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Transcriptional activity of estrogen-related receptor γ (ERRγ) is stimulated by the phytoestrogen equol

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ABSTRACT

Estrogen-related receptor γ (ERRγ) is an orphan nuclear receptor lacking identified natural ligands. The synthetic estrogen receptor ligands 4-hydroxytamoxifen and diethylstilbestrol have, however, been shown to bind to and abolish the constitutive transcriptional activity of ERRγ. Certain phytoestrogens were recently reported to act as agonists of the related ERRα. We investigated whether phytoestrogens also modulated the transcriptional activity of ERRγ. We analyzed a selection of phytoestrogens for their potential agonistic or antagonistic activity on ERRγ. In transiently transfected PC-3 and U2-OS cells equol stimulated the transcriptional activity of ERRγ and enhanced its interaction with the coactivator GRIP1. The agonistic effect of equol was abolished by 4-hydroxytamoxifen. Equol induced a conformational change in the ERRγ ligand-binding domain. Based on structural models of the ERRγ ligand-binding domain, we were able to introduce mutations that modulated the agonistic potential of equol. Finally, equol enhanced the growth inhibitory effect of ERRγ on the prostate cancer PC-3 cells. In conclusion, we have demonstrated that the phytoestrogen equol acts as an ERRγ agonist.

1. Introduction

Estrogen-related receptors (ERRα, ERRβ, and ERRγ) are orphan nuclear receptors whose physiological ligands have not yet been identified. Although ERRs are closely related to estrogen receptors (ERs) they do not respond to estrogens [1]. ERRs share a comparable modular structure with the classical ligand-regulated nuclear receptors such as steroid hormone receptors, thyroid hormone receptors, retinoid receptors, and vitamin D receptor. They thus consist of a centrally located conserved DNA-binding domain (DBD), a carboxyl-terminal ligand-binding domain (LBD), and a variable amino-terminal domain (NTD). The LBD folds into a three-layered sandwich structure composed of 12 α-helices (H1–12) forming a hydrophobic ligand-binding pocket [2]. In liganded nuclear receptors, ligand binding induces a conformational change in the LBD switching it from an inactive to an active conformation that allows interactions with transcriptional coactivator proteins. Interactions between the LBD and coactivator proteins involve the H12 containing the activation function-2 (AF-2) [3]. In contrast to the liganded nuclear receptors, structural studies have confirmed that the LBDs of ERRγ and ERRα adopt an active conformation and interact with coactivators in the absence of any bound ligand [4,5]. In line with this, ERRs activate transcription constitutively in reporter gene assays [1]. Their transcriptional activities are enhanced by the coactivator PGC-1 that was proposed to act as a protein ligand for ERRs [6]. In addition, the activity of ERRγ has also been shown to be modulated by cross-talk with other transcription factors [7–9]. Taken together, these observations suggest that the activities of ERRs are regulated by coactivator concentration or cross-talk with other transcription factors rather than by ligand binding.

The recently published crystal structures demonstrated that the ligand-binding pockets of ERRα and ERRγ are extremely small compared to the pockets in liganded nuclear receptors because the ERR ligand-binding pockets are partly filled by lipophilic side chains [4,5]. Ligand binding was predicted to displace H12 from the transcriptionally active conformation and thus to have an inhibitory rather than an activating effect on ERRs. Indeed, most of the identified synthetic ERR ligands act as inverse agonists. The ER agonist

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diethylstilbestrol (DES) deactivates all three ERRs and the selective ER modulator 4-hydroxytamoxifen (4-OHT) binds to and inactivates ERRβ and ERRγ [10–12]. The crystal structures of the ERRγ LBD in complex with DES and 4-OHT showed that binding of these inverse agonists resulted in rotation of the side chain of F435 and that the new rotamer of F435 displaced H12 from its agonistic position [13,14]. Recently, certain flavonoids were reported to inhibit the transcriptional activity of ERRγ by suppressing the interaction between ERRγ and the coactivator PGC-1α or by promoting the degradation of PGC-1α [15]. Zuercher et al. [16] reported however the identification of a synthetic ERRγ agonist GSK4716. Binding of GSK4716 to ERRγ caused rearrangements in the LBD that allowed the formation of a larger ligand-binding pocket and subsequently, ligand binding did not displace H12. Binding of GSK4716 led to global stabilization of the ERRγ LBD [14]. This demonstrated that it is possible to design and identify agonists for ERRγ. Although most ERRα ligands act as inverse agonists, certain phytoestrogens have been shown to be ERRα agonists [17]. Because of similarities between the ERR-family members, we have investigated whether these plant estrogens were also able to stimulate the transcriptional activity of ERRγ.

Phytoestrogens are plant-derived polyphenolic non-steroidal compounds that have estrogenic properties [18]. The main classes of phytoestrogens are lignans and isoflavones. Lignans, found in cereals, fruits, and vegetables, are metabolized by intestinal bacteria to enterolactone and enterodiol. Soybeans are a rich source of isoflavones. The principal isoflavones are genistein and daidzein. Daidzein is further converted to equol by gut microflora but this conversion has a high inter-individual variability [19]. Equol has been shown to have higher estrogenic activity than the precursor compound daidzein [20]. The interest in phytoestrogens has been increasing due to their suggested beneficial health effects. Phytoestrogen rich diet appears to reduce the risk of cardiovascular disease, osteoporosis as well as breast and prostate cancer [18].

ERRγ is expressed in several adult and embryonic tissues but its biological role is still largely unknown [21]. Overexpression of ERRγ in breast cancer correlates with a favorable outcome [22]. In addition, ERRγ has been reported to inhibit the growth of prostate tumor cells [23]. In this study we investigated whether phytoestrogens modulated the transcriptional activity of ERRγ. We analyzed a selection of phytoestrogens for their potential agonistic or antagonistic activities on ERRγ. Using reporter gene assays in transiently transfected U2-OS and PC-3 cells we identified equol as an ERRγ agonist. Based on structural models of the ERRγ LBD we were able to introduce mutations that modulated the agonistic effect of equol. Finally, the growth inhibitory effect of ERRγ on the prostate cancer cell line PC-3 was enhanced by equol. In conclusion, our data suggest that ERRγ is involved in mediating the biological effects of equol.

2. Materials and methods

2.1. Plasmid constructions and site-directed mutagenesis

The pCMX-PL1 vector was kindly provided by Dr. Ronald M. Evans (Howard Hughes Medical Institute, The Salk Institute for Biological Studies, La Jolla, CA). pCMX-ERRα, pCMX-ERRβ, and pCMX-ERRγ expression plasmids encoding wild-type mouse ERRα, ERRβ and ERRγ, respectively were generous gifts from Dr. Vincent Giguère (McGill University Health Center, Montréal, Quebec, Canada). pCMX-FLAG-ERRγ, pCMX-Gal4–ERRγ LBD, pCMX-Gal4–ERRγ H1, and pCMX-VP16-ERRγ H3–12 expression plasmids have been described previously [24]. pM-GRIP1(563–1121) was a gift from Dr. Jorma Palvimo (University of Kuopio, Finland), pCI-Gal4-RIP140 from Dr. Malcolm Parker (Imperial College London, UK), and pCDNA3-hPGC-1α from Anastasia Kralli (Scripps Research Institute, La Jolla, CA). The luciferase reporters ($pERRE_3$tk-LUC and MH100tk-LUC) used in transfection experiments were provided by Dr. Ronald M. Evans and contained three copies of the ERRE or four copies of the Gal4 binding sites, respectively, cloned upstream of the herpes simplex virus thymidine kinase gene minimal promoter. To monitor transfection efficiency, pCMX-βgal (from Dr. Ronald M. Evans) was used as an internal control.

ERRγ mutants were generated using QuickChange® Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions using pCMX-Gal4–ERRγ LBD as the template. The numbering of the ERRγ mutants used in this study is according to the ERRγ2 isoform. The oligonucleotides used were:

A272F:	5′-CCACACTCTGTGACTTGTTTGACCGAGAGTTGGTGG-3′
V313W:	5′-GGAGATTCTGATCCTCGGCTGGGTGTACCGATCGC-3′
Y326W:	5′-GAGGATGAACTTGTCTGGGCAGACGATTATATAATGG-3′
F435L:	5′-GCCCATGCACAAACTTCTTTTGGAAATGCTGGAG-3′

The bases coding for the mutated residues are underlined. The bacterial colonies obtained after transformation were screened by direct sequencing.

2.2. Cell culture and reporter gene assay

The cell lines were from American Type Culture Collection. PC-3 cells were maintained in F-12 medium supplemented with penicillin (25 U/ml), streptomycin (25 U/ml), and 10% (v/v) FBS. U2-OS and SaOs-2 cells were maintained in DMEM supplemented with penicillin (25 U/ml), streptomycin (25 U/ml), 10% (v/v) FBS, and L-glutamine. For reporter gene assays, the cells were seeded on 12-well plates (6×10^4 cells). Twenty four hours later, the cells were transfected using the FuGENE or FuGENE HD reagent (Roche Molecular Biochemicals) with 300 ng of the luciferase reporter plasmid, 100 ng of the pCMX-βgal internal control plasmid, and 50 ng of the expression vectors or empty vector. Twenty hours after the transfection, the cells received fresh medium containing 2% charcoal-stripped FBS and supplemented with 4-hydroxytamoxifen (4-OHT; Sigma), diethylstilbestrol (DES; Sigma), GSK4716 (Sigma), 17β-estradiol (E2; Sigma), equol, or DMSO (vehicle) when indicated. Equol was synthesized from daidzein by hydrogenation on palladium on charcoal (10%) in 96% ethanol. The crude product was purified by recrystallisation from dry benzene; equol white crystals, purity >99% (gas chromatography). The cells were harvested 24 h later, lysed, and assayed for luciferase and β-galactosidase activities. Values were computed as fold inductions after normalization to β-galactosidase activities. All transfection experiments were performed in triplicate dishes and each experiment was repeated at least three times with essentially identical results. The mean ± SEM of three to six independent experiments is shown.

2.3. Western blotting

PC-3 cells (150,000 cells/well) were transfected on 6-well plates with 2 μg of pCMX-FLAG or pCMX-FLAG-ERRγ expression vector using FuGene transfection reagent. Twenty hours after the transfection, the cells were treated with vehicle (DMSO), 20 μM equol, 1 μM GSK4716, or 1 μM 4OHT. The cells were harvested 24 h later. Whole cell extracts were prepared in a buffer containing 50 mM Tris–HCl [pH 7.8], 150 mM NaCl, 5 mM EDTA, 15 mM $MgCl_2$, 0.5% Nonidet P-40, 0.3% Triton X-100, 1 mM ditiothreitol, and 1:200 diluted protease inhibitor cocktail (Roche Diagnostics). Proteins were resolved by electrophoresis on a 10% polyacrylamide gel under denaturing conditions. Proteins were then transferred onto Hybond enhanced chemiluminescence nitrocellulose membrane

(Amersham Biosciences) and blotted with anti-ERRγ antibody (1 μg/ml; Perseus Proteomics Inc.). Detection of lamin B was used as a control for equal loading (0.2 μg/ml; Lamin B C-20 antibody, Santa Cruz Biotechnology). Horseradish peroxidase-conjugated anti-mouse immunoglobulin G (1:2000; Zymed Laboratories) and donkey anti-goat IgG HRP (1:4000; Santa Cruz Biotechnology) were used as secondary antibodies. Immunocomplexes were visualized with ECL Western blotting detection reagents from Amersham Biosciences. To analyze the expression of Gal4–ERRγLBD fusions, PC-3 cell were transfected with 2 μg of the expression vector for the wild-type pCMX-Gal4–ERRγLBD or it's mutated variants (A272F, F435L, V313W, and Y326W) using the Lipofectamine 2000 reagent (Invitrogen). The cells were harvested 48 h later and Western blot was carried out as described above using the anti-Gal4 DBD RK5C1 antibody from Santa Cruz Biotechnology (0.28 μg/ml).

2.4. Partial proteolysis of ERRγ

FLAG-ERRγ protein was produced by coupled *in vitro* transcription and translation in reticulosyte lysates according to the manufacturer's instructions (TNT® Quick Coupled Transcription/Translation Systems; Promega). 4 μl of the [^{35}S]methionine-labeled translation mixture was incubated in the presence of 4 mM equol, 4 mM enterodiol, or 100 μM 4-OHT for 10 min. Enterodiol was synthesized from enterolactone by hydride reduction [25,26], purity >99% (gas chromatography). The ERRγ protein was digested by trypsin (30 μg/ml or 50 μg/ml; Sigma) at room temperature for 10 min. The reactions were stopped by the addition of SDS-polyacrylamide gel sample buffer. The digestion products were resolved on SDS-PAGE, the gels were dried and the radioactive peptides were visualized by autoradiography.

2.5. Cell growth assay

PC-3 cells (4.5×10^4 cells/well) were plated on 12-well plates, and transfected 24 h later with 300 ng of pCMX-FLAG-ERRγ or empty pCMX-FLAG vector using the FuGENE HD reagent. Five hours later, the cells received fresh medium containing 10% charcoal-stripped FBS supplemented with DMSO (vehicle), 20 μM equol, or 5 μM GSK4716 when indicated. 5 h or 72 h post transfection, cells were trypsinized and the cell numbers counted by Coulter® Particle Counter (Beckman Coulter). The experiment was performed in duplicate or triplicate dishes and repeated four times. The mean ± SEM of four independent experiments is shown. Overexpression of ERRγ was confirmed from Flag and Flag-ERRγ transfected cells by RT-PCR. Shortly, the total RNA was isolated using Trizol reagent (Invitrogen) following cDNA synthesis with Superscript II (Invitrogen). RT-PCR was performed with *Taq* DNA polymerase (GE Healthcare). PCRs were denatured at 92 °C for 2 min and then amplified at 92 °C for 60 s, 55 °C for 60 s, and 72 °C for 60 s for 40 cycles. The PCR products were fractionated on 1% agarose gel. The primer sequences used were for ERRγ (forward) 5′-ATACAGCTGCCCTGCCACGAAT-3′ and (reverse) 5′-CTGGCCAGCTTCATAATCCTG-3′ and for G3PDH (forward) 5′-ACCACAGTCCATGCCATCAC-3′ and (reverse) 5′-TCCACCACCCTGTTGCTGTA-3′.

For siRNA experiments, PC-3 cells were plated on 12-well plates (6×10^4 cells/well). 24 h later the cells were transfected with 30 pmoles of siRNA duplexes targeted for human ERRγ (target sequence 5′-AATGGCCATCAGAACGGACTG-3′; Qiagen) or non-targeting Luciferase GL2 control siRNA (Qiagen) using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's recommendations. Four hours later, the cells received fresh medium containing 10% charcoal-stripped FBS supplemented with DMSO (vehicle) or 20 μM equol. 4 h or 72 h post transfection, the cells were trypsinized and the cell numbers counted by Cedex XS semi-automated cell counter (Roche Innovatis AG). The experiment was performed in triplicate dishes and repeated four times. Quantitative real-time PCR was used to monitor the suppression of ERRγ mRNA expression. Shortly, the total RNA was isolated using Trizol reagent (Invitrogen) following cDNA synthesis with Superscript II (Invitrogen). RT-PCR was performed using the LightCycler 480 instrument and SYBR Green I (Roche Applied Science). The RT-PCRs were performed in duplicates for four independent experiments and the results were normalized to the respective expression levels of G3PDH. The primer sequences were for ERRγ (forward) 5′-GCCCAAGAGACTGTGTTTAGTG-3′ and (reverse) 5′-TGATTTCACATTCATTCGTGGCA-3′ and for G3PDH (forward) 5′-ACCACAGTCCATGCCATCAC-3′ and (reverse) 5′-TCCACCACCCTGTTGCTGTA-3′. The mean ± SEM of four independent experiments is shown.

2.6. Molecular modeling

Geometric analysis and superposition of ERRγ X-ray structures were performed with Maestro 7.0 (Schrödinger, LLC: Portland, OR). The *apo* structure (1KV6) and the GSK4716-bound agonistic structure of ERRγ (2GPP) were prepared for docking with the Protein Preparation workflow in Maestro. Equol was docked to both structures using the XP protocol implemented in the Glide 4.0 program (Schrödinger, LLC: Portland, OR). Subsequent energy minimization of the highest scoring docking pose resulted in the final model used for comparison and mutant design.

2.7. Statistical analysis

All experiments were performed in duplicate or triplicate dishes and each experiment was repeated at least three times. Values were compared to the basal or vehicle treated samples and computed as fold inductions. The mean ± SEM of three to six independent experiments is shown. The statistical significance of differences between two groups were assessed by two-tailed Student's *t* test, unless the reference group was standardized to a constant value (=1) with no variability. In this case, one-sample *t* test was used. When multiple *t* tests were performed, the *p*-values were corrected by Bonferroni correction. Multiple groups were compared by one-way ANOVA followed by Scheffe's post hoc test to determine the significant differences among groups. $p < 0.05$ was considered to represent a statistically significant difference. All the statistical analyses were carried out by SPSS software (version 15.0).

3. Results

3.1. The phytoestrogen equol stimulates the transcriptional activity of ERRγ

Certain phytoestrogens were recently reported to act as ERRα agonists [17]. We investigated whether phytoestrogens modulated also the transcriptional activity of ERRγ. The transcriptional activity of ERRγ varies in different cell lines. We have previously observed that ERRγ activates transcription efficiently in osteoblastic U2-OS cells [24] and in the prostate cancer cell line PC-3 (data not shown). We analyzed a selection of phytoestrogens for their potential agonistic or antagonistic activity on ERRγ by cotransfecting U2-OS and PC-3 cells with the expression vector encoding a fusion between the yeast Gal4 DNA-binding domain and the ERRγ LBD (Gal4–ERRγ LBD) along with a reporter gene regulated by four Gal4-binding sites (MH100tk-Luc). Treatment of the transfected cells with equol stimulated the transcriptional activity of the ERRγ LBD whereas two other isoflavones, genistein and daidzein, and two lignans, enterodiol and enterolactone, had no effect (Fig. 1A and data not

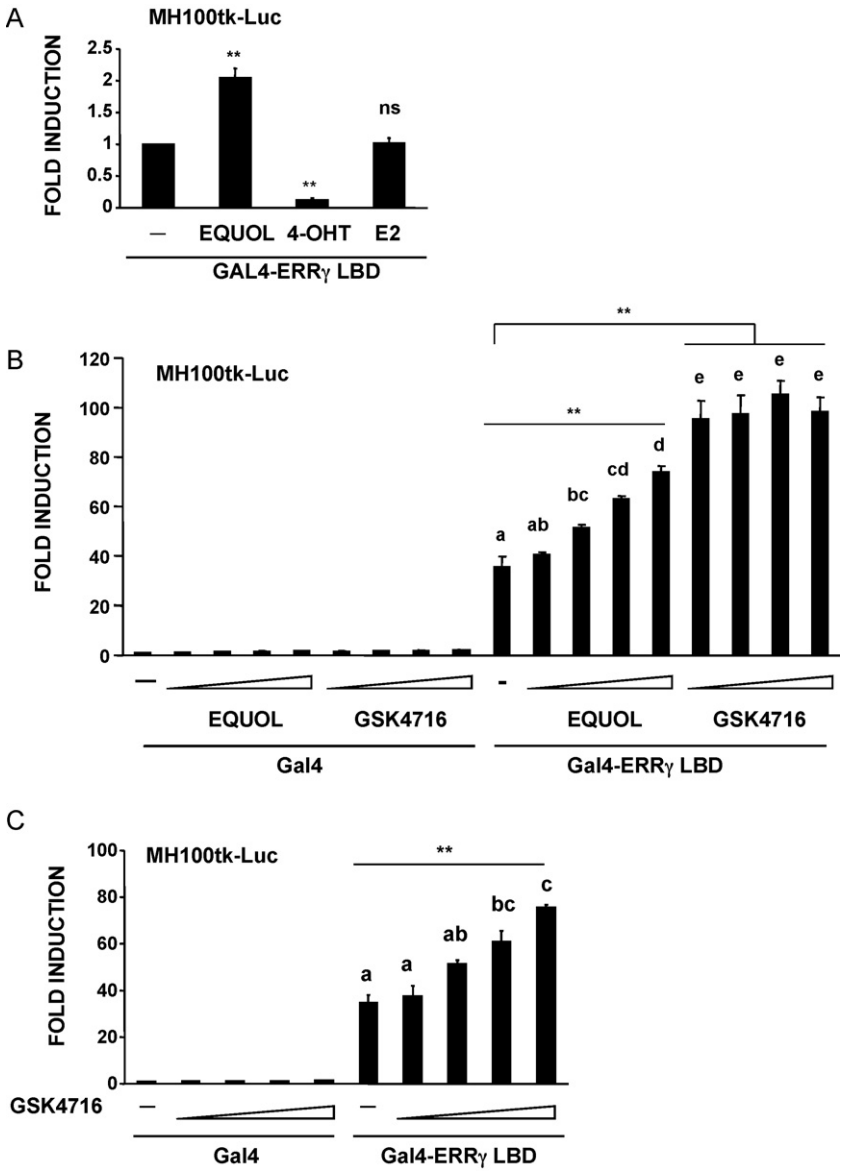

Fig. 1. The phytoestrogen equol stimulates the transcriptional activity of ERRγ.
(A) PC-3 cells were cotransfected with the expression vector for Gal4–ERRγ LBD along with the MH100tk-Luc reporter driven by four Gal4-binding sites. After transfection, the cells were treated with vehicle (DMSO), equol (20 μM), 4-OHT (1 μM), or E2 (10 nM) as indicated. *Asterisks* indicate statistically significant differences between the vehicle treated cells (–) and the cells treated with the ligands (**, $p < 0.01$; ns, non-significant, one-sample *t* tests with Bonferroni correction, $n = 5$). (B and C) PC-3 cells were cotransfected with the empty vector (Gal4) or the expression vector for Gal4–ERRγ LBD along with the MH100tk-Luc reporter. After transfection, the cells were treated with 1, 5, 10 and 20 μM of equol or the ERRγ agonist GSK4716 (B) or 0.01, 0.1, 0.5 and 1 μM of GSK4716 (C). *Asterisks* indicate statistically significant differences (**, $p < 0.01$, one-way ANOVA followed by Scheffe's multiple comparison tests, $n = 3$). Means without a common letter are significantly different ($p < 0.05$).

shown). As expected, 4-OHT inhibited the transcriptional activity of ERRγ whereas estradiol (E2) had no effect (Fig. 1A).

The agonistic potential of equol was compared to that of GSK4716 by cotransfecting PC-3 cells with the expression vector for Gal4–ERRγ LBD and the MH100tk-Luc reporter. The cells were subsequently treated with increasing concentrations (1, 5, 10, and 20 μM) of equol or GSK4716. Equol stimulated ERRγ-mediated transactivation in a dose-dependent manner and the maximum stimulation was 2 fold with the concentrations tested (Fig. 1B). GSK4716 stimulated ERRγ-mediated transactivation between 2.5–3 fold but showed dose-dependency at lower concentrations than equol (Fig. 1C).

Next, we examined whether equol exhibited selectivity for ERRγ over the other ERR family members. To address this PC-3 and SaOs-2 cells were cotransfected with the expression plasmids coding for the full-length ERRα, ERRβ, or ERRγ along with a reporter driven by three ERR response elements, ERREs ($ERRE_3$tk-Luc). The cells were subsequently treated with equol. Although ERRγ had high constitutive activity, equol stimulated ERRγ-mediated transactivation (Fig. 2A and B; $p < 0.05$). In contrast, ERRα and ERRβ failed to stimulate the reporter gene activity above the basal level measured in cells transfected with the empty vector. The response to equol was not different in the cells transfected with ERRα or ERRβ compared to the cells transfected with the empty vector (Fig. 2A and B). The stimulation in the basal reporter activity by equol treatment was most likely due to the endogenous ERRγ in PC-3 and SaOs-2 cells [[23], data not shown]. However, the effect of equol on ERRα and ERRβ could be different in an experimental design where these receptors were transcriptionally more active. To study this possibility, PC-3 cells were cotransfected with the expression vectors for the full-length ERRα, ERRβ, or ERRγ along with the expression vector for the coactivator PGC-1α and the $ERRE_3$tk-Luc reporter.

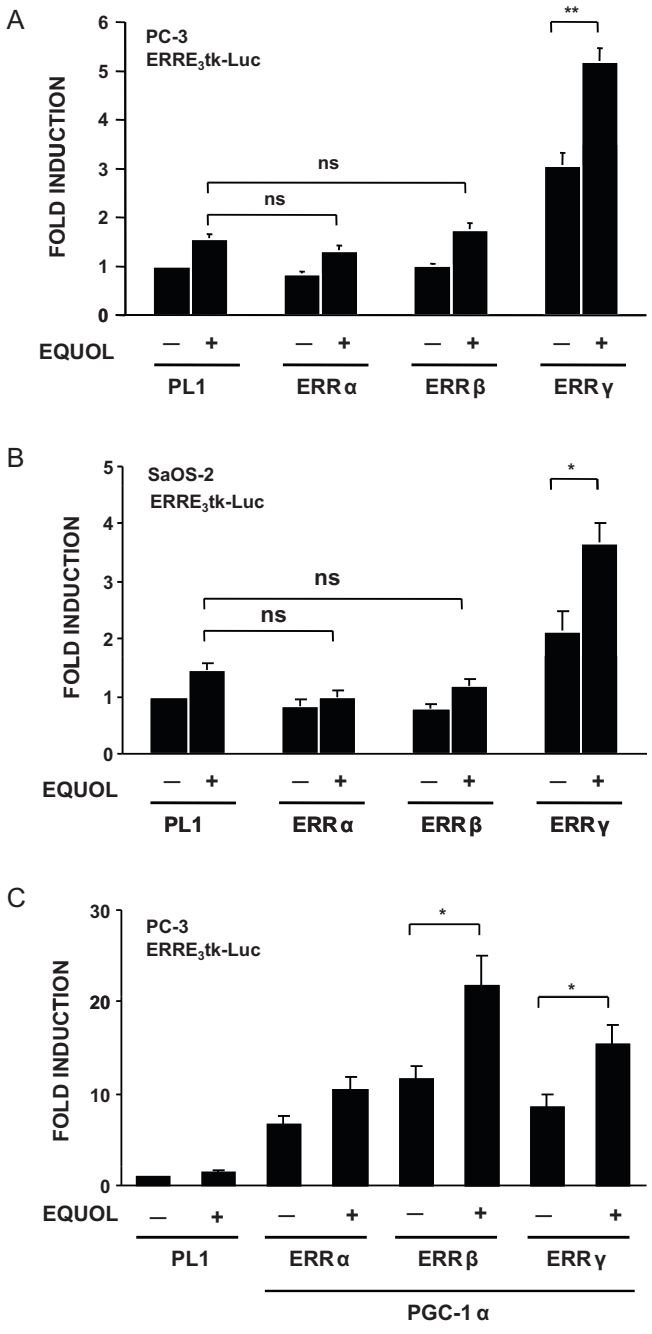

Fig. 2. The effect of equol on ERRα, ERRβ, and ERRγ.
(A) PC-3 and (B) SaOS-2 cells were transfected with empty vector (PL1) or the expression vectors for full-length ERRα, ERRβ, or ERRγ along with the $ERRE_3$tk-Luc reporter. The cells were subsequently treated with vehicle (DMSO) or equol (20 μM). *Asterisks* indicate statistically significant differences (*, $p < 0.05$; **, $p < 0.01$; ns, non-significant, Student's *t* test, $n = 3$). (C) PC-3 cells were transfected with empty vector (PL1) or the expression vectors for full-length ERRα, ERRβ, or ERRγ along with the expression vector for PGC-1α and $ERRE_3$tk-Luc reporter as indicated. The cells were subsequently treated with vehicle (DMSO) or equol (20 μM). *Asterisks* indicate statistically significant differences (*, $p < 0.05$, Student's *t* test, $n = 3$).

Under these experimental conditions, equol stimulated the transcriptional activity of both ERRγ and ERRβ whereas the modest stimulation of ERRα failed to reach statistical significance (Fig. 2C).

3.2. The stimulatory effect of equol on ERRγ is abolished by 4-OHT

The constitutive transcriptional activity of ERRγ is inhibited upon binding of 4-OHT [11,12]. We examined whether 4-OHT was

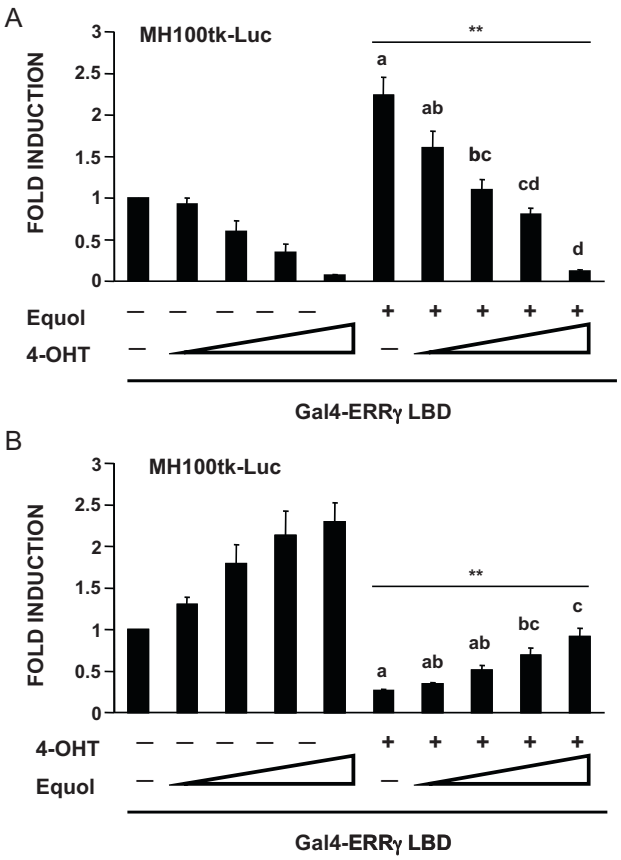

Fig. 3. The agonistic effect of equol on ERRγ is abolished by 4-OHT.
PC-3 cells were cotransfected with the expression vector for Gal4–ERRγ LBD along with the MH100tk-Luc reporter. (A) To examine the ability of 4-OHT to abolish the agonistic effect of equol on ERRγ, the cells were treated with increasing doses of 4-OHT (10, 50, 100 or 1000 nM) alone or in combination with 20 μM equol as indicated. (B) The ability of equol to prevent 4-OHT from inhibiting the transcriptional activity of ERRγ was examined by treating the cells with increasing doses of equol (1, 5, 10 or 20 μM) alone or in combination with 100 nM 4-OHT as indicated. *Asterisks* indicate statistically significant differences (**, $p < 0.01$, one-way ANOVA followed by Scheffe's multiple comparison tests, $n = 3$). Means without a common letter are significantly different ($p < 0.05$).

able to block the agonistic effect of equol on ERRγ. To that end, PC-3 cells were cotransfected with the expression vector for Gal4–ERRγ LBD together with the MH100tk-Luc reporter. First, the cells were treated with a constant concentration of equol along with increasing amounts of 4-OHT. As previously reported [11], 4-OHT inhibited the transcriptional activity of the ERRγ LBD (Fig. 3A). In addition, the agonistic effect of equol was inhibited by increasing doses of 4-OHT ($p < 0.01$). To address if equol could neutralize the inhibitory effect of 4-OHT, cells cotransfected with Gal4–ERRγ LBD and MH100tk-LUC were treated with a constant concentration of 4-OHT together with increasing concentrations of equol. Equol was able to abolish the antagonistic effect of 4-OHT (Fig. 3B; $p < 0.01$). Equol and 4-OHT thus abolish each others' effects on the activity of the ERRγ LBD implying that these ligands compete for binding to ERRγ.

3.3. Equol enhances the interaction between ERRγ and the coactivator GRIP1

Next, we studied the mechanism of equol action on ERRγ. One possibility could be that equol binding stabilizes the ERRγ protein and thereby enhances the transcriptional activity of ERRγ. To study this, PC-3 cells were transfected with the expression vector for ERRγ. The cells were subsequently treated with vehicle, equol, GSK4716, or 4-OHT and the amount of immunoreactive ERRγ pro-

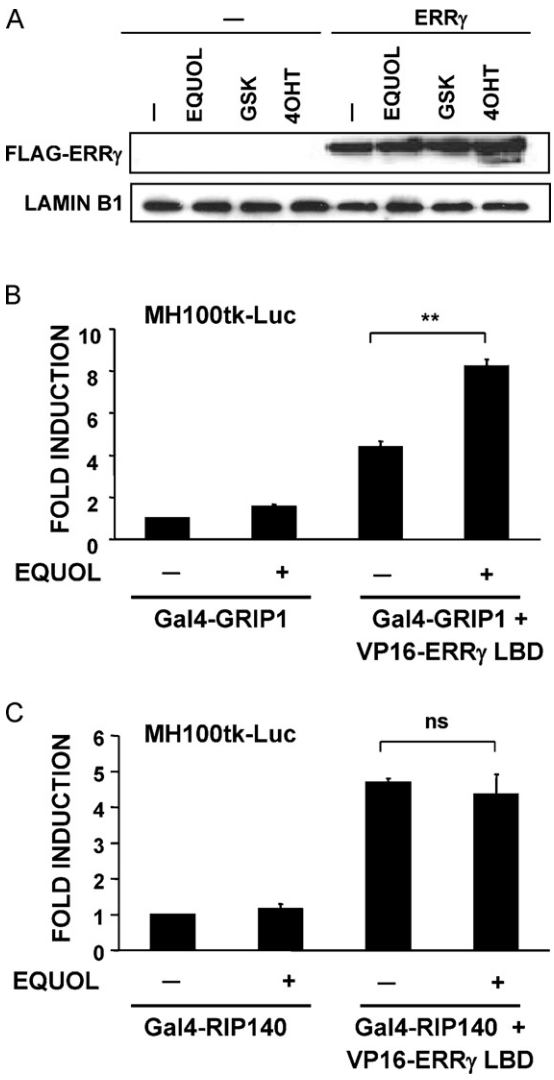

Fig. 4. Equol stimulates the interaction between ERRγ and the coactivator GRIP1. (A) PC-3 cells were transfected with an empty vector (–) or with the expression vector for FLAG-ERRγ. The cells were subsequently treated with vehicle (–), 20 μM equol, 1 μM GSK4716 (GSK), or 1 μM 4-OHT for 20 h. Immunoblot analysis of whole cell extracts was performed using anti-ERRγ and anti-Lamin B antibodies. (B) PC-3 cells were cotransfected with the expression vectors for Gal4-GRIP1 and VP16-ERRγ LBD along with the MH100tk-Luc reporter. The cells were subsequently treated with vehicle (–) or 20 μM equol. (C) PC-3 cells were cotransfected with the expression vectors for Gal4-RIP140 and VP16-ERRγ LBD along with the MH100tk-Luc reporter. The cells were subsequently treated with vehicle (–) or 20 μM equol. *Asterisks* indicate statistically significant differences (**, $p < 0.01$; ns, non-significant, Student's *t* test, $n = 3$).

tein was then analyzed in whole cell extracts. Treatment of the cells with 4-OHT slightly increased ERRγ expression whereas treatment with equol or GSK4716 did not clearly alter the expression level of ERRγ (Fig. 4A). This indicates that stabilization of the ERRγ protein is not the mechanism by which equol stimulates ERRγ-mediated transactivation.

Transcriptional activation by nuclear receptors involves interactions with transcriptional coactivator proteins [27]. ERRγ has been reported to interact *via* its AF-2 domain with the glucocorticoid receptor interacting protein 1 (GRIP1) [28]. To analyze whether equol enhanced the interaction between the ERRγ LBD and GRIP1, PC-3 cells were cotransfected with the expression vectors for Gal4-GRIP1 and VP16-ERRγ LBD along with the MH100tk-Luc reporter. The Gal4-GRIP1 plasmid codes for the GRIP1 residues 563–1121 that have previously been shown to interact with ERRγ [28]. The ERRγ LBD interacted with GRIP1 efficiently in the absence of any ligand and treatment of the cells with equol enhanced this interaction (Fig. 4B; $p < 0.01$). ERRγ has also been shown to interact with RIP140, a cofactor that interacts with several nuclear receptors in a ligand-dependent manner and acts in many cases as a transrepressor [14,29]. When cotransfected in PC-3 cells, RIP140 interacted with the ERRγ LBD but treatment of the cells with equol did not modulate this interaction (Fig. 4C). Equol binding thus increases the interaction between the ERRγ LBD and the coactivator GRIP1 but has no influence on the interaction between the ERRγ LBD and RIP140.

3.4. Equol binding induces a conformational change in the ERRγ LBD

Next, we wanted to assess whether equol binding induced a conformational change in the ERRγ LBD. This was studied using the assembly assay described by Pissios et al. [30]. In this assay, helix 1 of the nuclear receptor LBD is fused to the yeast Gal4 DNA-binding domain (Gal4-H1) and the remainder of the LBD, helices 3–12, is fused to the herpes simplex virus VP16 activation domain (VP16-H3–12). The efficiency of the interaction between H1 and H3–12 is measured as activation of a reporter gene driven by Gal4-binding sites. Ligand binding, corepressors, and other signals modulating the transcriptional activity of the LBD have been reported to influence this interaction [24,30,31]. When the assembly assay with the ERRγ LBD fragments was performed in PC-3 cells, interaction between Gal4-H1 and VP16-H3–12 was increased when the cells were treated with equol, GSK4716, DES, or 4-OHT (Fig. 5A; $p < 0.05$). This indicates that binding of these ligands induces a conformational change in the ERRγ LBD. GSK4716 was the most efficient ligand in this assay. As expected, E2 and enterodiol had no effect (Fig. 5A and B).

Partial proteolysis experiments were performed to address the conformational change *in vitro*. [^{32}S]Methionine-labeled full-length ERRγ was produced by coupled *in vitro* transcription and translation and subsequently incubated with vehicle, equol, enterodiol, or 4-OHT. After digestion with trypsin the reaction products were analyzed on SDS-polyacrylamide gels. Digestion of ERRγ in the presence of vehicle or enterodiol was complete (Fig. 5C). This is in line with the failure of enterodiol to modulate ERRγ-mediated transactivation in our reporter gene assays or to stimulate the interaction between H1 and H3–12 in the assembly assay (data not shown and Fig. 5B). In contrast, digestion in the presence of equol or 4-OHT yielded an approximately 48-kDa resistant fragment (Fig. 5C). Similar results were obtained when the ERRγ LBD was used in these experiments indicating that the protective effect of equol is mediated through the LBD (data not shown). In summary, these data imply that equol binds to and induces a conformational change in the ERRγ LBD.

3.5. Mutations in the ERRγ LBD modulate the response to equol and other ligands

The binding of equol in the ERRγ ligand-binding pocket was examined by generating a computer model of the ERRγ LBD in complex with equol and comparing it to X-ray structures of ERRγ with other ligands. 4-OHT and diethylstilbestrol (DES) act as inverse agonists by destabilizing the agonistic conformation of H12 in the ERRγ LBD (Fig. 6A and B) [13,14]. The ERRγ agonist GSK4716 binds to an enlarged cavity compared to the putative ligand-binding pocket in the unliganded structure (Fig. 6C) [14]. Here H12 is in its agonistic conformation. We docked equol in the *apo*-ERRγ structure and in the agonistic conformation induced by GSK4716. The *apo*-structure could not adopt the equol molecule whereas the larger pocket observed in the ERRγ–GSK4716 complex allowed equol binding in

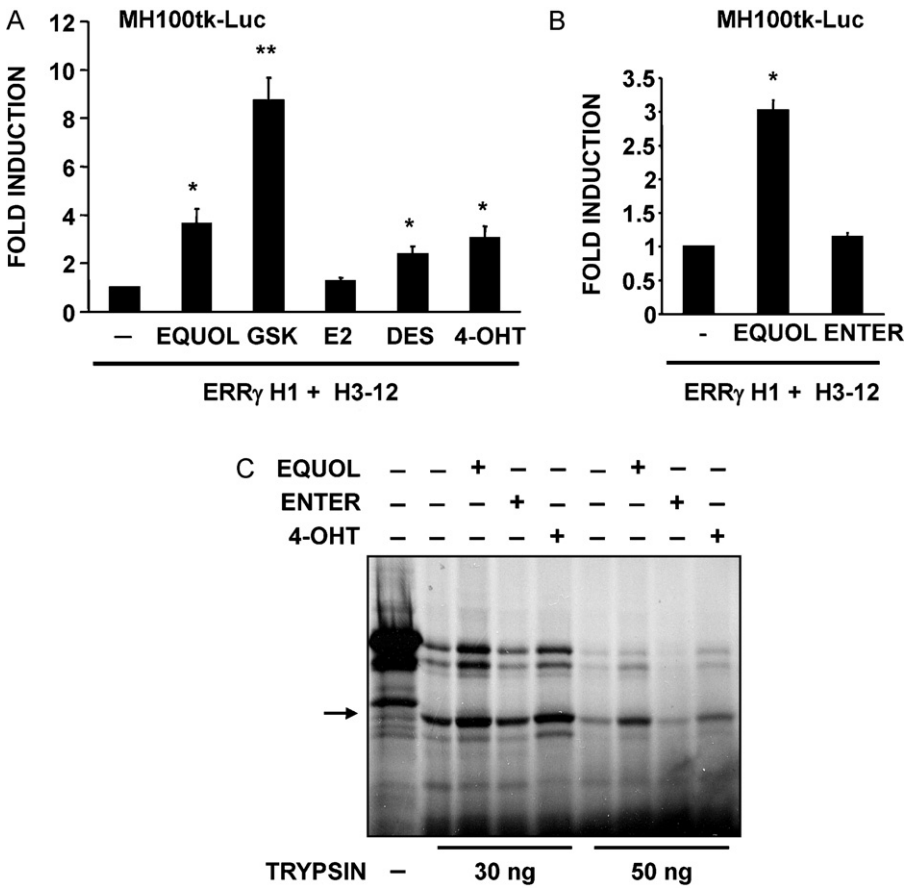

Fig. 5. Equol induces a conformational change in the ligand-binding domain of ERRγ.
(A and B) PC-3 cells were cotransfected with the expression vector for Gal4–ERRγ H1 along with VP16-ERRγ H3-12 and the MH100tk-Luc reporter. After transfection, the cells were treated with vehicle (DMSO), 20 μM equol, 1 μM GSK4716, 10 nM estradiol (E2), 10 μM DES, 1 μM 4-OHT or 20 μM enterodiol as indicated. *Asterisks* indicate statistically significant differences between the vehicle treated cells (–) and the cells treated with the ligands [*, $p < 0.05$; **, $p < 0.01$, one-sample *t* tests with Bonferroni correction, for figure (A) $n = 6$, for figure (B) $n = 3$]. (C) The ERRγ protein was produced by coupled *in vitro* transcription and translation in reticulocyte lysate. 4 μl of the translation mixture was incubated with vehicle (DMSO), 4 mM equol, 4 mM enterodiol or 100 μM 4-OHT and digested with 30 ng or 50 ng of trypsin as indicated. The digestion products were analyzed on SDS-PAGE and visualized by autoradiography. The protected fragment (48 kDa) is depicted by an arrow.

an agonistic conformation of the ERRγ LBD (Fig. 6D). This binding mode correlates well with the agonistic effects of equol observed in our experiments.

The binding mode of equol to ERRγ was further examined by introducing several point mutations in the ligand-binding pocket based on our computer model of the ERRγ LBD in complex with equol. The effects of the ligand-binding domain mutations on the responses to the different ligands were examined using reporter gene assays. PC-3 cells were cotransfected with the Gal4–ERRγ LBD expression vector or its mutated variants along with the MH100tk-Luc reporter. Subsequently, the cells were treated with equol, GSK4716, 4-OHT, or DES. The X-ray structures of the ERRγ LBD in complex with GSK4716, 4-OHT, or DES were then compared to our model of the ERRγ LBD in complex with equol in order to elude the structural basis of the effects that the different mutations had on the responses to these ligands. All the ERRγ LBD mutants were expressed at levels similar to or slightly higher than the wild-type ERRγ LBD (Fig. 7A). All the mutants remained constitutively active (Fig. 7B).

First, A272 which is in close proximity to all four tested ligands was mutated with the intention to block the binding cavity. A272F did not significantly respond to any of the tested ligands (Fig. 7D).

F435 was mutated to a smaller leucine in order to create more space in the binding pocket. The antagonistic effect of DES on ERRγ has been shown to involve a change in the conformation of F435 which then leads to displacement of H12. Mutating F435 to leucine changed DES to a weak agonist (Fig. 7E; $p < 0.05$). When the F435L mutation was studied in the assembly assay by introducing this mutation in the VP16-ERRγ H3–12 construct, DES stimulated the interaction between H1 and H3–12 very efficiently (Fig. 7F; $p < 0.01$). 4-OHT that has been shown to displace H12 directly with its basic side chain still had an antagonistic effect on the F435L mutant (Fig. 7E; $p < 0.01$). Equol and GSK4716 maintained their agonistic effects ($p < 0.01$ and $p < 0.05$, respectively). When the F435L mutation was studied in the assembly assay, equol was able to efficiently enhance the interaction between H1 and H3–12 (Fig. 7F; $p < 0.05$).

V313 is in close contact with both equol and GSK4716 but makes little contacts with 4-OHT and DES (Fig. 6). When a larger tryptophan was substituted for V313 the effect of GSK4716, 4-OHT, and DES on the transcriptional activity of ERRγ was abolished whereas equol acted in a similar way as with the wild-type ERRγ (Fig. 7G).

GSK4716 forms a hydrogen bond with the backbone of the highly conserved Y326 and has lipophilic interactions with its side chain (Fig. 6) [14]. Mutating Y326 to tryptophan reduced the agonistic effect of GSK4716 and also abolished the antagonistic effects of 4-OHT and DES (Fig. 7H). In contrast, equol remained as an agonist ($p < 0.05$).

In summary, the mutations in the ligand-binding pocket influenced the ability of equol to modulate the activity of ERRγ in

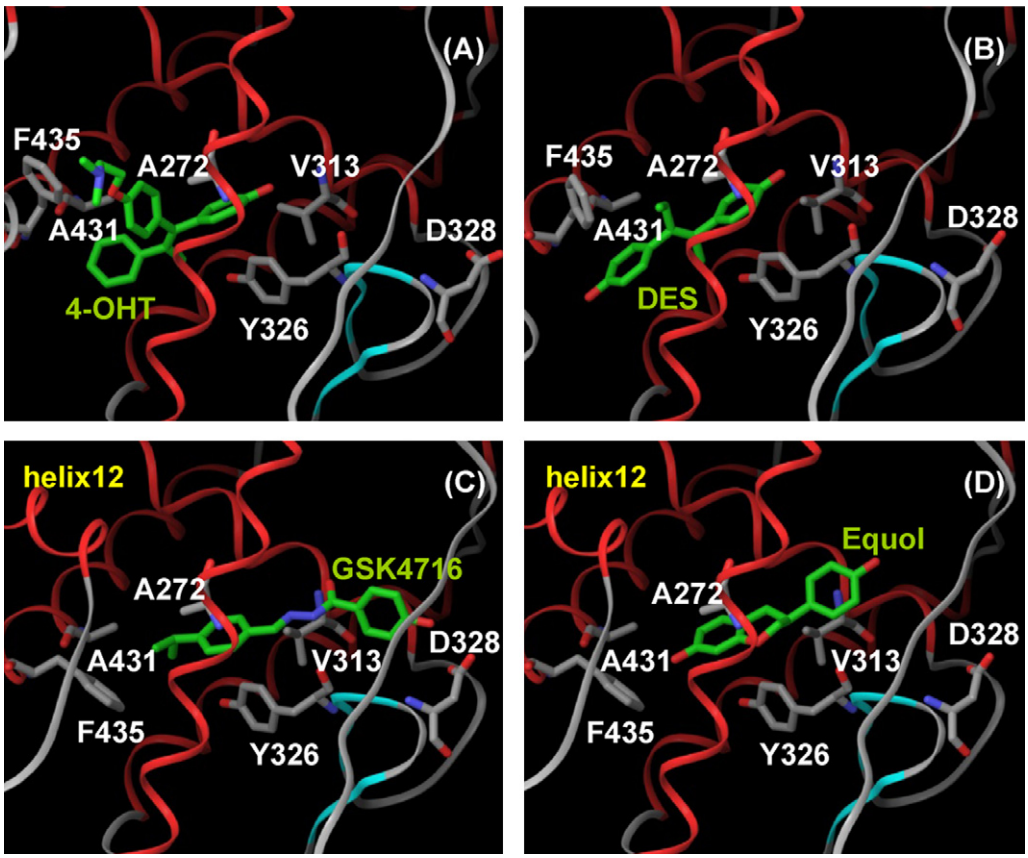

Fig. 6. The ligand-binding pocket of ERRγ with different ligands.
Only side chains of the residues subjected to mutation in the present study are shown. All structures have been superimposed so that the view is similar. (A) 4-hydroxytamoxifen (4-OHT; PDB code 2GPU), (B) diethylstilbestrol (DES; PDB code 1S9P), (C) GSK4716 (PDB code 2GPP), and (D) equol docked into the structure 2GPP.

a different fashion compared to GSK4716, 4-OHT, and DES. This, together with our structural model, indicates that equol binds to the ERRγ ligand-binding pocket in a slightly different manner than the other ligands.

3.6. The antiproliferative effect of ERRγ on prostate cancer cells is enhanced by equol

Next we wanted to study the agonistic effect of equol on ERRγ function in PC-3 cells. To identify an ERRγ target gene in PC-3 cells that could be used as a measure of ERRγ signaling, we treated PC-3 cells with the ERRγ agonist GSK4716 or transfected the cells with siRNA targeted for ERRγ. The expression of several potential ERRγ target genes was then analyzed by quantitative RT-PCR (ERRα, PGC-1α, PGC-1β, p21, p27, PDK2, PDK4, glucocorticoid receptor, Plk2, MAO-B, ApoD) [32–39]. To our surprise, none of these genes were induced by GSK4716 or repressed by ERRγ siRNA in PC-3 cells (data not shown). We were thus unable to study the role of equol as an ERRγ agonist by analysing the regulation of these previously suggested ERRγ target genes.

Both equol and ERRγ have been reported to inhibit the growth of human prostate cancer cells *in vitro* [23,40,41]. Therefore, we examined whether equol was able to further enhance the growth inhibitory effect of ERRγ. To that end, PC-3 cells were transfected with the expression vector for ERRγ and 5 h later, the cells were treated with equol. PC-3 cells express endogenous ERRγ and the expression level of ERRγ mRNA was substantially increased upon ERRγ overexpression (Fig. 8A). Cells transfected with the empty vector proliferated significantly between 5 and 72 h post transfection (Fig. 8; $p < 0.01$). Treatment of the mock-transfected cells with equol reduced the cell number 72 h post transfection and the number of cells was further diminished when the cells were transfected with the expression vector for ERRγ (Fig. 8A; $p < 0.01$). To study if the growth inhibitory effect of equol in mock-transfected cells was mediated by the endogenous ERRγ in PC-3 cells, we transfected the cells with siRNA targeted for ERRγ. This reduced the expression of endogenous ERRγ mRNA to about 50% and abolished the growth-inhibitory effect of equol (Fig. 8B). These results indicate that ERRγ is involved in mediating the antiproliferative effects of equol. GSK4716 also reduced the number of the PC-3 cells when the cells were transfected with the ERRγ vector (Fig. 8C; $p < 0.05$).

4. Discussion

A major step in defining the biological functions of an orphan nuclear receptor is the identification of ligands that can be used to modulate its activity in cell cultures and *in vivo*. Identification of physiological orphan receptor ligands provides also information about the signaling pathways the receptor is involved in. Recently, several synthetic ERR ligands have been identified. Studies utilizing the synthetic ERRα inverse agonist XCT790 facilitated the elucidation of the role of ERRα as a regulator of oxidative phosphorylation [42,43]. Synthetic ligands modulating the activities of ERRβ and ERRγ have been recently identified but the biological functions of these receptors are not as well characterized. The identified ERRγ ligands such as 4-OHT and DES imply, however, that ERRγ potentially impinges on estrogen signaling pathways. In addition, the endocrine disruptor bisphenol A (BPA) which is an estrogenic chemical with only a weak binding ability to ERs was recently reported to bind to ERRγ and prevent 4-OHT from antagonizing the intrinsic transcriptional activity of ERRγ [44]. Most of the iden-

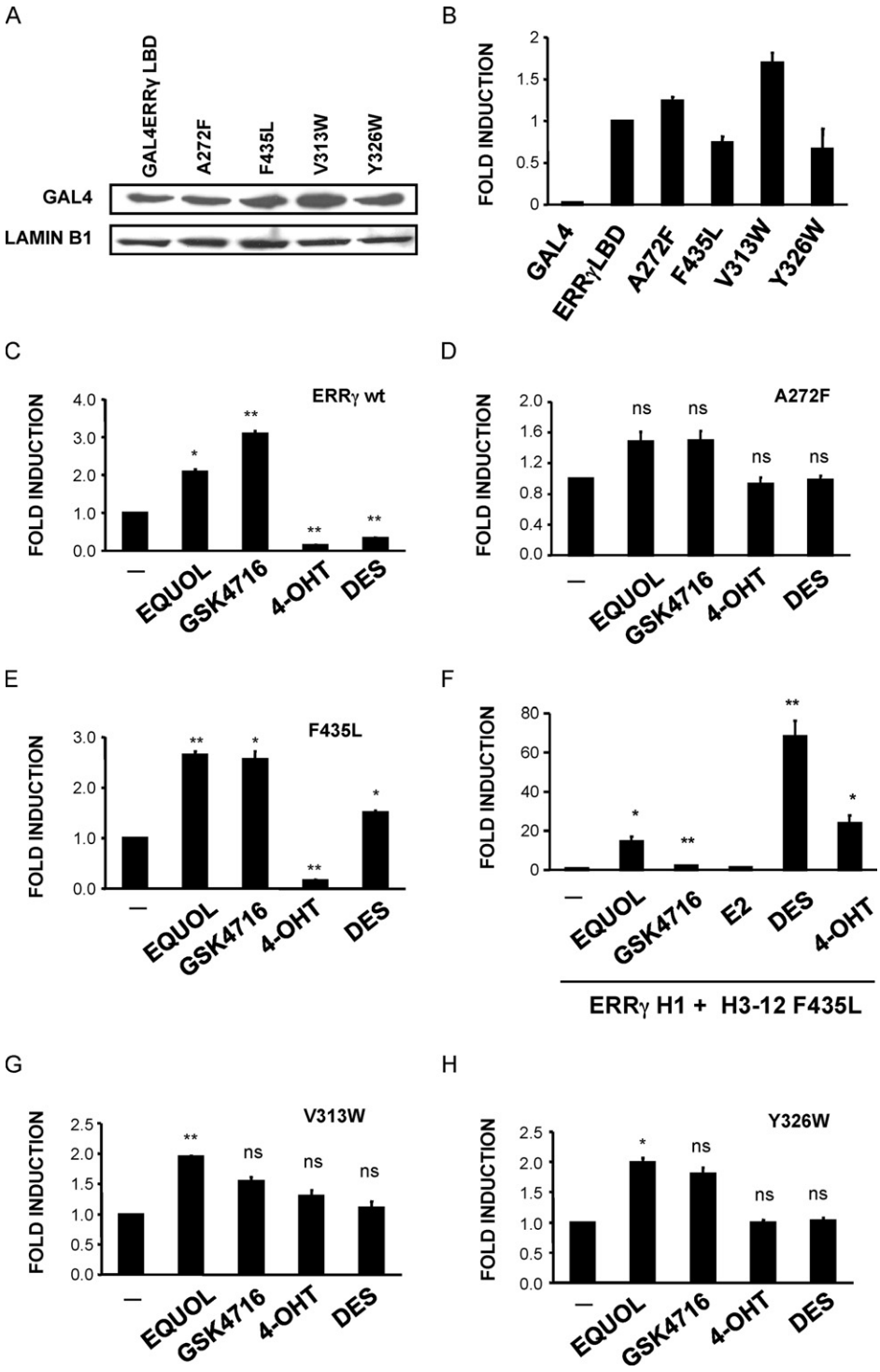

Fig. 7. The agonistic effect of equol is modified by mutations in the ERRγ ligand-binding pocket.
(A) PC-3 cells were transfected with the expression vectors for Gal4–ERRγ LBD or its mutated variants. The cells were harvested 48 h later and immunoblot analysis of whole cell extracts was performed using anti-Gal4 and anti-Lamin B antibodies. (B) PC-3 cells were transfected with the MH100tk-Luc reporter and the expression vectors for Gal4–ERRγ LBD or its mutated variants as indicated. The differences in the reporter gene activation elicited by the wild-type Gal4–ERRγ LBD and the mutants did not reach statistical significance (one-sample *t* tests with Bonferroni corrections, $n = 3$). (C–E) PC-3 cells were transfected with the MH100tk-Luc reporter and the expression vector for Gal4–ERRγ LBD or its mutated variants as indicated. The cells were subsequently treated with vehicle (–), 20 μM equol, 1 μM GSK4716, 1 μM 4-OHT, or 10 μM DES as indicated. *Asterisks* indicate statistically significant differences between the vehicle treated cells (–) and the cells treated with the ligands (*, $p < 0.05$; **, $p < 0.01$; ns, non-significant, one-sample *t* tests with Bonferroni corrections, $n = 3$). (F) PC-3 cells were cotransfected with the expression vector for Gal4–ERRγ H1 along with VP16-ERRγ H3-12 F435L and the MH100tk-Luc reporter. After transfection, the cells were treated with vehicle (DMSO), 20 μM equol, 1 μM GSK4716, 10 nM estradiol (E2), 10 μM DES, or 1 μM 4-OHT as indicated. *Asterisks* indicate statistically significant differences between the vehicle treated cells (–) and the cells treated with the ligands (*, $p < 0.05$; **, $p < 0.01$, one-sample *t* tests with Bonferroni correction, $n = 6$). (G, H) PC-3 cells were transfected with the MH100tk-Luc reporter and the expression vector for the indicated Gal4–ERRγ LBD variants. The cells were subsequently treated with vehicle (–), 20 μM equol, 1 μM GSK4716, 1 μM 4-OHT, or 10 μM DES as indicated. *Asterisks* indicate statistically significant differences between the vehicle treated cells (–) and the cells treated with the ligands (*, $p < 0.05$; **, $p < 0.01$; ns, non-significant, one-sample *t* tests with Bonferroni corrections, $n = 3$).

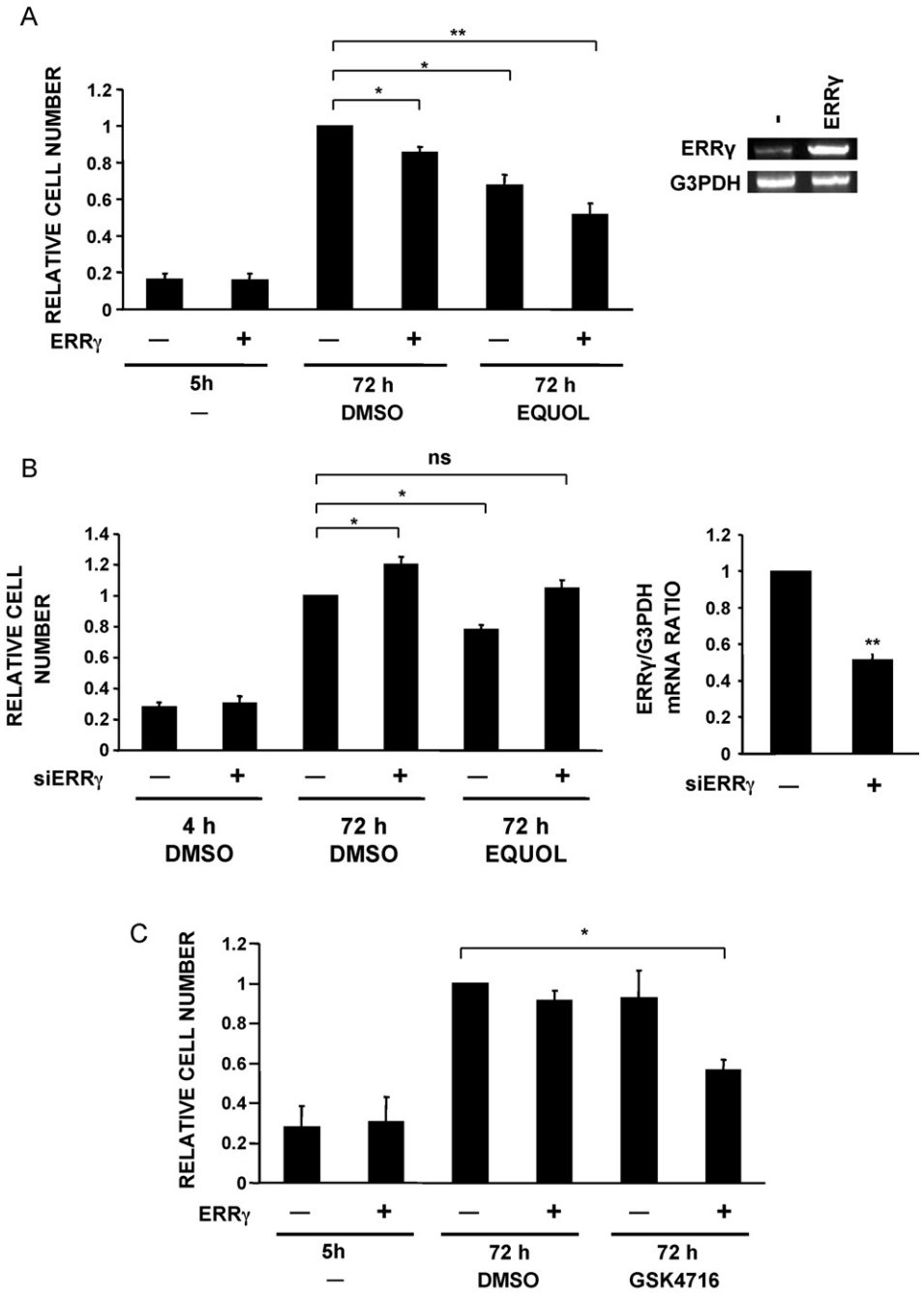

Fig. 8. The effect of equol and ERRγ on the proliferation of the PC-3 prostate cancer cells.
PC-3 cells were transfected with the expression vector for ERRγ and subsequently treated with vehicle (DMSO), 20 μM equol (A) or 5 μM GSK4716 (C) as indicated. The expression of endogenous and transfected ERRγ was analyzed by RT-PCR. The cell numbers were counted 5 and 72 h post transfection. *Asterisks* indicate statistically significant differences (*, $p < 0.05$; **, $p < 0.01$, one-sample *t* tests with Bonferroni corrections, $n = 4$). (B) PC-3 cells were transfected with siRNA targeted for ERRγ or with control siRNA and subsequently treated with vehicle (DMSO) or 20 μM equol as indicated. The cell numbers were counted 4 and 72 h post transfection. *Asterisks* indicate statistically significant differences (*, $p < 0.05$; ns, non-significant, one-sample *t* tests with Bonferroni corrections, $n = 4$). The effect of siRNA treatment on ERRγ mRNA expression was studied from control (−) and ERRγ siRNA transfected PC-3 cells by quantitative RT-PCR. The levels of ERRγ mRNA were normalized with respect to the corresponding expression levels of G3PDH. *Asterisk* indicates statistically significant difference (**, $p < 0.01$, one-sample *t* test, $n = 4$).

tified ERR ligands act as inverse agonists. However, the activities of ERRs can also be stimulated by ligand binding as indicated by the identification of the synthetic ERRβ/γ agonists GSK4716 and DY131 [14,16,45].

The best-documented biological actions of phytoestrogens are mediated by ERs [18]. Some isoflavonoids modulate also steroid metabolism for example by inhibiting the key steroidogenic enzymes such as 5α-reductase, 17β-hydroxysteroid oxidoreductase, and aromatase [46–48]. Phytoestrogens have been reported to act as ligands for also other nuclear receptors than ERs such as PPARγ and ERRα [17,49]. In addition to their hormonal effects, phytoestrogens have many other actions including antioxidant activity and inhibition of tyrosine kinases and topoisomerase [50–52].

We report here that the transcriptional activity of ERRγ is stimulated by the isoflavan equol. In contrast, ERRα and ERRβ failed to transactivate the $ERRE_3$tk-Luc reporter in PC-3 or SaOs-2 cells and equol did not add to their transcriptional activity (Fig. 2A and B). The intrinsic transcriptional activity of ERRα and ERRβ is very

low in many cell lines most likely due to differential expression of transcriptional coactivator proteins in different cell lines [24,53]. When PC-3 cells were cotransfected with the expression vectors for ERRs and the coactivator PGC-1α, all three ERRs transactivated the $ERRE_3$tk-Luc reporter efficiently. However, equol was able to further enhance the activity ERRγ and ERRβ but not that of ERRα (Fig. 2C). These results suggest that equol is selective for ERRγ and ERRβ as has been reported for GSK4716 [16].

Structural studies and site-directed mutagenesis experiments were performed to examine the binding modes of equol, GSK4716, 4-OHT, and DES to ERRγ. The effect of the different mutants on ligand-responsiveness was studied using reporter gene assays. All the tested mutants maintained constitutive transcriptional activity indicating that they were able to adopt an active conformation and to interact with transcriptional cofactor proteins despite the mutations (Fig. 7B).

Enlarging the ligand-binding pocket by reduction of the size of the residue F435 switched DES to an agonist indicating that F435 is involved in mediating the antagonistic effect of DES (Fig. 7E) [13]. The conformation of the smaller side chain of L435 was either not altered by DES or was too small to displace H12 from its agonistic conformation. Diminishing the size of the ligand-binding pocket by enlarging the pocket-forming residue A272 reduced the effect of all the ligands (Fig. 7D). However, introduction of an additional large side chain to position 431 in order to decrease the size of the binding pocket even more did not show stronger effects than the single mutant (data not shown). This indicates that binding to the pocket might be completely blocked with the single mutant and that the residual increase of activity induced by equol and GSK4716 probably originates from effects not related to the pocket. Activation *via* residues outside the classical ligand-binding pocket has been described for mineralocorticoid receptor [54]. However, this possibility remains to be addressed as a mutation (D328A) in the corresponding region of ERRγ did not abolish the agonistic effect of equol (data not shown). Reducing the size of the ligand-binding pocket by mutating V313 to a larger tryptophan did not influence equol responsiveness (Fig. 7G). Based on the structural models it seems plausible that this mutation creates unfavorable interactions with the phenolic group in 4-OHT and DES thus reducing the effects of these ligands. In contrast, equol could make stacking interactions with the indole of W313 and therefore maintains its agonistic effect. Y326 is highly conserved in several nuclear receptors and is located within the ligand-binding pocket of ERRγ. Y326 belongs to an area whose correspondent residues in mineralocorticoid receptor seem to be involved in aldosterone binding and receptor activation [54]. Diminishing the size of the ligand-binding pocket by mutating Y326 reduced the effects of GSK4716, 4-OHT and DES but had no effect on equol (Fig. 7H). In summary, mutations in the ERRγ ligand-binding domain modified the response to equol in a slightly different fashion than the responses to the other tested ligands. Our structural model and data of the mutagenesis experiments thus indicate that equol binds to the ERRγ ligand-binding pocket in a slightly different manner than the other examined agonist GSK4716 and the inverse agonists 4-OHT and DES. However, further structural studies are required to confirm these modeling studies.

The concentration of equol required to stimulate the activity of ERRγ in our cell-based assays is fairly high. However, plasma concentrations of phytoestrogens in soya-consuming populations can reach levels as high as 1 μM and even higher levels can be achieved by single-dose administration of the purified compounds [55,56]. Furthermore, equol and some other phytoestrogens have been shown to reach much higher concentrations in for example prostate fluid than in plasma [57,58]. Modulation of the transcriptional activity of nuclear receptors in transient transactivation assays or modulation of nuclear receptor functions *in vitro* often require ligand concentrations that exceed those required to modulate the receptor functions *in vivo*. Based on these observations, the concentrations of equol may well be sufficient to stimulate ERRγ in humans.

Epidemiological investigations have indicated that phytoestrogens have many beneficial health effects such as a cancer-protective effect [18]. Several studies have indicated that equol possesses higher biological activity than its precursor compound daidzein [20]. According to our current study, equol enhances the transcriptional activity of ERRγ whereas daidzein has no effect. The higher bioactivity of equol could thus be at least partially explained by its ability to activate ERRγ in addition to ERs. Equol was recently shown to inhibit the growth of human breast and prostate cancer cells *in vitro* [40,41]. Only less than half of the human population is so called equol producers whose intestinal bacteria convert daidzein to equol [19]. The ability to produce equol and high serum equol levels has been associated with a reduced prostate cancer risk indicating an important role for equol in prostate cancer prevention [20]. ERRγ has been shown to suppress prostate cancer cell proliferation and tumor growth [23]. In addition, ERRγ has been indicated as a marker of favorable prognosis in human prostate cancer [59]. In this report, we have shown that the growth inhibitory effect of ERRγ on human prostate cancer cells is enhanced by equol (Fig. 8). The antiproliferative effects of equol on prostate and breast cancer cells may thus be at least partially mediated by ERRγ. In summary, our data demonstrate that equol acts as an ERRγ agonist and that at least some of the beneficial health effects of equol may be mediated by ERRγ.

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