Retinoid X Receptor γ Is Necessary to Establish the S-opsin Gradient in Cone Photoreceptors of the Developing Mouse Retina

Melanie R. Roberts,¹,² Anita Hendrickson,² Christopher R. McGuire,² and Thomas A. Reh²

PURPOSE. The retinoid X receptors (RXRs) are members of the family of ligand-dependent nuclear hormone receptors. One of these genes, RXRγ, is expressed in highly restricted regions of the developing central nervous system (CNS), including the retina. Although previous studies have localized RXRγ to developing cone photoreceptors in several species, its function in these cells is unknown. A prior study showed that thyroid hormone receptor β2 (TRβ2) is necessary to establish proper cone patterning in mice by activating medium-wavelength (M) cone opsin and suppressing short-wavelength (S) cone opsin. Thyroid hormone receptors often regulate gene transcription as heterodimeric complexes with RXRs.

METHODS. To determine whether RXRγ cooperates with TRβ2 to regulate cone opsin patterning, the developmental expression of RXRγ was examined, and cone opsin expression in RXRγ-null mice was analyzed.

RESULTS. RXRγ was expressed in postmitotic cones and was transiently downregulated at the time of S-opsin onset in both mouse and human cones. RXRγ-null mice expressed S-opsin in all cones, similar to the TRβ2-null mice. Unlike TRβ2-null mice, which did not express M-opsin, RXRγ-null mice had a normal pattern of M-opsin expression.

CONCLUSIONS. RXRγ is essential (along with TRβ2) for suppressing S-opsin in all immature cones and in dorsal cones in the mature retina, but it is not necessary for M-opsin regulation. These results demonstrate a critical role for RXRs in regulating cell differentiation in the CNS and highlight a remarkable conservation of opsin regulation from Drosophila to mammals.


The retinoid X receptors (RXRs) are members of the family of ligand-dependent nuclear hormone receptors that regulate gene expression either as homodimers or more frequently as heterodimeric complexes with other nuclear hormone receptors (for review, see Ref. 1). Studies of mice with targeted gene mutations in each of the three RXR isoforms—α, β, and γ—have demonstrated that RXRs regulate several developmental processes, including cardiogenesis and neurogenesis.²,³ Although these studies have revealed some redundancy of function, they also have demonstrated some isoform-specific functions. For example, RXRα heterodimerizes with retinoic acid receptors (RARs) to regulate retinal growth and cardiac development.⁴

Although all three isoforms are expressed in the developing nervous system, RXRγ has the most restricted and developmentally regulated expression. It is expressed in the developing striatum, part of the tectum, the pituitary, the ventral horns of the spinal cord,⁵,⁶ and the retina.⁷ RXRγ-null mice show isoform-specific defects in both striatal and hippocampal function, although TRβ can partially compensate for the loss of RXRγ in the striatum.⁸ RXRγ has also been identified in the developing retina of Xenopus, chicks, and mice.⁹,¹⁰ It has a highly restricted pattern of expression; and, in both chicks and mice, RXRγ is expressed in developing cones. Despite the conserved expression pattern in developing cone photoreceptors, the function of RXRγ in cone development is not known.

We have shown that thyroid hormone receptor β2 (TRβ2), another member of the same family of nuclear hormone receptors, has a role in the developmental “choice” of which opsin gene to express.¹¹ Like most mammals, mice have two opsin proteins that respond maximally to different wavelengths: S-opsin to short wavelengths and M-opsin to longer wavelengths. However, the ratio and distribution of cone opsins varies dramatically among species (for review, see Ref. 12). For example, S-opsin is excluded from the central fovea of human retinas.¹³,¹⁴ In mice, however, there is an opposing gradient of M- and S-opsin, so that M-opsin expression is highest in dorsal cones, whereas S-opsin predominates in the ventral retina.¹⁵ TRβ2-null mice show a profound disruption in the cone opsin gradient. These mice also show a profound disruption in the cone opsin gradient. TRβ2-null mice mice express S-opsin, which indicates that TRβ2 is also necessary to suppress S-opsin. Because TRs often regulate transcription as heterodimers with RXRs, we hypothesized that RXRγ may also play a part in the determination of cone opsin expression.

To determine whether RXRγ is necessary for proper cone photoreceptor development, we examined the expression of RXRγ during retinal development and analyzed the cone phototype of RXRγ-deficient mice. We found that RXRγ is expressed in developing cones in both the mouse and human retina and that RXRγ is downregulated at the time of S-opsin onset in both species. Like TRβ2-deficient mice, RXRγ-deficient mice express S-opsin in every cone. Unlike TRβ2-deficient mice, however, RXRγ-deficient mice have a normal M-opsin gradient. Thus, RXRγ is involved in the regulation of the expression of S-opsin expression, but not that of M-opsin, which suggests that M- and S-opsin are independently regulated by different nuclear hormone receptor complexes.

METHODS

Animals

RXRγ-null mice (C57BL/6; from Ronald Evans, The Salk Institute, La Jolla, CA) were genotyped with separate DNA primer pairs for the 1Graduate Program in Neurobiology and Behavior and the 2Department of Biological Structure, University of Washington, Seattle, Washington.

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wild-type (5’-AATCAGGGTGGTCCAGGTCTGGA/GCTGGGATTGGT3’) and the RXR-null allele (5’-GACAAGAATGGGACTGT/CAACTGTGGATTTGGG3’). Reactions containing all four primers were amplified for 28 cycles (30 seconds at 94°C, 1 minute at 60°C, and 1 minute at 72°C). The day of birth was designated postnatal day (P)0. Human fetal eyes were obtained from the Human Embryology Laboratory (University of Washington School of Medicine) or from ABR, Inc. (Alameda, CA). All experiments were conducted in accordance with the institutional guidelines of the University of Washington School of Medicine and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Tissue Preparation
For immunohistochemistry, retinas were dissected in cold phosphate-buffered saline (PBS), fixed for 2 hours in 4% paraformaldehyde, rinsed in PBS, and equilibrated in 30% sucrose/PBS overnight at 4°C. Retinas were frozen in optimal cutting temperature compound (OCT, Tissue-Tek; Sakura Finetek, Torrance, CA) and cryosectioned at 12 μm (mouse) or 20 μm (human).

Immunohistochemistry and Flatmounts
Slides were incubated overnight in primary antibodies—1:200 rabbit RXRγ (sc-555, lot A1111), 1:150 goat S-sensitive opsin (sc-14356); Santa Cruz Biotechnology, Santa Cruz, CA), 1:500 rabbit S-opsin or M-opsin (AB5407, AB5745; Chemicon International, Temecula, CA), 1:500 rabbit S-opsin or M-opsin (AB5407, AB5745; Chemicon International, Temecula, CA), 1:10,000 rabbit TR (a gift from Lily Ng and Douglas Forrest), or 1:200 rat RXR (mouse) or 20 m (human). Levels of RXRγ in mouse and human cones were determined by quantitative PCR, as described elsewhere. We analyzed the expression of RXRγ in mouse retinas, RXRγ antibodies labeled nuclei in the developing ganglion cell layer and at the scleral surface (developing photoreceptor layer) as early as embryonic day (E)14.5. RXRγ expression extended along the length of the retina in these regions by E15.5 (Fig. 1A). In situ hybridization for RXRγ mRNA showed a pattern of expression similar to that observed with the antisera (Fig. 1B). The scleral surface of the developing retina contains both dividing progenitor cells and immature photoreceptors. To test directly whether the cells that express RXRγ are progenitors or immature photoreceptors, we double-labeled retinal sections from E16 mice with RXRγ and an antibody to phosphohistone H3, which is expressed specifically in mitotic progenitors (Fig. 1C). We did not find any phosphohistone-expressing cells at the scleral surface of E16 retinas that also expressed RXRγ, indicating that RXRγ is not expressed in progenitors. Thus, the RXRγ-positive cells were most likely immature photoreceptors.

There are no known markers to discriminate between immature rods and cones in mouse retinas. However, we suggest that these immature photoreceptors are cones, based on two lines of evidence. First, by E16, most of the cones but only a small percentage of the rods have been generated. Although we cannot rule out the possibility that a few of the early-born rods express RXRγ, many of the RXRγ-expressing photoreceptors must be cones. Second, as the photoreceptors matured, we were able to confirm that RXRγ is expressed specifically in cone nuclei by double-labeling with peanut lectin, which binds specifically to cone outer segments, and with cone-specific opsins (described later). Every lectin-positive cone had an RXRγ-positive nucleus (Fig. 1D) and RXRγ was not present in rod nuclei (not shown).

We also analyzed the expression of RXRγ in human retinas, where it is much easier to distinguish immature cones from rods because of differences in their morphology and protein expression. An early marker of cone photoreceptors in human retina, tubby-like protein 1 (TULP1), is expressed in cones before the onset of cone opsin expression. By double-label immunofluorescence, we found that all TULP1-expressing cones had RXRγ-positive nuclei by fetal week (FW)16 (Fig. 2A). α-Transducin is another cone-specific marker that labels both S- and M-opsin-expressing cones in fetal human retina. At FW16, α-transducin was visible in the cytoplasm of cones across much of the retina. These α-transducin expressing cones also have RXRγ nuclei (Fig. 2B). Thus, our data suggest that RXRγ is an early cone-specific marker.

Dynamic Expression of RXRγ during Cone Development
To analyze further the developmental time course of RXRγ expression, we labeled mouse retinal sections from animals at ages E12.5 through adult. We found RXRγ expression in a few newly generated cones at the scleral surface and also in the ganglion cell layer beginning at E14.5 (Fig. 3A). By E15.5, RXRγ was not present in any cones.
The downregulation in RXRγ persisted in cones through adult stages, though the intensity of expression was somewhat reduced after the first postnatal week (Fig. 3F).

The transient downregulation of RXRγ in cones correlated with the onset of S-opsin expression, which begins at E18. We hypothesized that downregulation of RXRγ is necessary to allow induction of S-opsin. To determine whether the expression of these genes is consistent with the hypothesis, we labeled retinal sections with both S-opsin and RXRγ antisera at E19 (Figs. 3G–I). Although we occasionally observed cells that expressed both S-opsin and RXRγ at this age, nearly all of the cones expressed either RXRγ or S-opsin, but not both. Most S-opsin-expressing cones were found in regions where RXRγ had been downregulated. Moreover, cones expressing the highest levels of S-opsin were usually RXRγ− or expressed very low levels of RXRγ. This downregulation was transient; by P5, RXRγ was re-expressed in all S-opsin-expressing cones (Figs. 3J–L). The double-labeling results in mouse retina are consistent with the hypothesis that a transient downregulation of RXRγ in perinatal cones promotes the onset of S-opsin.

In human retinas, we also observed a transient downregulation of RXRγ in S-opsin-expressing cones at the time of S-opsin onset. At the beginning of S-opsin expression at FW14 and FW16 (Fig. 4), the S-cones had undetectable or low levels of RXRγ. However, by FW18, when S-opsin expression has reached the edge of the retina,13 most S-opsin-expressing cones also re-expressed RXRγ (not shown).

Patterns of RXRγ and TRβ2 during Cone Maturation

We reported earlier that TRβ2 is expressed in developing cones.11 To determine whether TRβ2 has the same developmental pattern as RXRγ, we labeled retinal sections and mounts with TRβ2 antiserum, which was raised against an N-terminal polypeptide of mouse TRβ2 (Lily Ng, Iwan Jones, and Douglas Forrest). We observed TRβ2 in postmitotic cones at E13.5, corresponding with the time of RXRγ onset (data not shown). By E15.5, TRβ2 and RXRγ were uniformly expressed in developing cones (Fig. 5A, compare with RXRγ in Fig. 1A). However, between E19 and P2, we observed a downregulation of TRβ2 in cones, similar to that described for RXRγ (Fig. 5B). At P5, TRβ2 was re-expressed in S-opsin-positive cones (Figs. 5C, 5D). TRβ2 expression declined after the first postnatal week and was present at very low levels in adult cones (not shown).

was expressed uniformly across the retina (Fig. 1A) and remained robust until E17.5 (Fig. 3B). However, there was a dramatic decline of RXRγ expression in cones just before birth. From E19 (Fig. 3C) to P2 (Fig. 3D), we observed only small patches of isolated RXRγ-positive cones. We could not detect a consistent spatial pattern in the downregulation of RXRγ, and the patches of RXRγ varied in number and location, even between littersmates. The downregulation in RXRγ was transient. By P5 all cones re-expressed RXRγ (Fig. 3E). RXRγ expression persisted in cones through adult stages, though the intensity of expression was somewhat reduced after the first postnatal week (Fig. 3F).

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S- and M-opsin Expression in RXRγ-Deficient Mice

To determine whether RXRγ has a role in the regulation of cone opsin expression, we analyzed RXRγ-null mice for changes in opsin expression. TRβ2-null mice do not express M-opsin, but all cones express S-opsin.11 Because RXRγ heterodimerizes with TRβ2 and both receptors are expressed in developing cone nuclei, we hypothesized that RXRγ is necessary, along with TRβ2, to regulate cone opsin expression and predicted that RXRγ-null mice would have a phenotype similar to that of TRβ2-null mice.

In wild-type mice, the S-opsin-expressing cones were concentrated in the ventral retina, and there were few S-opsin-positive cones in the most dorsal retina. This gradient is established during development. Even in P0 retinas, many more ventral cones than dorsal cones expressed S-opsin (Figs. 6A, 6C). By contrast, P0 RXRγ-null mice had a profound disruption in the normal pattern of S-opsin expression: S-opsin was expressed at nearly equal levels in both dorsal and ventral cones (Figs. 6B, 6D). The ectopic expression of S-opsin was maintained in RXRγ-null adult retinas. In adult RXRγ-null retinas, nearly all cones expressed S-opsin (Figs. 6F, 6H), whereas in wild-type retinas, few dorsal cones expressed S-opsin (Figs. 6E, 6G). This phenotype was similar to that of TRβ2-null mice, and supports our hypothesis that a TRβ2-RXRγ heterodimer is necessary to establish the S-opsin gradient by suppressing its expression in a subset of dorsal cones.

Because mice deficient in TRβ2 lack M-opsin, we also examined RXRγ-null mice for changes in M-opsin expression. Remarkably, we did not find the same M-opsin phenotype in RXRγ-null retinas that we had in TRβ2-null retinas. M-opsin expression in RXRγ-null retinal sections (Figs. 6J, 6L) was indistinguishable from the gradient in wild-type sections (Fig. 6I, 6K). We also used quantitative PCR to confirm that the level of RXRγ was downregulated at the onset of S-opsin expression in mouse retinas. (A–F) Retinal sections at various developmental time points were labeled with RXRγ antiserum. RXRγ protein was detected in cone nuclei at E14.5 (A). The number of RXRγ-expressing cones increased until E17.5 (B). RXRγ was transiently downregulated between E19 (C) and P2 (D). RXRγ was re-expressed in all cones by P5 (E) and persisted in adult cones (F). At E19 (G–I), S-opsin (green) turned on in cones with downregulated RXRγ (red, arrowheads). Cells that retained high levels of RXRγ (arrows) had not yet turned on S-opsin. By P5 (J–L), RXRγ (red) was re-expressed in all S-opsin-positive (green) cones.
M- and S-opsin in developing cone photoreceptors is regulated by different nuclear receptor complexes.

FIGURE 4. RXRγ was downregulated in S-opsin-expressing cones in developing human retinas. Sections from FW16 human retinas were costained with RXRγ and S-opsin antisera. A three-dimensional confocal image of the photoreceptor surface revealed numerous RXRγ+/− nuclei (red). However, S-opsin-expressing cones (green) did not express RXRγ(Δ). Scale bar, 10 μm.

of M-opsin expression is similar between wild-type and RXRγ-null retinas (data not shown).

To quantify the opsin gradients in wild-type, RXRγ+/−, and RXRγ-null retinas, we coimmunolabeled retinal flatmounts with an opsin antibody and FITC-peanut lectin (PNA), which labeled all cones (Figs. 7A–D). We determined the percentage of cones that expressed S-opsin in the dorsal, central, and ventral regions of the retina (Fig. 7E). In wild-type retinas, 19% of dorsal cones, 63% of central cones, and 86% of ventral cones expressed S-opsin. By contrast, almost all cones in RXRγ-null retinas expressed S-opsin; 93%, 98%, and 96% of cones expressed S-opsin in the dorsal, central, and ventral retina, respectively. We found a significant gene-dosage effect of RXRγ inactivation on the S-opsin gradient. In RXRγ+/− animals, S-opsin was expressed in 40% of dorsal, 86% of central, and 92% of ventral cones. Counts of M-opsin-expressing cones showed that 94%, 79%, and 34% of wild-type cones expressed M-opsin, compared with 88%, 63%, and 34% of RXRγ-null cones in the dorsal, central, and ventral retina, respectively (Fig. 7E).

RXRs and RXRβ are also expressed in the retina and have been shown to compensate for RXRγ function in other systems. To determine whether other RXR isoforms are upregulated in RXRγ-null retinas, we performed quantitative PCR to compare the levels of RXRα and RXRβ in wild-type and RXRγ-null retinas at P5. RXRα expression was not affected in RXRγ-null retinas. We found a small increase (5.6-fold) of RXRβ in RXRγ-null retinas. However, because of high variation between animals, this increase was not significantly significant. To examine further whether RXRβ compensates for RXRγ in M-opsin regulation, we counted the number of M-opsin-expressing cones in RXRγ−/− double-null mice. M-opsin expression in RXRγ−/− double-null retinas was still graded, but M-opsin expression was significantly reduced compared to RXRγ-null retinas (data not shown).

Taken together, our results indicate that both RXRγ and TRβ2 are necessary to establish the ventral–dorsal gradient of S-opsin by suppressing its expression in a subset of dorsal cones. However, there is a need for TRβ2, but not for RXRγ, in the regulation of M-opsin expression. Thus, the expression of M- and S-opsin in developing cone photoreceptors is regulated by different nuclear receptor complexes.

FIGURE 5. TRβ2 had an expression profile similar to that of RXRγ in the developing mouse retina. TRβ2 expression was examined by immunohistochemistry (compare with RXRγ expression in Figs. 1, 2). (A) By E15.5, TRβ2 was expressed in immature cone nuclei (arrows). Note that, unlike RXRγ, TRβ2 was not expressed in the ganglion cell layer (GCL). Downregulation of TRβ2 was coincident with S-opsin onset. (B) In an E19 retinal section, only a few patches of TRβ2 were visible at the scleral surface (arrows). (C) By P5, TRβ2 (red) was re-expressed in all S-opsin-expressing cones (green). Scale bar: (A) 100 μm; (D) 25 μm.

DISCUSSION

Other reports have shown expression of RXRγ in cone photoreceptors of several species. To determine whether RXRγ has a role in cone development, we examined its spatial and temporal expression and the effect of RXRγ loss of function on opsin expression in the mouse retina. We found that RXRγ is necessary for the developmental determination of cone opsin
expression. Mice deficient in RXRγ express S-opsin in nearly every cone, resulting in a loss of normal cone opsin patterning. Although we confirmed the previous reports that RXRγ is expressed in developing and adult cone photoreceptors, we

**FIGURE 6.** RXRγ−/− mice had upregulated S-opsin and normal M-opsin expression. The expression of S-opsin and M-opsin was examined by immunohistochemistry. At P0 (A–D), S-opsin was visible in the cell body and processes of the cones. P0 wild-type retinas had few S-opsin-expressing cones in the dorsal retina (A) and many in the ventral retina (C). By contrast, P0 RXRγ−/− retinas had many S-opsin-expressing cones in both the dorsal (B) and ventral (D) retina. In adult retinas (E–L), opsins were mostly localized to cone outer segments (arrows), though S-opsin is also visible in a few cell bodies (arrowhead). Adult wild-type retinas have few S-opsin-expressing cones in the dorsal retina (E) and many in the ventral retina (G), whereas RXRγ−/− retinas had many S-opsin-expressing cones in both dorsal (F) and ventral (H) retina. M-opsin expression (I–L) appeared to be similar in both wild-type (I, K) and RXRγ−/− (J, L) retinas. In both, M-opsin was highest in the dorsal retina (I, J) and lowest in the ventral retina (K, L).
A striking, but transient, downregulation of RXRγ and TRβ2 in the perinatal retina that coincided with S-opsin onset. We also found that RXRγ was expressed in developing human cone photoreceptors, suggesting that the function of this gene in cone development is conserved.

We propose that RXRγ and TRβ2 cooperate to regulate the pattern of S-opsin expression in developing cones. Studies in many systems have shown that TR/RXR heterodimers can regulates transcription.21–24 We have reported that mice deficient in TRβ2 show a profound disruption in the normal pattern of opsin expression in cone photoreceptors.3 We now show that both TRβ2 and RXRγ are present in developing cones and that loss of either receptor results in a similar disruption in the pattern of S-opsin expression. Thus, it is likely that heterodimers of RXRγ and TRβ2 normally establish the pattern of S-opsin expression by inhibiting it in many of the dorsal retinal cones. Of interest, both RXRγ and TRβ2 were transiently downregulated at S-opsin onset in both mice and humans, suggesting that this downregulation is necessary for the timing of S-opsin expression.

In contrast with our previous analysis of the TRβ2-null mice, we found that RXRγ was not necessary for activation of M-opsin expression. Although TRβ2-null cones do not express M-opsin, RXRγ-null retinas have normal M-opsin expression. Thus, S- and M-opsin must be regulated by different nuclear receptor complexes. Because RXRβ and α are also expressed in the retina,4 we hypothesized that one of these isoforms may dimerize with TRβ2 and compensate for RXRγ’s loss, much as RXRβ does in the striatum.4 However, we did not find an upregulation of either RXRα or β in RXRγ-null retinas and found that M-opsin expression was graded but reduced in RXRβ-/double-null retinas. We could not examine M-opsin in RXRα-null mice because they die in utero.5 Thus, we cannot rule out that RXRα alone is sufficient to compensate for the loss of both RXRγ and β without being significantly upregulated. Incomplete compensation by RXRα could explain the small but significant decrease of M-opsin in the central retina of RXRγ-null mice.

Our model of how RXRγ fits into photoreceptor development is shown in Figure 8. Cones are generated in mice from multipotent progenitors from E10.5 to E16.18 Shortly after withdrawal from the cell cycle, both rods and cones express photoreceptor-specific transcription factors such as crx and NeuroD1.25 Several nuclear hormone receptors are involved in regulating the next stages of photoreceptor differentiation. Nr2e3 is an orphan nuclear receptor that is expressed in rods.26,27 Mutations in Nr2e3 underlie the human enhanced S-syndrome, and mouse mutants in this gene have an increased number of S-cones.28–30 Targeted deletion of Nrl, a leucine zipper transcription factor upstream of Nrl2e3, has an even more extreme phenotype. Rods fail to develop and nearly all photoreceptors become S-opsin-expressing cones.31 We have shown that TRβ2 is critical for the developmental choice between S- and M-opsin in cones,11 and now we show that RXRγ is also a critical regulator of this step. In our model, RXRγ/γ/TRβ2 heterodimers suppress S-opsin expression in a subset of cones, whereas M-opsin is regulated by either a TRβ2 homodimer or TRβ2 with an unknown heterodimeric partner.

As noted in the introduction, mice have an opposing gradient of M- and S-opsin, so that M-opsin expression is highest in the dorsal retina, whereas S-opsin-expressing cones predominate in the ventral retina.12,13 By contrast, the human central fovea lacks S-opsin-expressing cones.13,14 Although several studies have described gradients in all-trans-retinoic acid,34,35 the ligand for RARs, the distribution of RXR ligands in the retina has not been reported. Nevertheless, there is evidence that ligand-dependent RXR activation occurs normally in the developing retina,36 consistent with the possibility that a gradient in an RXR ligand controls the graded pattern of opsin expression.

The transcriptional mechanisms that control photoreceptor specification are remarkably well conserved among species. Notably, the Drosophila homologue of RXR, ultraspiracle (usp), regulates opsin transcription in a heterodimeric complex with the ecdysone receptor, the fly homologue of thyroid hormone receptor. Flies have eight photoreceptors (R1–8) that express one of five opsins. The R7 photoreceptor expresses either Rh-3 or Rh-4 opsin (for review, see Ref. 36). Remarkably, R7 photoreceptors in usp-null flies express only Rh-3.37,38 Thus, usp may suppress Rh-3 in fly photoreceptors, just as RXRγ suppresses S-opsin in mice. Because the transcriptional machinery controlling opsin expression is well conserved, RXR, TRβ2, and their ligands may be used to generate highly varying photoreceptor patterns in a multitude of species, from flies to humans.

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