Crystal Structure of the Human RORα Ligand Binding Domain in Complex with Cholesterol Sulfate at 2.2 Å*

Joerg Kallen§§, Jean-Marc Schlaepf†, Francis Bitsch‡, Isabelle Delboni¶, and Brigitte Fournier*†

From the Discovery Technologies, Protein Structure Unit, the Discovery Technologies, Biomolecules Production Unit, and the Arthritis and Bone Metabolism, Bone Metabolism Unit, Novartis Pharma AG, CH-4002 Basel, Switzerland

The retinoic acid-related orphan receptor α (RORA) is an orphan member of the subfamily 1 of nuclear hormone receptors. Our recent structural and functional studies have led to the hypothesis that cholesterol or a cholesterol derivative is the natural ligand of RORA. We have now solved the x-ray crystal structure of the ligand binding domain of RORα in complex with cholesterol-3-O-sulfate following a ligand exchange experiment. In contrast to the 3-hydroxyl of cholesterol, the 3-O-sulfate group makes additional direct hydrogen bonds with three residues of the RORα ligand binding domain, namely NH-Gln289, NH-Tyr290, and NH1-Arg370. When compared with the complex with cholesterol, seven well ordered water molecules have been displaced, and the ligand is slightly shifted toward the hydrophilic part of the ligand binding pocket, which is ideally suited for interactions with a sulfate group. These additional ligand-protein interactions result in an increased affinity of cholesterol sulfate when compared with cholesterol, as shown by mass spectrometry analysis done under native conditions and differential scanning calorimetry. Moreover, mutational studies show that the higher binding affinity of cholesterol sulfate translates into an increased transcriptional activity of RORα. Our findings suggest that cholesterol sulfate could play a crucial role in the regulation of RORα in vivo.

The group of retinoic acid-related orphan nuclear receptors (RORs) is encoded by three different genes (α, β, and γ) (1). RORA has been implicated in numerous age-related phenotypes such as atherosclerosis, cerebellar atrophy, immunodeficiency, and bone metabolism (2). RORα was still considered an orphan receptor until we recently reported the first crystal structure of the RORα LBD. It had revealed a ligand that was unexpectedly present, namely cholesterol (3). We also had shown that the transcriptional activity of RORα could be modulated by changes in intracellular cholesterol level or mutation of residues involved in cholesterol binding. This has led to the hypothesis that RORα could play a key role in the regulation of cholesterol homeostasis and thus represents an important drug target in cholesterol-related diseases. Despite the relatively high homology between RORα LBD and RORβ LBD (63%), cholesterol seems not to be a ligand for the RORβ isoform, as reported recently by Stehlin-Gaon et al. (4). This indicates a possible distinct function for RORβ and RORα. An inspection of the x-ray structure of the complex between RORα LBD and cholesterol had shown that in the hydrophilic part of the LBP, there is space for a substituent attached to the hydroxy group of cholesterol, if water molecules are displaced (3). The presence of three arginines (Arg392, Arg379, and Arg367) and of two free backbone amide nitrogens (NH-Gln289 and NH-Tyr290) strongly suggested a negatively charged substituent with at least two hydrogen-bond acceptor functionalities. Docking studies led to the prediction that cholesterol sulfate should have higher affinity than cholesterol, and might be, because of its excellent fit and optimized interactions, the actual natural ligand of RORα (instead of cholesterol itself). Here, we present the x-ray crystal structure of the RORα LBD-cholesterol sulfate complex. We show a comparison with the x-ray structure of the RORα LBD-cholesterol complex, which suggests that cholesterol sulfate has a higher affinity than cholesterol. Indeed, as shown by mass spectrometry analysis, the exchange of cholesterol with cholesterol sulfate is practically irreversible under the conditions used. In addition, DSC analysis revealed that cholesterol sulfate increased the phase transition temperature for RORα LBD by 9 °C, relative to cholesterol. We also showed that cholesterol sulfate shows increased (versus cholesterol) transcriptional activation, which is reduced by a point mutation that affects the binding of cholesterol sulfate more than that of cholesterol. We speculate that cholesterol sulfate plays a role in the regulation of RORα in vivo.

MATERIALS AND METHODS

RORA LBD Protein Preparation for Crystallization and ESI-MS Exchange Experiment—The RORα LBD protein (residues 271–523, flanked by an N-terminal hexa-His tag and a PreScission™ cleavage site) was expressed in the baculovirus system (SF-9 cells) and purified as described previously (3). The exchange of cholesterol by cholesterol sulfate was done at 37 °C and confirmed by ESI-MS-analysis as reported previously (5). Briefly, cholesterol sulfate was dissolved at 50 mM in Me2SO and added at 1.0 mM final concentration to the (His)6-RORα LBD271–523 solution at 73 °C in 50 mM AcONH4, 150 mM NaCl, 50 mM imidazole, and 1 mM DTT. The resulting solution was incubated overnight at 37 °C and further purified by size exclusion chromatography on an SPX75 column before concentration to 17.6 mg/ml for crystallization trials. MS determination of the native complex was done as described previously (5). A control experiment was done by incubating the same amount of RORα LBD protein with 5% Me2SO under identical conditions. The protein concentration was 15 µM in 50 mM AcONH4, ...
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pH 7.0. Both spectra were recorded under identical conditions with Vc = 20 volts.

Differential Scanning Calorimetry—The RORα LBD complexes with cholesterol and cholesterol sulfate, respectively, were obtained as described above. After the exchange experiment, the excess of cholesterol sulfate was removed on 5-ml HiTrap desalting columns. Protein concentration was determined by high pressure liquid chromatography described above. After the exchange experiment, the excess of cholesterol and cholesterol sulfate, respectively, were obtained as de-

Crystalization, Data Collection, and Structure Determination—Crystals were obtained at 4 °C by the vapor diffusion method in 2-μl hanging drops containing equal volumes of protein (17.6 mg/ml) and crystallization buffer (0.2 M MgCl2, 16% w/v polyethylene glycol 4000, 0.1 M Tris HCl, pH 8.5). The crystal form obtained was similar to the one for the complex with cholesterol (3). Diffraction data at 100 K were collected at the Swiss Light Source (beamline X06SA) using a Marresearch CCD detector and an incident monochromatic x-ray beam with 0.2990-A wavelength. In total, 226 images were collected with 1.0° rotation each, using an exposure time of 9 s/frame and a crystal-to-detector distance of 150 mm. Raw diffraction data were processed and scaled with the HKL program suite version 1.96.1 (6). The estimated B-factor by Wilson plot analysis is 32.9 Å2. The structure was determined using as starting model the coordinates of the complex RORα LBD-cholesterol refined to 1.63-Å resolution (3). The program REFMAC version 5.0 (7, 8) was used for refinement. Bulk solvent correction, an initial anisotropic B factor correction, and restrained isotropic atomic B-factor refinement were applied. The refinement target was the max-

Overall Structure of the Complex RORα LBD-Cholesterol Sulfate—The results of the crystallographic refinement are summarized in Table I. The nomenclature of the secondary structure elements is based on the IRR LBD crystal structure (13) and is identical to the one used for RORα LBD (3). In general, the electron density is of excellent quality, except for amino acids 461–466 (loop L9–10), which had only weak density. The protein part of the refined model consists of the last two His amino acids from the His tag followed by the PreSciss-

Results and Discussion

Table I

| Table I | Crystallographic summary |
|---------|--------------------------|
| **Diffraction data** | **Space group** |
| | P21 |
| Unit cell dimensions | a = 54.4 Å, b = 49.9 Å, c = 60.7 Å, β = 97.8°, 1 complex/ |
| Resolution range | 20.0–2.2 Å (2.28–2.20 Å) |
| No. of observations | 57,993 |
| No. of unique reflections | 16,514 |
| I(σ(I)) | 16.2 |
| Rsym on intensities | 0.079 |
| Completeness | 99.7% (99.4%) |
| Refinement | |
| Resolution range | 20.0–2.20 Å |
| R cryst | 0.194 |
| Rfree | 0.219 |
| Protein atoms | 2,067 |
| Ligand atoms | 33 |
| Solvent atoms | 255 |
| Average B-factor | 40.0 Å2 |
| r.m.s.d. from target values | |
| Bond lengths | 0.014 Å |
| Bond angles | 1.41° |


d asymm unit (2.20 Å resolution) reached maximal dimensions of up to 0.2 mm in hanging drops at 4 °C within 6 weeks. The structure was solved using the coordinates of the complex of RORα LBD with cholesterol (3).

Structure Determination of the Complex RORα LBD-Cholesterol Sulfate—The overall structure of the RORα LBD-Cholesterol Sulfate is as follows: the classical charge clamp is bound to the sulfate group (with a concomitant movement of the respective side chains), thus improving the interactions with the sulfate group. The side chain of Ile327 has moved slightly, to prevent a steric clash with the terminal isopropyl group of cholesterol sulfate.

Cholesterol Sulfate in the LBP of RORα—Cholesterol sulfate is bound in the LBP of RORα. The sulfate group, which is...
located in the hydrophilic part of the LBP, makes direct hydro-
gen bond interactions with NH-Gln 289 (3.0 Å), NH-Tyr 290 (2.9 Å), and a bidentate interaction with NH1-Arg 370 (3.0 Å, 3.1 Å). This confirms the docking hypothesis, which had led to the proposal of cholesterol sulfate as a ligand with an improved affinity relative to cholesterol (3). In addition, a water-mediated interaction is made with NH1-Arg 367 (Fig. 1A). The comparison shows that cholesterol sulfate and cholesterol have a similar overall mode of binding, but cholesterol sulfate is displaced slightly toward the hydrophilic and positively charged part of the LBP (Fig. 1A). This can be explained by the optimization of electrostatic and hydrogen-bond interactions made by the sulfate group. Interestingly, seven well ordered water molecules present for cholesterol sulfate in the hydrophilic part of the LBP have been displaced in the complex with cholesterol sulfate (Fig. 1B). Only one conserved water molecule (red arrow) is still present, which mediates interactions from the sulfate group to NH1-Arg 367 and O-Ala 330. The sulfate group makes direct hydrogen bond interactions with NH-Gln 289, NH-Tyr 290, and NH1-Arg 370.

The average B-value for the ligand (28.7 Å²) is lower than the average B-value for the protein (40.0 Å²), consistent with the fact that excellent electron density for all non-hydrogen atoms of cholesterol sulfate is visible. Cholesterol sulfate adopts thus (like cholesterol) a single, well defined position in the LBP. The following amino acids have a non-hydrogen atom closer than 4 Å to the ligand cholesterol sulfate: Cys 288 (loop H1-H2), Gln 289 (loop H1-H2), Tyr 290 (loop H1-H2), Trp 320 (H3), Cys 323.

**Fig. 1. Crystal structure of the complex between human ROR LBD and cholesterol sulfate.** A, an overview of the interactions made by cholesterol sulfate (cyan) with the LBP of ROR LBD (yellow). Selected hydrogen bonds are shown as dotted lines. The sulfate group makes direct hydrogen bonds with NH-Gln 289, NH-Tyr 290, NH1-Arg 370, and a water-mediated hydrogen bond with NH1-Arg 367. Also shown is a superposition with the x-ray structure of ROR LBD-cholesterol (white/magenta). Water molecules for the complexes with cholesterol sulfate and cholesterol are shown as green and white spheres, respectively. Only the regions comprising Gln 289, Tyr 290 (loop L1–2), and Ile 327 show significant changes (black arrows). Gln 289 and Tyr 290 move toward the sulfate group to improve the interactions of their main chain NH-moieties with the sulfate group. CD1-Ile 327 has moved to avoid a steric clash (red line, 3.2 Å) with the isopropyl group of cholesterol sulfate (true distance is 4.2 Å, blue line). B, a comparison of ROR LBD/cholesterol (left) and ROR LBD/cholesterol sulfate (right) in the hydrophilic part of the corresponding LBPs. Selected hydrogen bonds are shown as dotted lines. Seven well ordered water molecules (six of which are shown as gray spheres) present for cholesterol (left) have been displaced in the complex with cholesterol sulfate (right). Only one conserved water molecule (red arrow) is still present, which mediates interactions between the sulfate group and NH1-Arg 367 and O-Ala 330. The sulfate group makes direct hydrogen bond interactions with NH-Gln 289, NH-Tyr 290, and NH1-Arg 370.
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Fig. 2. Cholesterol sulfate has a higher affinity than cholesterol for RORα LBD, as confirmed by differential scanning calorimetry. DSC scans of RORα LBD complex with cholesterol (dotted line, curve 1; Tm = 61.6 °C) and RORα LBD complex with cholesterol sulfate (solid line, curve 2; Tm = 70.6 °C) are shown. The shoulder around 62 °C for curve 2 is due to a remaining fraction of non-exchanged cholesterol. Temperature scan rate was 250 °C/h, and protein concentration was 20 μM. The +9 °C Tm shift indicates a higher stability for the complex with cholesterol sulfate.

H3), Ala324(H3), Lys325(H3), Ile327(H3), Ala330(H3), Val364(H5), Arg365(H5), Met366(H5), Arg367(H5), Ala371(H5), Val379(s1), Tyr380(s1), Phe381(s1), Phe391(H6), Leu394(H6), Val403(H7), and His484(H11).

Cholesterol Sulfate Is Bound More Tightly than Cholesterol—We used non-denaturing ESI-MS to confirm the exchange of cholesterol by cholesterol sulfate in the RORα LBD. Exchange yield using cholesterol sulfate was greater than 95%. As shown previously (5), the corresponding protein-ligand complex also showed a higher stability to collision-induced dissociation in the electrospray ionization interface. Moreover, the exchange of bound cholesterol sulfate could not be reversed by adding back an excess of cholesterol or other cholesterol derivatives such as hydroxycholesterols (data not shown). In addition, differential scanning calorimetry was used to assess the stabilities of the RORα LBD complexes with cholesterol and cholesterol sulfate, respectively. Fig. 2 shows that cholesterol sulfate dramatically increased the phase transition temperature of RORα LBD by 9 °C, relative to cholesterol. Overall, these results confirm that, as predicted by the x-ray structures, cholesterol sulfate has a higher affinity than cholesterol for RORα LBD.

Sulfonation of Cholesterol Improves RORα Transcriptional Activity—To further characterize the possible biological role of cholesterol sulfate for RORα, a series of mutations was designed and evaluated for their effects on the transcriptional activities. The double mutation K339A,E509A (i.e. a “knockout” of the charge clamp) was made as a negative control. Indeed, this double mutant triggered a transcriptional activity similar to the one obtained with an empty vector (Fig. 3A). The triple mutant A330Q, A371Q, C323L (designed to prevent binding of cholesterol and cholesterol sulfate in the LBP) showed a reduced activity (Fig. 3A) consistent with the results obtained previously for the single mutants A330L, A371Q, and C323L (3). A mutation that might prevent binding of cholesterol sulfate more than that of cholesterol was designed based on the crystal structure. We hypothesized that the mutation C288Q would modify the position of Arg in and/or of loop L1–2 (Fig. 1B) and thus would preferentially lower the affinity of cholesterol sulfate versus cholesterol. We first tested this mutation in cells for which the intracellular cholesterol level was not manipulated. Most likely, as shown in normal epithelial cells, the ratio between cholesterol and cholesterol sulfate in these cells is about 500:1 (15). Under these experimental conditions, the transcriptional activity of the mutant RORα C288Q was found to be similar to the one elicited by RORα wild type (Fig. 3A). This unaffected transcriptional activity of the C288Q mutant probably reflects the binding of cholesterol to the mutated RORα.

The intracellular cholesterol level in U2OS cells can be reduced by using lovastatin (hydroxymethylglutaryl-CoA reductase inhibitor) and cyclodextrin, as described previously (3). Under these conditions, using RORα wild type, we have shown that cholesterol sulfate elicited an increased transcriptional activity of 160% when compared with cholesterol. In contrast, the mutant C288Q did not display this improved transcriptional activity (with respect to cholesterol) when cells were treated with cholesterol sulfate (Fig. 3B). This is consistent with the prediction that the C288Q mutant preferentially reduces the affinity of cholesterol sulfate. We cannot exclude that the only partial cholesterol depletion in these cells might reduce this observed effect on cholesterol sulfate. It would be interesting to investigate RORα transcriptional activity in cells for which the cholesterol:cholesterol sulfate ratio is more drastically modified such as in keratinocytes during differentiation, where this ratio is as low as 5:1, or in ichthyosis-derived cells, where this ratio is even further reduced to 1:1 (16, 17).

Recently, there has been an increasing interest in steroid sulfonation, in particular for their potential involvement in breast and prostate cancer (18). It is postulated that estrone sulfate in the breast tumors (19) could play a role in regulating the level and the activity of 17β-estradiol and that disruption of estrogen sulfotransferase could lead to modulation of the estrogen pathway (20). In our study, we show that sulfonation of cholesterol allows improved binding to RORα, reflected by an increased transcriptional activity. The possible exchange of cholesterol with cholesterol sulfate inside the cells could represent a mode of regulation of intracellular cholesterol level since it has been shown that cholesterol sulfate inhibits cholesterol esterification (21) and that cholesterol sulfate could potently modulate hydroxymethylglutaryl-CoA, the rate-limiting enzyme for cholesterol synthesis (22). The identification of
FIG. 3. Effect of RORα mutations on transcriptional activities induced by cholesterol sulfate or cholesterol. A, transcriptional activity of RORα wild type (WT) and mutants. Cos7 cells were seeded at a density of $1 \times 10^5$ cells/cm$^2$ in 12-well plates and transiently transfected with 0.50 μg of expression vector for RORα wild type or RORα mutants (C288Q and K339A,E509A) together with 1.0 μg of ROREtkluc and as indicated under “Materials and Methods.” 24 h after transfection, cells were collected with 250 μl of passive lysis buffer, and luciferase activity was determined in duplicate on 20 μl of cell extracts. Luminescence data were corrected with β-galactosidase activity and expressed as relative luminescence unit/β-galactosidase unit. Results are expressed as percentage of induction when compared with the activity of wild type RORα. Western blot analysis of extracts from cells transfected was performed and showed comparable expression of RORα wild type and mutants (data not shown). B, U2OS cells were transfected as described under “Materials and Methods” with RORα wild type and RORα C288Q. After transfection, cells were treated with hydroxypropylcyclodextrin (10 mM) in presence of 5 μM lovastatin for 4 h in a medium containing 10% low density lipoprotein-free serum. After four h, the medium was removed, and cells were treated with vehicle (ethanol or Me2SO) or cholesterol or cholesterol sulfate at 10 μM in presence of 5 μM lovastatin. Twenty-four hours after transfection, cells were collected with 250 μl of passive lysis buffer, and luciferase activity was determined in duplicate on 20 μl of cell extract from three separate culture dishes. Luminescence data were corrected with β-galactosidase activity and expressed as relative luminescence unit/β-galactosidase unit. Results are expressed as the percentage of induction of cholesterol sulfate when compared with cholesterol for RORα and RORα C288Q.
RORα target genes possibly involved in these mechanisms could contribute to a better understanding of these regulations by cholesterol sulfate.

Despite the fact that cholesterol sulfate is widely distributed in human tissues, its physiological role is not well understood (for a review, see Ref. 23). In skin, an important role for cholesterol sulfate emerged, e.g. in recessive X-linked ichthyosis for which a genetic deficiency in steroid sulfatase leads to the accumulation of cholesterol sulfate in stratum corneum (16). In addition, the role of cholesterol sulfate in keratinocyte differentiation has been well documented (24–27). It would therefore be interesting to reconsider RORα function in organs such as skin and testis, in which cholesterol sulfate was found to be abundant and which display the strongest expression of RORα (28).

In addition, cholesterol sulfate is also a possible precursor of important sulfonated adrenal steroids such as dehydroepiandrosterone sulfate and pregnenolon sulfate (23, 29). It is worth noting that pregnenolon sulfate, now considered as an essential neurosteroid, is actively synthesized in brain, in particular in Purkinje cells (30). This information could thus also shed new light on the well described cerebellar phenotype of the RORα mutant mice (31).

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