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Intracellular localization of RORα is isoform and cell line-dependent

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Abstract

The retinoid-related orphan receptor α (RORα) belongs to the nuclear receptor superfamily and comprises four isoforms generated by different promotor usage and alternative splicing. To better understand its function, the subcellular distribution of RORα was investigated. We could show that subcellular distribution of RORα is cell line and isoform-dependent. Isoform specific differences were mediated by the A/B domains which with the exception of RORα1 contain a signal that mediates cytoplasmic localization. The lack of this signal in RORα1 results in a complete nuclear localization and prevents cell membrane association observed for RORα2, 3, and 4. The region responsible for membrane association was identified as the C-terminal α-helix 12. Furthermore, the hinge region/ligand binding domain mediates nuclear localization. Our results show that isoform specific activity of RORα is not only regulated by different expression and DNA binding affinities but also by different subcellular distribution. Different access to the nucleus reveals an important mechanism regulating the activity of this constitutively active nuclear receptor.

Keywords: RORα; Cholesterol; 293 HEK cell; HeLa cell; Cellular distribution; Nuclear receptor

1. Introduction

The retinoid-related orphan receptor α (RORα) belongs to the superfamily of nuclear receptors consisting of structurally related, ligand-dependent transcription factors, which control numerous processes involved in the maintenance of homeostasis, in development, growth, cell differentiation, proliferation, and apoptosis [1]. They transduce extracellular hormone signals via binding to specific cis-acting DNA sequences in the promoter region of target genes, known as response elements. Besides receptors for ligands such as glucocorticoids, estrogens, thyroid hormone, retinoic acid, and vitamin D, a large number of so-called orphan receptors with so far unknown ligands have been identified based on domain organization and sequence conservation [2,3]. All nuclear receptors share a common modular structure with several independent functional domains [4,5]. The very variable N-terminal A/B domain is followed by the highly conserved DNA binding domain (DBD) which encompasses two zinc finger motifs, a flexible hinge region, and a C-terminal ligand binding domain (LBD). The transcriptional activity of nuclear receptors can be regulated by ligand binding, by the regulation of their gene expression [6], by posttranscriptional mechanisms including phosphorylation [7,8], acetylation and ubiquitinylation as well as by the modulation of their intracellular localization [9].

The subcellular distribution of nuclear receptors is the result of a dynamic and controlled shuttling between cytosol and nucleus as demonstrated for the progesterone receptor [10]. Balancing nuclear import and export can be an important mechanism to regulate the transactivation potential of nuclear receptors, especially in the case of constitutively active receptors. Depending on their intracellular localization, nuclear receptors can be divided into three categories: (I) exclusively cytoplasmic receptors like the glucocorticoid receptor [11], (II) both cytoplasmic and nuclear like the mineralocorticoid receptor [12], and (III) exclusively nuclear like the progesterone receptor [13,14]. Active translocation of a cytoplasmic receptor into the nucleus is mostly mediated by ligand binding [15–18]. It depends on nuclear localization signals (NLS), typically short sequences rich in basic amino acids [18]. It is believed that ligand binding induces

Abbreviations: CLSM, confocal laser scan microscopy; DBD, DNA binding domain; EGFP, enhanced green fluorescent protein; LBD, ligand binding domain; NES, nuclear export signal; NLS, nuclear localization signal; NR, nuclear receptor; ROR, retinoid-related orphan receptor; RORE, RORα response element

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conformational changes of the receptor leading to NLS exposure which subsequently becomes accessible to nuclear import factors that facilitate translocation through the nuclear pore complex [19]. Certain nuclear receptors have also been shown to undergo export from the nucleus. A nuclear export signal present in the DNA binding domain of diverse receptors has been identified within the glucocorticoid receptor [20]. Furthermore, membrane-associated forms of several nuclear receptors have been described [21–24]. It is supposed that these membrane-associated receptors mediate rapid, non-genomic effects of steroids such as stimulation of signaling cascades via modulation of kinase activities and regulation of second messengers including cAMP.

The retinoid-related orphan receptor subfamily of nuclear receptors consists of the three ROR subtypes α, β, and γ (NR1F1, NR1F2 and NR1F3 [25]) which are encoded by different genes. The ROR subtype α is believed to be a constitutive activator of transcription in the absence of exogenous ligands [26]. Recently, cholesterol was found in the ligand binding pocket of the crystallized ligand binding domain suggesting that it might be a natural ligand of RORα [27]. By now, four human isoforms of RORα were identified [28,29]. These isoforms are a result of different promoter usage and alternative splicing and differ only in their N-terminal domains, resulting in different DNA binding properties [28]. They show different tissue distribution with RORα1 and RORα4 expressed more ubiquitously [29,30], while RORα2 and RORα3 are expressed in a more restricted manner. RORα3 was only found in human testis [31], RORα2 could be detected in human testis as well as in cells of the immune system [31,32]. The precise role of RORα in various tissues still remains to be elucidated. However, it has been shown that RORα negatively regulates inflammatory responses [33]. Homozygous RORα-deficient mice as well as the so-called staggerer mice that express a RORα truncation mutant, show a severe cerebellar ataxia phenotype [31,34–36], abnormalities in social and sexual behavior, and an increased risk in developing atherosclerosis [37] and osteoporosis [38,39]. Furthermore, RORα together with Rev-erbα was recently found to be essentially involved in the maintenance of the circadian rhythm [40].

In this study, we investigated the cell type- and isoform-specific intracellular localization of RORα. We decided to use green fluorescent protein chimeras to analyze intracellular localization since GFP was shown to work as a functional tag for many nuclear receptors [11,12,14,16–18,20]. We demonstrate that the A/B domains of RORα2, 3 and 4 but not that of RORα1 confer a cytoplasmic localization suggesting either nuclear export or cytoplasmic retention and that the common part of the four isoforms mediates nuclear localization. Furthermore, the role of the C-terminal part of RORα, containing helix 12, for the observed membrane localization was elucidated.

2. Materials and methods

2.1. Cell culture

293 HEK, HeLa, MCF-7, and SH-SY5Y cells were cultured as monolayers in DMEM (Invitrogen). Medium was supplemented with 10% FCS, 100 μg/ml streptomycin and 100 U/ml penicillin. If indicated, charcoal-stripped FCS was used.

2.2. RT-PCR analysis

Extraction of total RNA from cultured cell lines was performed according to the method of Chomczynski and Sacchi [41]. β-mercaptoethanol was added to a final concentration of 2%. Putative genomic DNA was digested with DNase at 37 °C for 1 h. The enzyme was removed by chloroform–phenol extraction.

Reverse transcription was performed with Superscript Plus reverse transcriptase (Invitrogen) according to manufacturer's instructions using oligo-d$(T)_{12–18}$ primer. PCR primer pairs and conditions for touch down PCR are listed in Table 1.

2.3. Construction of EGFP chimeras

pEGFP-N1 and pEGFP-C2 (Clontech) were chosen for cloning and expression. Expression vectors for RORα isoforms pCMX-RORα1, pCMX-RORα2, pCMX-RORα3, and pSG5-RORα4 were kindly provided by C. Carlberg. Sequences of oligonucleotides used for site-directed mutagenesis are listed in Table 2.

To generate a vector expressing a fusion protein with the C-terminus of RORα isoforms fused to the N-terminus of EGFP, the stop codons of the RORα isoforms were replaced by site-directed mutagenesis to sequences coding for alanine. cDNAs encoding the isoforms RORα1–3 were released with *Kpn*I and *Bam*HI and inserted into pEGFP-N1 at these sites. The RORα4-cDNA was cut with *Eco*RI and *Bam*HI and inserted into pEGFP-N1 at these sites. The resulting constructs were denoted as RORα1/EGFP, RORα2/EGFP, RORα3/EGFP, and RORα4/EGFP, respectively. In the resulting fusion proteins, the C-terminus of RORα is fused to the N-terminus of EGFP through a GDPPVAT linker sequence.

To generate a vector expressing a fusion protein where the N-terminus of RORα4 is linked to the C-terminus of EGFP, pSG5-RORα4 was digested with *Hin*dIII and *Eco*RI. The released RORα4-cDNA fragment was inserted into pEGFP-C2 at these sites. A stop codon in the linker region between EGFP and receptor at position −1 of RORα4 was replaced by site-directed mutagenesis to a sequence coding for alanine. The resulting plasmid was denoted as EGFP/RORα4. In the fusion protein, EGFP is linked to RORα4 through a SPHRAA linker.

Sequences coding for the A/B domains of the RORα isoforms were generated by PCR using oligonucleotides inserting *Kpn*I and *Bam*HI sites and the pCMX- or pSG5-expression vectors as templates. Fragments were inserted into the *Kpn*I and *Bam*HI sites of pEGFP-C2 resulting in the expression vectors EGFP/RORα1-A/B (aa 1–72), EGFP/RORα2-A/B (aa 1–105), EGFP/RORα3-A/B (aa 1–97), and EGFP/RORα4-A/B (aa 1–17). The isoform-specific A/B region of RORα4 was deleted from RORα4/EGFP by site-directed mutagenesis resulting in the construct RORα-ΔA/B/EGFP.

To generate a RORα4 truncation mutant that lacks the C-terminal α-helix (aa 448–468), a stop codon was inserted by site-directed mutagenesis with the

Table 1
Oligonucleotide pairs and amplification conditions used for RT-PCR analysis

Transcript		Sequence (5′→3′)	Annealing	Cycles
RORα1	432 bp	AGAGC TATTC CAGCA CCAGC AG CGTTG GCCGA GATGT TGTAG GT	61–47 °C, ΔT=−0.4 °C	35
RORα2	442 bp	CAGAA TGGCA AGCCA CCATA TTC CGTTG GCCGA GATGT TGTAG GT	59–45 °C, ΔT=−0.4 °C	35
RORα3	502 bp	TGTAG CTCCC TGAGC AGGCT GTT CGTTG GCCGA GATGT TGTAG GT	61–47 °C, ΔT=−0.4 °C	35
RORα4	405 bp	TGTAT TTTGT GATCG CAGAG CGTTG GCCGA GATGT TGTAG GT	48–41 °C, ΔT=−0.2 °C	35
β-actin	844 bp	GAGGA GCACC CCGTG CTGCT GA CTAGA AGCAT TTGCT GTGGA CGATG GAGGG GCC	60 °C	24

Table 2
Oligonucleotide pairs used for construction of EGFP chimeras

Construct	Sequence (5′→3′)
EGFP/RORα1-A/B	GGGGT ACCGC ATGGA GTCAG CTCTG GCAGC
	CGGGA TCCCT GGAAT AATTT CAATT TGAGA TGTAT GTGTC TT
EGFP/RORα2-A/B	GGGGT ACCGC ATGAA TGAGG GGGCC CCAGG
	CGGGA TCCCT GGAAT AATTT CAATT TGAGC ATTCA TGTAT
EGFP/RORα3-A/B	GGGGT ACCGC ATGAA TGAGG GGGCC CCAGG
	CGGGA TCCCT GGAAT AATTT CAATT TGAGC TTTTC TCAAT
EGFP/RORα4-A/B	GGGGT ACCAA ATGAT GTATT TTGTG ATCGC AGAGA
	CGGGA TCCAT GGAAT AATTT CAATT TGAGC TTTCA TCT
RORα4-ΔA/B/EGFP	CCGCA CCGCG ATTAA ATGCA AATTG AAATT ATTC
	GAATA ATTTC AATTT GCATT TAAGC GCGGT GCGG
EGFP/RORα4-Δh12	CCAGA CATTG TGCGA CTTCA TTAAC CTCCA TTATA CAAGG AG
	CTCCT TGTAT AATGG AGGTT AATGA AGTCG CACAA TGTCT GG

indicated primer pair (Table 2) and EGFP/RORα4 as template DNA. The new construct was termed EGFP/RORα4-Δh12.

All constructs were confirmed by sequencing. A scheme of the generated fusion proteins is given in Fig. 4A.

2.4. Analysis of intracellular distribution of EGFP fusion proteins

To determine the intracellular distribution of the different EGFP-RORα receptor constructs, cells were grown on glass cover slips. For seeding of SH-SY5Y cells, cover slips were coated with gelatin. Cells were transiently transfected by calcium phosphate transfection [42] and fixed or lysed 17 h after transfection. In experiments were NR ligands should be eliminated from the medium, cells were washed with PBS after transfection and covered with DMEM that was supplemented with 10% charcoal-stripped and silica-treated FCS. Cholesterol depletion was carried out for 6 h with 10 mM 2-hydroxypropyl-β-cyclodextrin and 5 μM cerivastatin. Transfected cells growing on glass cover slips were rinsed with PBS, fixed for 10 min with ice-cold methanol, and washed 3 times with PBS. Cover slips were embedded on slides in mounting solution (50% glycerol in PBS) and sealed with nail polish. Images were taken by confocal laser scan microscopy (CLSM; Leitz DM-IRB; 488 nm, TCS 4D). For costaining of the nucleus, cells were stained with TO-PRO-3 iodide (Molecular Probes). 24 h after transfection cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min and stained for 20 min with 1 mM TO-PRO-3 iodide in PBS with 1% BSA.

2.5. Western blot analysis

To obtain whole cell lysates, cells were seeded in 100 mm dishes containing a glass cover slip for the control of transfection efficiency, transfected as described above and harvested at 80–90% confluency. Cells were washed with PBS, resuspended in ice-cold lysis buffer (50 mM Tris–HCl, pH=7.4, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 2 mM EGTA, 40 mM β-glycerophosphate, 50 mM NaF, 10 mM sodium pyruvate) and disrupted by drawing them 10 times through a narrow-gauge syringe. Debris was removed by centrifugation, protein amount was estimated according to Bradford. SDS-PAGE was carried out with 50 μg protein per lane as described by Laemmli [43]. PBS with 0.05% Tween-20 and 5% dry milk powder was used for blocking and antibody incubations. For the investigation of full-length expression of EGFP-tagged RORα fusion proteins, both primary (mouse-anti-GFP, Sigma) and secondary antibodies (goat-anti-mouse-AP, Sigma) were diluted at 1:5,000. Membranes were incubated in primary antibody for 3 h, in secondary antibody for 1 h. After antibody incubation they were washed 3 times with PBS/0.05% Tween-20. Detection was carried out with nitroblue tetrazolium and 5-bromo-4-chloro-indolyl phosphate.

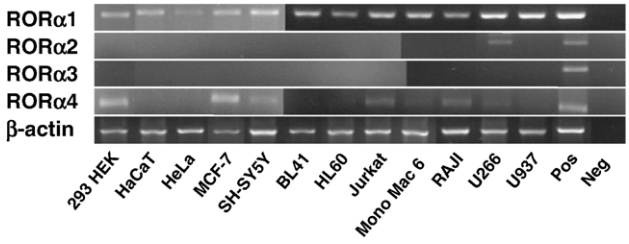

Fig. 1. RT-PCR analysis of RORα isoform expression in different human cell lines. Total RNA of the indicated cell lines was extracted, cDNA was synthesized with oligo-d(T) priming and RT-PCR analysis of RORα isoforms and β-actin was performed as described in Materials and methods.

2.6. Reporter gene analysis

The reporter gene plasmid RORE$\alpha 2_1$-TKLUC, containing one copy of the RORα2 response element (ATAACT AGGTCA), was kindly provided by V. Giguère [28]. Two further copies of the same response element were inserted into the *Sal*I and *Hin*dIII sites resulting in the construct RORE$\alpha 2_3$-TKLUC. 40,000 HeLa or 100,000 293 HEK cells were seeded in 24-well plates using DMEM without phenol red, and the following day the cells were cotransfected with 400 ng reporter gene plasmid RORE$\alpha 2_3$-TKLUC and 40 ng expression vector for the EGFP-RORα fusion protein by standard calcium phosphate method [42]. Cells were incubated with the precipitate for 16 h before medium was replaced. 24 h after transfection, cells were harvested and processed for luminescence measuring using the Steady-Glo-kit (Promega) in a Microlumat Plus LB96V (EG&G Berthold) luminometer. Transfection efficiency was normalized by cotransfection of 20 ng pCMV-SEAP to determine the secreted placental alkaline phosphatase activity using Phospha-Light™-kit (Tropix). Induction is related to cotransfection with the corresponding empty vector, pEGFP-N1 or pEGFP-C2, given as mean+S.E. of three experiments, each performed in triplicate.

3. Results

3.1. RORα isoforms are differentially expressed in human cell lines

The endogenous RNA expression of RORα isoforms in a number of human cell lines was analyzed on a qualitative level by RT-PCR analysis. Due to the very low expression level, at present, data on cellular RORα protein expression cannot be

Table 3
Cellular localization of different RORα proteins

Construct	Cell line			
	HeLa	293 HEK	MCF-7	SH-SY5Y
RORα1/EGFP	N	N	N	N
RORα2/EGFP	N	N+C+M	N+C	N
RORα3/EGFP	N	N+C+M	N+C	N
RORα4/EGFP	N	N+M	N	N
EGFP/RORα4	N	–	–	–
EGFP/RORα1-A/B	N+C	N+C	–	–
EGFP/RORα2-A/B	C	C	–	–
EGFP/RORα3-A/B	C	C	–	–
EGFP/RORα4-A/B	C	C	–	–
RORα-ΔA/B/EGFP	N	N	–	–
EGFP/RORα4-Δh12	–	N	–	–

The localization is given as N—nuclear, C—cytoplasmic, and M—membrane associated.

obtained. Thus, RNA expression as a prerequisite for protein expression was analyzed. Amplification of β-actin transcripts served as a control for RNA quality and cDNA synthesis. In general, RORα is expressed at a low level in the tested cell lines, since detection of RORα transcripts required PCR with at least 30 amplification cycles. As shown in Fig. 1, the most prominent isoform was RORα1 as it was detected in a wide range of cell lines, reflecting a nearly ubiquitous tissue distribution. RORα4

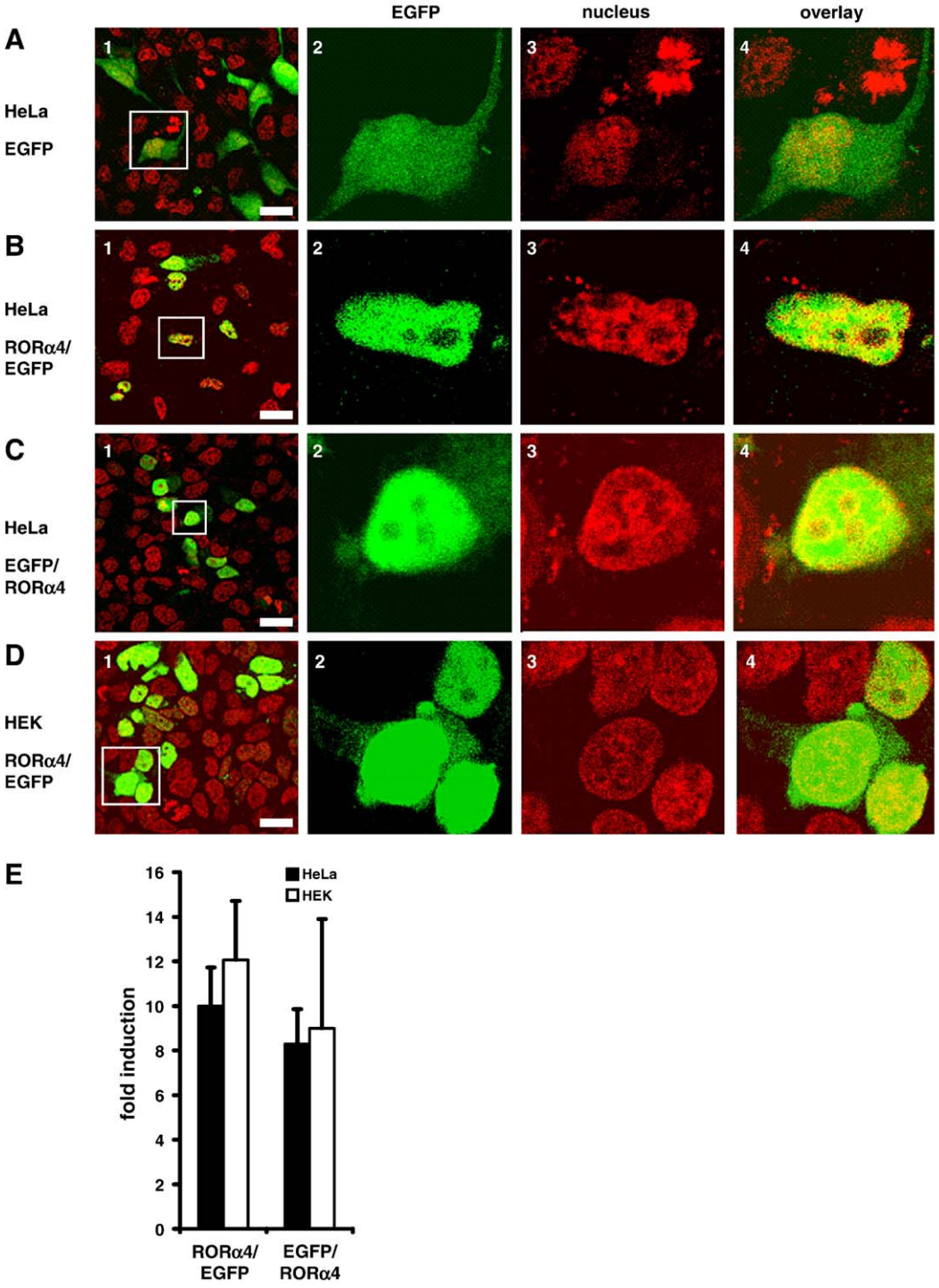

Fig. 2. Functionality of RORα4 tagged with EGFP. (A–D) Subcellular distribution of EGFP fusion proteins (green) and costaining of the nuclei with TO-PRO-3 (red). HeLa or 293 HEK cells were transiently transfected with the indicated expression vectors and images taken by CLSM. The bar represents 10 μm. (A) pEGFP-C2 in HeLa; (B) RORα4/EGFP in HeLa; (C) EGFP/RORα4 in HeLa; (D) RORα4/EGFP in 293 HEK. For each construct panel (1) shows an overview, panels (2–4) show magnified sections of the indicated boxes with (1) EGFP in green; (2) TO-PRO-3 in red; and (3) overlay. (E) Transactivation by RORα. HeLa (black columns) or 293 HEK cells (white columns) were transiently transfected with RORE$\alpha 2_3$-TKLUC reporter gene construct and the indicated expression vector. Cells were harvested 24 h after transfection and reporter gene activity was measured. Induction is related to transfection with the corresponding empty vector pEGFP-N1 or pEGFP-C2, given as mean+S.E. of three experiments each performed in triplicate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this arcticle.)

transcripts were found in fewer of the tested cell lines. In addition, its expression seems to be more tightly regulated, as for instance the two Burkitt's lymphoma derived cell lines BL-41 and RAJI show different RORα4 expression patterns. In line with the very restricted expression of RORα2 and RORα3, transcripts of these isoforms were not detected in any of the tested cell lines with the exception of low levels of RORα2 in U266 cells (Fig. 1).

To investigate the subcellular localization of RORα we chose four different adherent cell lines with different endogenous RORα expression patterns—HeLa, expressing low levels of RORα1 isoform; 293 HEK and MCF-7, expressing both RORα1 and RORα4; and SH-SY5Y, expressing RORα1 and low levels of RORα4 (Fig. 1).

3.2. EGFP is a functional tag to investigate subcellular RORα distribution

To analyze subcellular distribution of the four RORα isoforms, cDNAs encoding chimerical RORα proteins fused to EGFP were constructed. These constructs were transiently transfected into four different cell lines. In all experiments the transfection efficiency was in the range of 20–50%. All data concerning the subcellular distribution of EGFP-tagged proteins are summarized in Table 3. We tested whether RORα affects the subcellular distribution of EGFP and if addition of EGFP impairs the functionality of RORα4. The addition of RORα to the green fluorescent protein changed the subcellular distribution of EGFP, which alone was uniformly distributed throughout cells of all lines used in this study (data shown for HeLa cells in Fig. 2A). N-terminal fusion of EGFP to the RORα4 induced redistribution, with a green fluorescence clearly restricted to the nucleus (Fig. 2B). As shown in Fig. 2A, the green fluorescence of the EGFP-RORα4 chimera clearly colocalizes with the nuclear staining (panels 2, 3 and 4). To determine if the addition site might influence the subcellular localization, a second vector containing C-terminally EGFP-tagged RORα4 was constructed and transiently transfected into HeLa cells. Both chimeras displayed identical fluorescence distribution, indicating that the fusion site (N- or C-terminal) does not alter the subcellular distribution of RORα4 (Fig. 2C).

To verify that the addition of EGFP to RORα4 does not influence its transcriptional activity, a luciferase reporter gene assay was performed in HeLa and 293 HEK cells. Cotransfection of the expression vector for the RORα/EGFP fusion protein with a

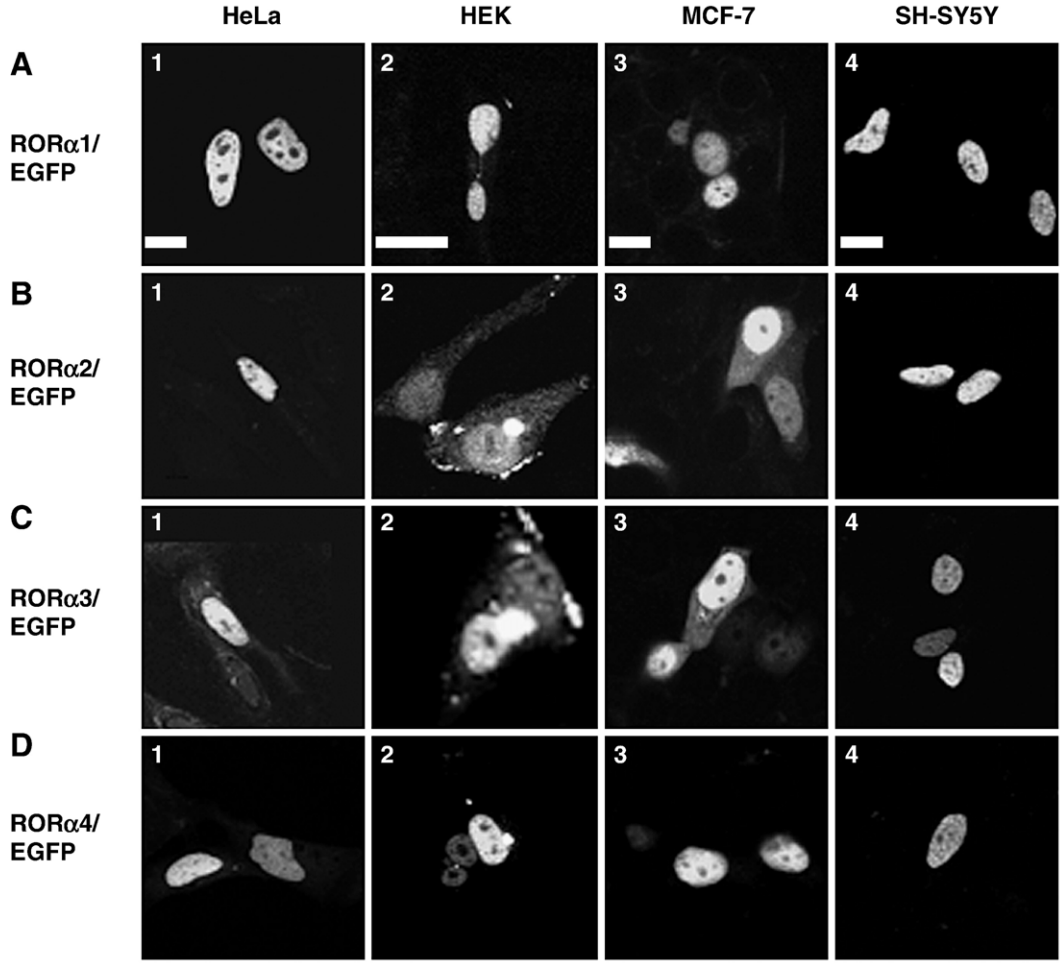

Fig. 3. Subcellular distribution of RORα isoforms in different cell lines. Cells were transiently transfected with the indicated expression vectors and images taken by CLSM. The bar, given for each cell line in panel A, represents 10 μm. Expression of (A) RORα1/EGFP; (B) RORα2/EGFP; (C) RORα3/EGFP; (D) RORα4/EGFP in (1) HeLa; (2) 293 HEK; (3) MCF-7; (4) SH-SY5Y cells is shown.

reporter gene plasmid containing three RORα response elements upstream of the TK promoter and luciferase as reporter gene revealed that EGFP-RORα4 is able to activate transcription (Fig. 2D) with an efficiency comparable to untagged RORα4 (12.8-fold). The transactivation potential is not cell line-dependent. The EGFP fusion site (N- or C-terminal) did not significantly influence the transactivation potential of RORα4.

Having shown the functionality of the EGFP-tag in our system, we used EGFP-tagged RORα proteins to characterize the subcellular distribution of the four RORα isoforms in different cell lines.

3.3. Subcellular localization of RORα is cell line-dependent and isoform-specific

As shown in Fig. 3, the subcellular distribution of transiently expressed EGFP-RORα fusion proteins revealed both cell line-dependency as well as isoform-specificity (see also Table 3). Only GFP-tagged RORα1 was completely restricted to the nuclear compartment in all four tested cell lines (Fig. 3A). GFP-tagged RORα2 and RORα3 were completely nuclear in HeLa and SH-SY5Y cells, but showed a lower restriction to this compartment when expressed in 293 HEK and MCF-7 cells (Fig. 3B, C). GFP-tagged RORα4 was mostly located in the nucleus (Fig. 3D).

3.4. Expression of different EGFP-RORα fusion proteins

To further address the isoform-specific localization we were interested in the impact of different protein domains on the subcellular distribution of RORα. Therefore, a series of EGFP-tagged mutated RORα proteins was investigated (Fig. 4A). The integrity of all chimeric proteins was checked by immunoblotting with an anti-GFP antibody. All proteins were detected at the expected

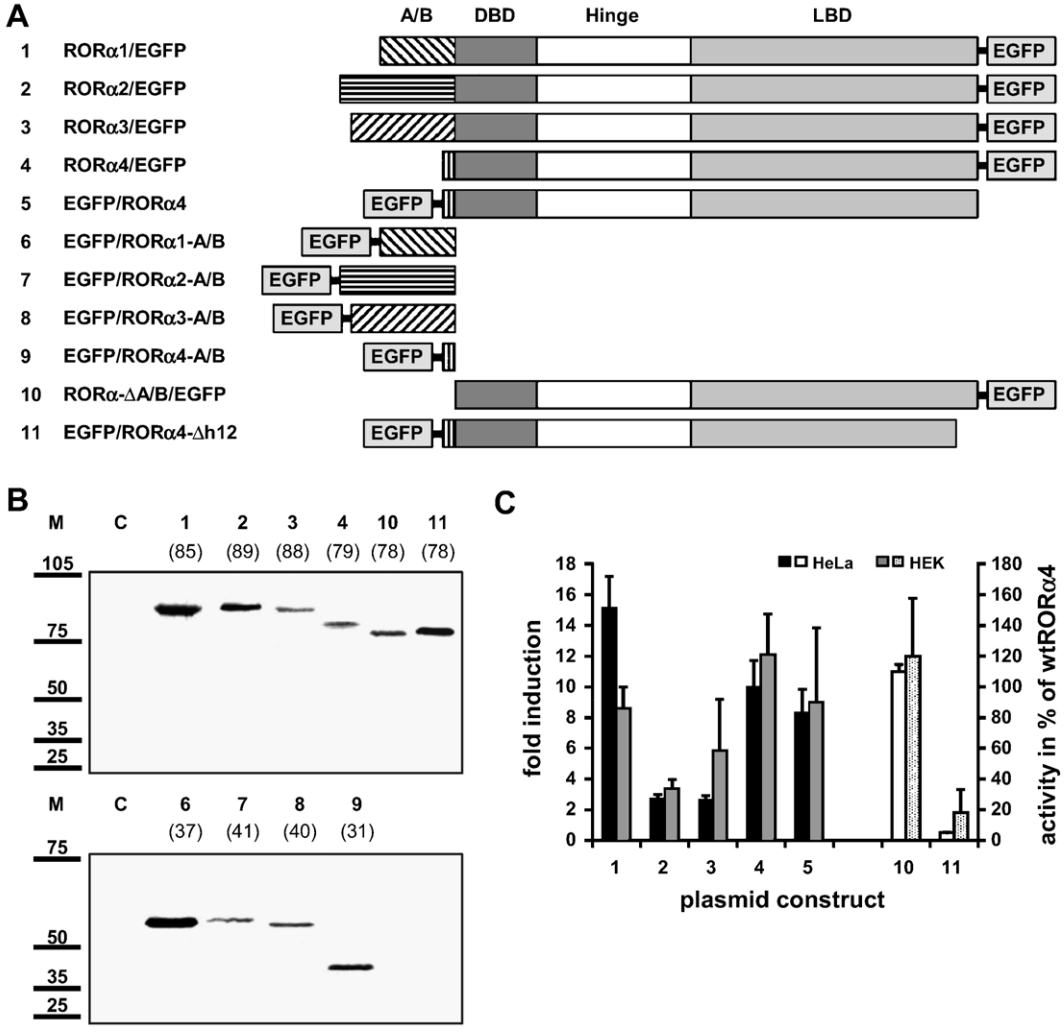

Fig. 4. Characterization of the RORα-EGFP fusion proteins. (A) Structure of the RORα-EGFP fusion proteins. (B) Immunoblotting analysis. HeLa cells were transiently transfected with the indicated expression vectors and harvested after 17 h. Cell lysates were analyzed by SDS-PAGE, blotting and detection with an anti-GFP antibody. Bands of the molecular weight marker are indicated in lane M. Lysate from untransfected cells was used as control (lane C). Constructs are numbered as in (A) and the expected size (in kDa) is given in brackets. (C) Reporter gene analysis. HeLa (black and white columns) or 293 HEK cells (grey columns) were transiently transfected with RORα2$_3$-TKLUC reporter gene construct and the appropriate expression vector. Reporter gene activities were measured 24 h after transfection. Induction (black and dark grey columns) is related to transfection with the corresponding empty expression vector pEGFP-N1 (constructs 1–4) or pEGFP-C2 (construct 5), activity of mutant constructs 10 and 11 is given in % of the wild type RORα4 construct (white and light grey columns). All data are given as mean+S.E. of three experiments done in triplicate.

sizes, no degradation products containing intact GFP (27 kDa) were found (Fig. 4B). Therefore we can exclude the possibility, that the observed fluorescence reflects degradation products of these proteins. Furthermore, the transactivation potential of all proteins still containing a DNA binding domain was determined by reporter gene assay within two cell lines. We could show that all four EGFP-tagged isoforms are active in the reporter gene assay (Fig. 4C). Our results on the transactivation potential of the isoforms correspond with findings by Giguère et al. [28], where RORα2 is much less active on the RORE used here compared to RORα1. The transactivation potential does not significantly differ between HeLa and 293 HEK cells. Only RORα1 showed a higher transactivation potential in HeLa cells as in 293 HEK cells. Differences in transcriptional activity between the four isoforms can only be mediated by their different A/B domains. Interestingly, the A/B domain of RORα4, being the shortest of the four A/B domains, does not contribute to RORα4 transactivation, since the truncated RORα4 protein missing this domain displays 110–120% of the RORα4 activity (Fig. 4C, construct 10). The truncated RORα4 protein missing helix 12 did not show any transactivation potential (Fig. 4C, construct 11). This was expected since helix 12 is known to contain a glutamic acid residue which is essential for the transcriptional activity by the recruitment of coactivators [44].

3.5. The A/B domains of RORα2, 3, and 4 mediate cytoplasmic localization

Differences in the subcellular localization of the four RORα isoforms should be conferred by their N-terminal A/B domains, since the isoforms differ only in these domains. To investigate the role of the A/B domains, we expressed the four different A/B domains as well as the common part of the RORα isoforms, lacking the A/B domain and containing only DBD, hinge region and LBD. As shown in Fig. 5, only the GFP-tagged A/B domain of RORα1 was evenly distributed in the cells. (Fig. 5A1, B1), whereas the other three A/B domains were exclusively found in the cytosol. With a molecular mass of less than 40 kDa, these proteins are probably able to pass the nuclear pore complex by simple diffusion. Therefore, the even distribution of the RORα1 A/B domain indicates that it should not contain any localization signal, while the A/B domains of the other three isoforms should possess a signal for nuclear export or cytoplasmic retention.

In contrast, the common part of the four isoforms, lacking an A/B domain, was predominantly restricted to the cell nucleus (Fig. 5A5, B5). These data indicate that the common part of the RORα isoforms mediates nuclear localization.

The complete restriction of the untruncated RORα1 protein to the nuclear compartment was most probably enabled by the lack of a signal inducing cytosolic localization within its A/B domain.

3.6. RORα can associate with the cell membrane

Besides a clear nuclear and cytosolic distribution, GFP-tagged RORα isoforms 2, 3, and 4 expressed in 293 HEK cells were additionally detected at cellular membranes (Fig. 6A1–3). In case of RORα4 this membrane association was only observed in cells that grew in medium supplemented with stripped FCS.

We found that deletion of the C-terminal part of RORα4 containing helix 12 resulted in the loss of ability of membrane association. As shown in Fig. 6A4, the membrane association of RORα4 found in 293 HEK cells was not detected in this truncation mutant, which suggests that membrane association is mediated by helix 12.

At cellular membranes RORα might gain direct access to its putative ligand cholesterol which could regulate the functions of the membrane associated receptor form. Therefore, we investigated whether cholesterol depletion influences the subcellular localization of RORα2, RORα3, and RORα4. To deplete cholesterol from the cell, medium supplemented with charcoal-stripped, delipidated FCS was used. Free cholesterol was depleted using 2-hydroxypropyl-β-cyclodextrin, intracellular cholesterol synthesis was inhibited with cerivastatin for 6 h. Under these conditions,

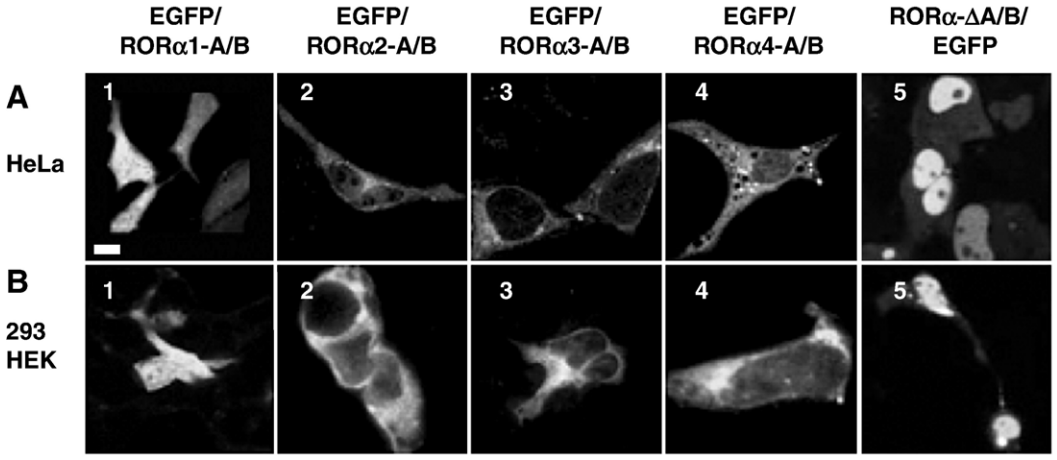

Fig. 5. Impact of the isoform specific A/B domains on the subcellular distribution of RORα. Cells were transiently transfected with the indicated expression vectors and images taken by CLSM. The bar represents 10 μm. Expression of (1) EGFP/RORα1-A/B; (2) EGFP/RORα2-A/B; (3) EGFP/RORα3-A/B; (4) EGFP/RORα4-A/B; (5) RORα4-ΔA/B/EGFP in (A) HeLa and (B) 293 HEK cells is shown.

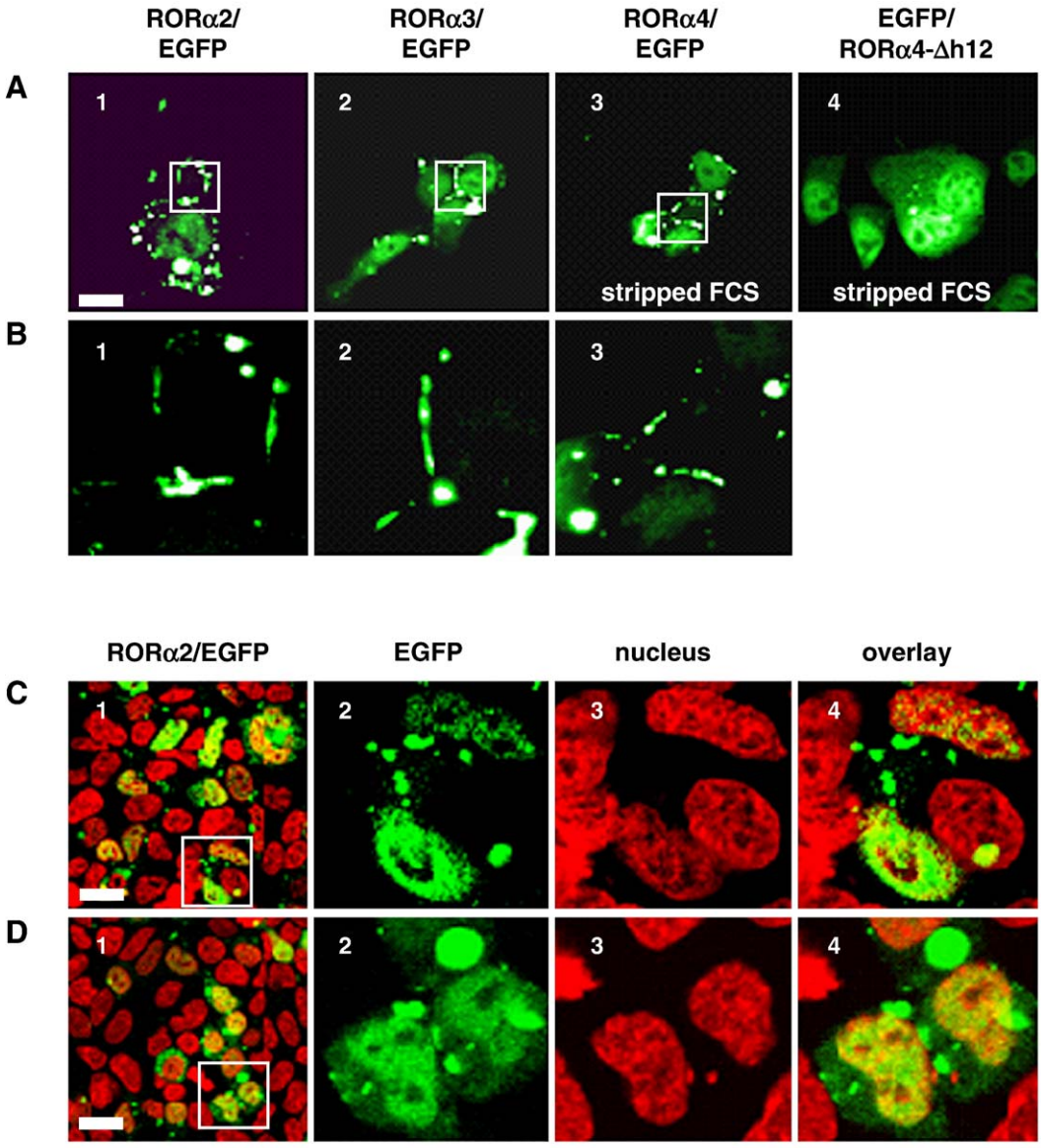

Fig. 6. Plasma membrane association of RORα in 293 HEK cells. Cells were transiently transfected with the indicated expression vectors and images taken by CLSM. The bar represents 10 μm in panel A. Panel B represents a magnified section of the indicated box in panel A. As indicated, cells were cultured in stripped medium after transfection. A more detailed view of RORα2/EGFP localization is given in panels (C) and (D). In (D) cells were cholesterol depleted after transfection for 6 h. Panel (1) shows an overview, panels (2–4) show magnified sections of the indicated boxes with (2) EGFP in green; (3) TO-PRO-3 in red; and (4) overlay. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

subcellular localization was not altered (data shown for RORα2, Fig. 6C and D).

4. Discussion

RORα displays an isoform-specific expression in different tissues, due to different promoter usage and alternative splicing. Transcripts of RORα4 were found in a wide range of human organs and tissues including brain, heart, lung, and skin with highest levels detected in peripheral blood leukocytes [29]. The expression of RORα1 is likewise ubiquitous with differences in expression levels compared to RORα4. For instance, in mouse liver cells, RORα4 expression is predominant compared to RORα1 [45]. Within human vascular cells, the expression of RORα1 is higher in endothelial cells, while aortic smooth muscle cells and normal arteries express higher levels of RORα4 [46]. As described for different human tissues, RORα1 and RORα4 were found to be widely expressed in human cell lines with differing expression levels.

In contrast to RORα1 and 4, the expression of the two human isoforms RORα2 and RORα3 is very restricted. RORα3 could only be detected in the tissue where it was cloned from, in human testis [28]. In addition to the expression in human testis, RORα2 was recently found in cells of the immune system [32]. In line with these data, we could not detect RORα3 in any of the tested cell lines, while RORα2 was found in the B cell line U266, corresponding with its detection in CD19+ B cells [32]. Although RORα2 was also found in human T cells, none of the tested T cell lines showed an expression of this isoform. As described for

different human tissues, RORα1 and RORα4 were found to be widely expressed in human cell lines with differing expression levels.

Furthermore, we could show in this study that the RORα isoforms are not only differentially expressed but they also differ in their subcellular localization representing an additional mechanism for the regulation of RORα transcriptional activity. Many nuclear receptors are known to shuttle between the cytosol and the nucleus [10]. The regulation of their subcellular distribution depends on nuclear localization signals and nuclear export signals located in different receptor domains.

Nuclear translocation is due to the presence of NLS, short sequences rich in basic amino acids, often found at the junction of DBD and hinge region [47]. For many ligand-activated receptors, an additional NLS in the LBD was described, which functions in a ligand-dependent manner [15]. However, the sequence of these ligand-dependent NLS still remains unknown. For the orphan nuclear receptor Rev-erbα, closely related to RORα, an unusual NLS was identified recently within the DNA binding domain [47]. This NLS is based on two basic residues not present in RORα excluding the presence of this signal in the DBD of RORα. We could show that the common part of the four isoforms comprising the DBD, the hinge region and the LBD mediates nuclear localization.

Nuclear export signals are often sequences rich in leucine residues [48]. However, for diverse nuclear receptors missing such a leucine-rich NES an alternative nuclear export signal within the DBD was identified [20]. It encompasses two conserved phenylalanine residues located between the two zinc fingers. Such a NES might also be present in RORα, since its DBD was found to be exclusively located in the cytosol of COS-1 cells [47] and double mutation of these two residues resulted in a complete restriction of the protein to the nucleus of 293 HEK and HeLa cells (data not shown). Furthermore, we identified a signal mediating cytoplasmic localization in the A/B domains of the RORα isoforms 2, 3, and 4, whereas the A/B domain of RORα1 does not affect cellular localization. Thereby, RORα is the first nuclear receptor for which an isoform-specific localization is described, that results from differential localization signals within the isoform-specific A/B domains. Whether the strict cytoplasmic localization mediated by the A/B domain is due to nuclear export or cytoplasmic retention remains to be elucidated. For many nuclear receptors, cytoplasmic localization was shown to be dependent on a nuclear export signal [17,20]. However, nuclear export can also occur without this NES [49,50].

Furthermore, we could identify RORα as one of the nuclear receptors that can also associate to cellular membranes. An association with the plasma membrane was recently described for the vitamin D receptor [24]. For the estrogen receptor it was shown that the membrane-associated form has rapid non-genomic effects—it is involved in signaling cascades from the cellular membrane [21,22]. We could show that the C-terminal helix 12, known to be essential for the interaction of nuclear receptors with coactivator proteins [49], is involved in the membrane association of RORα4. The missing membrane association of RORα1 might be explained by the lack of cytoplasmic localization, the NLS in the hinge region/LBD probably prevents its cytosolic localization and subsequently its membrane association. The functional role of the observed membrane association remains to be elucidated. We hypothesized that at cellular membranes RORα might gain direct access to its putative ligand cholesterol which could regulate the functions of the membrane associated receptor form. However, cholesterol depletion of 293 HEK cells did not alter RORα localization.

Altogether, our results and previous data in the literature suggest that RORα activity is regulated at multiple levels. Usage of different promoters and alternative splicing results in differential isoform-specific tissue expression. Here, we could show that the isoform-specific A/B domains mediate different access of RORα to the nucleus resulting in regulation of transcriptional transactivation of this constitutively active nuclear receptor. Thus, in addition to their different DNA binding properties, cellular distribution seems also to contribute to the differences between the isoforms in receptor function.

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