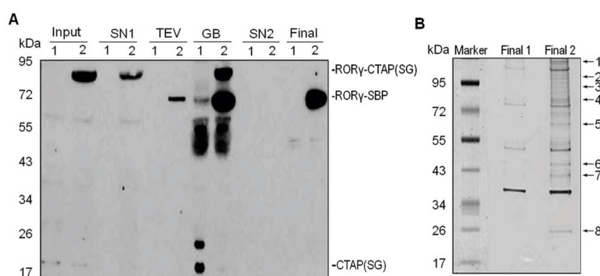

Fig. 3. Isolation and identification of RORγ protein complex. (A) Tracing of the SBP tag throughout the TAP procedure. SBP tag was tracked throughout the entire TAP procedure by immunoblotting using an anti-SBP-tag antibody. Input, total lysate; SN1, supernatant after IgG beads incubation; TEV, eluate after TEV protease cleavage; GB, samples left on IgG beads; SN2, supernatant after streptavidin beads incubation; Final, the final eluate. Lanes: 1, pMSCVpuro CTAP(SG)-transfected HepG2 (control group); 2, pMSCVpuro RORγ-CTAP(SG)-transfected HepG2. Bands specific to RORγ-CTAP(SG), RORγ-SBP and CTAP(SG) are marked on the right. (B) Silver staining of purified protein complexes. Proteins purified from Protein G/SBP-tagged RORγ were separated on a 4-12% gel and silver-stained. Final 1, control purification from HepG2 cells transfected with pMSCVpuro CTAP(SG); Final 2, purification from HepG2 cells transfected with pMSCVpuro RORγ-CTAP(SG).

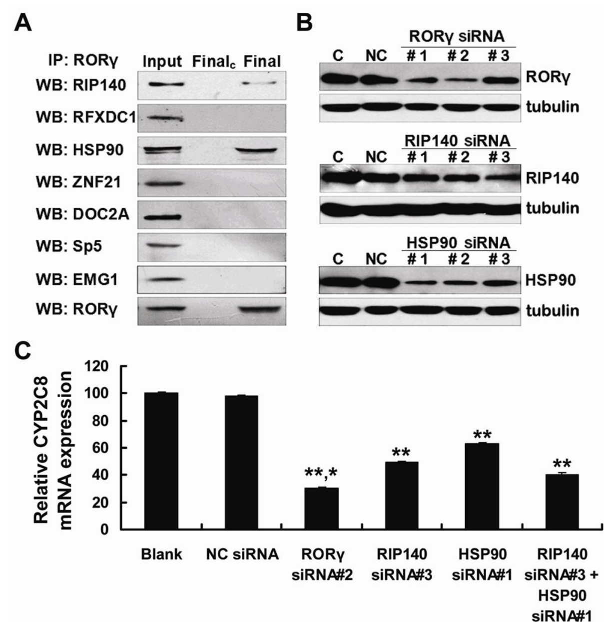

Fig. 4. Confirmation of RORγ protein complex. (A) Total extracts were prepared from HepG2 cells and immunoprecipitated by anti-RORγ antibody. Proteins bound to the beads were analyzed by Western blot using the indicated primary antibodies. Input, total lysate; Finalc, isotype IgG control; Final, the immunoprecipitated output by anti-RORγ antibody. (B) Western blot assay validating RORγ, RIP140 and HSP90 knockdown by siRNA. C, HepG2 cell control; NC, the irrelevant siRNA negative control. Tubulin was used as the internal standard for sample loading. (C) The CYP2C8 mRNA expression level relative to GAPDH in siRNA transfected HepG2 cells was examined by quantitative reverse transcription polymerase chain reaction. The siRNAs used for transfection were indicated. Data are shown as fold induction relative to blank HepG2 cells and are the mean ± SD. Error bars indicate standard deviation of three independent experiments. *P < 0.04 vs groups RIP140/siRNA#3, HSP90/siRNA#1, and RIP140/siRNA#3/HSP90/siRNA#1; **P < 0.0.1 vs Blank.

co-immunoprecipitated with RORγ protein, confirming the interaction among RIP140, HSP90 and RORγ in the HepG2 cells (Fig. 4A).

We further determined whether RIP140 and HSP90 had functional relevance with RORγ protein by using RNAi strategy. Western blot assay indicated that introduction of RORγ siRNA-2, RIP140 siRNA-3 and HSP90 siRNA-1 in HepG2 cells resulted in the dramatic decrease of RORγ, RIP140 and HSP90 protein levels, respectively, in contrast to other siRNAs (Fig. 4B). We then examined the effects of the decreased RORγ, RIP140 and HSP90 gene expressions on the RORγ-transcribed CYP2C8 gene expression in HepG2 cells by quantitative reverse transcription polymerase chain reaction. Results showed that the interference of RIP140 or/and HSP90 caused a dramatic decrease of CYP2C8 gene expression (Fig. 4C). Results further demonstrated that silencing of RORγ gene caused a profound decrease of CYP2C8 expression compared with the effects of silencing of RIP140 or/and HSP90, indicating the RORγ-dependent format for CYP2C8 expression, and that both CYP2C8 and HSP90 might be the functional co-activators of transcription factor RORγ (Fig. 4C).

DISCUSSION

RORγ has been characterized for its critical functions in development, immunity and metabolism (6, 7). Considering RORγ probably function via forming protein-protein complex, thus it will be very beneficial to explore the RORγ-interacting proteins by directly capturing and dissecting such complex. In this study, to investigate the protein complexes involved in RORγ transcriptional regulation, we used the TAP strategy, a two-step affinity purification protocol to capture the endogenous RORγ-interacting proteins in a protein complex format. Finally, we got 7 candidate RORγ-interacting proteins as confirmed by MS/MS analysis. Among the seven candidates, RIP140 and HSP90 proteins were finally verified as the RORγ-interacting proteins in a complex as determined by co-IP/Western-blot assays. We further confirmed the functional relevance between RORγ and RIP140/HSP90 by using RNAi strategy.

RIP140 is a coregulator for nuclear receptors and other transcription factors, which represses catabolic pathways in metabolic tissues. Although RIP140 functions as a corepressor for most nuclear receptors, mounting evidence points to RIP140 as a dual coregulator that can repress or activate different sets of genes (18). In HepG2 cells, we demonstrate that RIP140 functions as a co-activator of RORγ because interfering of RIP140 shows about 50% down-regulation of RORγ-transcribed CYP2C8 gene expression (Fig. 4C). Besides RIP140, we also identified another interacting protein of RORγ, HSP90. HSP90 was an ATPase-dependent chaperone and responsible for managing protein folding and quality control in the crowded environment inside the cell. It has been demonstrated to participate in activating and stabilizing more than 200 proteins involved in post-translational folding, protein stability, activation, and maturation of cellular proteins, which are all essential events in cell cycle control and signaling (19, 20). Hence, HSP90 may act as a chaperone to help RORγ complete its functions *in vivo*. Expectedly, we confirm that RORγ-transcribed CYP2C8 gene expression is markedly decreased by interfering of HSP90. Further study is needed to discover whether RORγ directly or indirectly contacts with HSP90 in the complex in HepG2 cells.

We further demonstrate that interference of RORγ gene expression by the synthetic siRNA targeted to RORγ causes significantly decreased expression of the CYP2C8, the RORγ target gene. Considering the transfection efficiency for the current commercial siRNA transfection reagent is about 70-80%, the results thus have verified the RORγ-dependent format for CYP2C8 expression, in accordance with the observation by Chen and colleague (9). Collectively, these observations indicate that both RIP140 and HSP90 may be the functional co-activators of the transcription factor RORγ.

However, despite we have identified two novel RORγ-interacting proteins RIP140 and HSP90 in this study, the TAP strategy did not detect the previously reported RORγ-interacting protein Mi-2β yet. There might be several possibilities for this result: (1) the previously reported Mi-2β-RORγ interaction is verified in rabbit reticulocyte; in contrast, in this study, we have identified the components of RORγ-interacting complex in HepG2 cells. There might be different Mi-2β protein expression formats between both cell types because the suppressor Mi-2β of RORγ protein might highly express and function only in certain physiological condition other than the HepG2 tumor cells, thus we cannot capture its interaction with RORγ protein in this study. (2) Due to the highly stringent condition of TAP strategy that efficiently excludes false positive products, it thus probably accompanied by losing some faint interacting proteins in the mean time. In this study, during the gel resolution step to isolate RORγ-interacting proteins, some faint bands might be overlooked, and thus the corresponding interacting protein partner was missed. This problem will be resolved by using LC/MS or consecutive cutting of the gel for MS/MS analysis in the future.

In conclusion, in this study we have identified and verified RIP140 and HSP90 as the interactors of RORγ in a complex format in HepG2 cells and confirmed that the functional relevance between RIP140/HSP90 and RORγ-mediated CYP2C8 gene expression. These findings support the hypothesis that RORγ is a coregulator-dependent transcription factor and functions in a complex. HSP90 may act as a chaperone helping RORγ execute its regulatory roles while RIP140 might function as a co-activator of RORγ in HepG2 cells.

MATERIALS AND METHODS

Cloning of RORγ gene

Total RNA was extracted from HepG2 cells, converted to complementary DNA (cDNA), and used as template to amplify the

RORγ gene with the following primers: forward (F) 5′-AGCCA GGACGGCACCAAGG-3′, and reverse (R) 5′-CCATAGGGAG AGGCAAGGAGTCC-3′. PCR parameters were set as follows: 30 cycles of 98°C for 15 sec, 68°C for 10 sec, 72°C for 90 sec. The amplicon was then subcloned into the pMD19-T vector (TaKaRa, China) and verified by double-digestion with *EcoR I* and *Hind III* restriction enzymes (TaKaRa) and sequencing.

Plasmids

The plasmid pCeMM CTAP (SG) was kindly provided by EUROSCARF (University of Frankfurt, Germany). The RORγ cDNA was amplified from the pMD19-T RORγ plasmid by using the following primers: F5′-GCGAATTCATGGACAGGGCC CCAC-3′ and R5′-CCGTTTAAACTTCTTGGACAGCCCCACA G-3′. The amplified product was cloned into the *EcoR I* and *Pme I* sites of pCeMM CTAP(SG) to construct the pCeMM RORγ-CTAP(SG) plasmid. To obtain a stable transfected cell line selected by puromycin, the RORγ-CTAP(SG) or CTAP(SG) fragments were cloned into the *Xho I* and *EcoR I* sites of pMSCVpuro vector (Clontech, USA), using the following primer pairs, respectively: F5′-GCCTCGAGATGGACAGGGCCC CAC-3′ and R5′-GCGAATTCCTATTCAGTGACAGTG-3′; or F5′-GCCTCGAGATGAAGTTTAAACGG-3′, R5′-GCGAATTCC TATTCAGTGACAGTG-3′. The resultant plasmids were designated as pMSCVpuro RORγ-CTAP(SG) and pMSCVpuro CTAP(SG). All constructs were confirmed by sequencing.

Cell culture, siRNA and transfection

HepG2 cell line (ATCC, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, Uruguay), containing 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Transfections were performed using Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's instructions.

The siRNA duplexes targeting RORγ, RIP140 and HSP90 were designed and synthesized (Invitrogen, China), and the siRNA sequences for RORγ, RIP140 and HSP90 genes could be found in Supplementary Table S2. For transient transfection, cells were collected 24 hr later. For stable transfection, cells were selected 36 hr later by addition of 1.5 μg/ml puromycin (Sangon, China) to the complete culture medium, followed by refreshing the selective complete culture medium every two to three days.

Western-blot analysis

Equal volumes with equal protein concentration of samples were separated by 4-12% NuPAGE gel electrophoresis (Invitrogen), then transferred onto polyvinylidene fluoride membranes for immunoblotting. After blocking, the membranes were incubated with one of the following primary antibodies: mouse anti-RORγ monoclonal antibody (Abcam, CA, USA), mouse anti-SBP tag monoclonal antibody (Santa Cruz, CA, USA), mouse anti-protein G monoclonal antibody (Santa Cruz), rabbit anti-RIP140 polyclonal antibody (Santa Cruz), rabbit anti-RFXDC1 polyclonal antibody (Santa Cruz), mouse anti-HSP90 monoclonal antibody (Santa Cruz), mouse anti-ZNF21 monoclonal antibody (Santa Cruz), goat anti-DOC2 polyclonal antibody (Santa Cruz), goat anti-Sp5 polyclonal antibody (Santa Cruz), rabbit anti-EMG1 polyclonal antibody (Santa Cruz) or mouse anti-Tubulin antibody (Santa Cruz). After 1 hr incubation at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody, specific protein bands on the membranes were visualized by the Super Signal West Femto kit (Pierce Chemical, USA) according to the manufacturer's instructions.

Immunofluorescence staining

Cells were fixed, permeabilized, blocked, and incubated with mouse anti-SBP tag monoclonal antibody overnight at 4°C. After washing, the cells were incubated for 1 hr at room temperature in the presence of goat anti-mouse CY3 secondary antibody, followed by counterstaining with the nuclear probe DAPI and observation under fluorescence microscope.

Tandem affinity purification

Tandem affinity purifications of RORγ protein complex were conducted according to the published method (17). Briefly, the lysates of HepG2 (5×10^8) cells were cleared by ultracentrifugation and the supernatant was incubated with 200 μl IgG beads at 4°C for 4 hr. After washing, the bound proteins were subjected to TEV-protease cleavage. The TEV-protease cleavage product was incubated at 4°C for 2 hr with 100 μl Streptavidin beads (Ultralink Immobilized Streptavidin Plus; Pierce). The final bound protein samples were eluted and run on a Ready gel (Invitrogen, USA), and detected by silver-staining.

Mass spectrometry

Protein bands were excised from the SDS-PAGE gel and digested by trypsin (Promega, USA). Peptides were dissolved in 0.1% formic acid and analyzed by HPLC-Ion Trap-Chip-MS/MS (Agilent 6300 Series, USA). Peptide and protein identifications were run automatically using the accompanying Spectrum Mill software from Agilent (RevA.03.03.078).

Immunoprecipitation

Cells were lysed and precleared for 1 hr at 4°C with Protein A/G Plus Agarose (Santa Cruz). Precleared lysates (1 mg) were then incubated overnight at 4°C with 4 μg of control mouse IgG (Santa Cruz) and mouse anti-human RORγ antibody (Abcam, USA); the following day, a 2 hr incubation was carried out with Protein A/G Plus beads and four washes with RIPA buffer. Proteins remaining bound to the beads were then analyzed by Western blot.

Quantitative reverse transcription polymerase chain reaction

Total RNA was extracted and used to synthesize cDNA with Reverse Transcription kit (TaKaRa, Japan) according to the man-

ufacturer's instructions. GAPDH was used as internal control. Using specific primers for CYP2C8 (F5′-AGATCAGAATTTTCT CACCC-3′; R5′-AACTTCGTGTAAGAGCAACA-3′), PCR was carried out in 25 μl of total volume with 0.5 mM primers via a SyBr Green kit (TaKaRa) for 40 cycles on Rotor-Gene 6000 (Gene Company Limited, Hong Kong). We used the $2^{-\Delta\Delta}$ CT method for quantifying expression relative to the GAPDH housekeeping control.

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