Thyroid hormone receptors TRα1 and TRβ differentially regulate gene expression of *Kcnq4* and prestin during final differentiation of outer hair cells

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Summary

Thyroid hormone (TH or T3) and TH-receptor β (TRβ) have been reported to be relevant for cochlear development and hearing function. Mutations in the TRβ gene result in deafness associated with resistance to TH syndrome. The effect of TRα1 on neither hearing function nor cochlear T3 target genes has been described to date. It is also uncertain whether TRα1 and TRβ can act simultaneously on different target genes within a single cell. We focused on two concomitantly expressed outer hair cell genes, the potassium channel *Kcnq4* and the motor protein prestin *Slc26a5*. In outer hair cells, TH enhanced the expression of the prestin gene through TRβ. Simultaneously *Kcnq4* expression was activated in the same cells by derepression of TRα1 aporeceptors mediated by an identified TH-response element, which modulates KCNQ4 promoter activity. We show that T3 target genes can differ in their sensitivity to TH receptors having the ligand either bound (holoreceptors) or not bound (aporeceptors) within single cells, and suggest a role for TRα1 in final cell differentiation.

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Key words: KCNQ4, Prestin, Final differentiation, TR aporeceptors

Introduction

Thyroid hormone (TH or T3) regulates many processes in the development of mammals before the onset of function in a variety of organ systems. This pleiotropic nature of TH is mediated through interactions with ligand-modulated nuclear receptors encoded by two genes *TRα* and *TRβ* (Wu and Koenig, 2000) that can either positively or negatively regulate target genes in response to T3 (Lazar, 2003). To date it is unknown whether T3 target genes can differ in their sensitivity to distinct thyroid hormone receptor (TR) isoforms or to TRs having the ligand bound (holoreceptors) or not bound (aporeceptors). This is a crucial question considering the pathologies associated with hypothyroidism or TR mutations (O'Shea and Williams, 2002). The presumptive redundant effects of coexpressed TRs could definitively mask the individual TR responsiveness of genes and dramatically define the pathological phenotype. To unravel the question of TR specificity at the single-cell level, we focused on two cochlear outer hair cell (OHC) genes that are concomitantly expressed during a crucial time period during which hypothyroidism leads to irreversible hearing deficits in adults (Deol, 1973; Uziel et al., 1985). Neurosensory deafness in humans and rodents is presumed to be related to mutations in the TRβ, leading to resistance to thyroid hormone (RTH), caused by a transdominant negative transcriptional effect (Refetoff et al., 1993; Weiss and Refetoff, 2000). A role of TRα1 for auditory function was suggested in a study of TRα2-deficient mice exhibiting increased expression of TRα1, which was proposed to compensate for missing TRβ activity (Ng et al., 2001). However, no T3 target genes have been identified in the cochlea at the level of TR-isoform-specific transcriptional regulation.

During a developmental period before the onset of hearing, the gene that encodes the outer hair cell motor protein prestin, *Slc26a5* (Zheng et al., 2000), is expressed from approximately postnatal day (P) 3 in euthyroid rats, whereas its expression is delayed under conditions of hypothyroidism (Weber et al., 2002). The voltage-dependent K^+ channel KCNQ4, which is responsible for the predominant K^+ conductance, $I_{K,n}$, of mature OHCs (Marcotti and Kros, 1999) is expressed in rodents from approximately P6 onwards (present study) (Kharkovets et al., 2000). In order to study the specific effect of TH on the expression of these genes, a variety of animal models of hypothyroidism and several TR mutant mice were used to examine their TH dependency. These included (1) animals with goitrogen-induced hypothyroidism; (2) animals that acquired hypothyroidism as a consequence of a naturally occurring point mutation in the thyrotropin receptor (*Tshr*^{hyt} mutants) (for a review, see Walsh and McGee, 2001); (3)

Table 1. Mouse mutants used

Strain	Molecular characterization	Pathophysiology	Reference
Pax8$^{-/-}$	Targeted inactivation of the *Pax8* gene	Athyroidism Absence of thyroid follicular cells Deafness, degeneration of outer hair cells	Christ et al., 2004 Mansouri et al., 1998
Tshr$^{hyt/hyt}$	Naturally inherited, autosomal recessive P556L point mutation in the TshR	Primary, congenital hypothyroidism Hypoplasia, retarded growth, infertility Deafness	Beamer et al., 1981 Sprenkle et al., 2001a Sprenkle et al., 2001b Sprenkle et al., 2001c Stein et al., 1994
TRα1$^{-/-}$	Replacement of TRα1-specific coding region with that of TRα2	Abnormal heart rate Reduced body temperature	Wikstrom et al., 1998
TRβ$^{-/-}$	Targeted inactivation of the *TRβ* gene	Recessive resistance to thyroid hormone Hyperthyroidism Deafness	Forrest et al., 1996b
TRα1$^{-/-}$β$^{-/-}$	Compound mutant mice, generated by crossing TRα1 and TRβ mice	Hyperthyroidism Retarded growth Retarded bone maturation Female infertility Deafness	Gothe et al., 1999 Rusch et al., 1998
TRα1$^{m/+}$	Dominant-negative R384C mutation introduced in TRα1	Tenfold reduced ligand binding Retarded postnatal development and growth Cardiac function abnormalities	Tinnikov et al., 2002

athyroid, *Pax8*$^{-/-}$ mutants (Christ et al., 2004; Macchia, 2000; Mansouri et al., 1998; Tell et al., 1999); (4) *TRα1*$^{-/-}$ (Rusch et al., 1998; Wikstrom et al., 1998), *TRβ*$^{-/-}$ (Forrest et al., 1996a; Forrest et al., 1996b; Gauthier et al., 1999), *TRα1*$^{-/-}$/*β*$^{-/-}$ (Gothe et al., 1999; Rusch et al., 2001) mutants; and (5) euthyroid *TRα1*$^{m/+}$ mice carrying a dominant-negative TRα1 R384C point mutation (Tinnikov et al., 2002). The different mutant strains used in this study are listed and summarized in Table 1.

Both the prestin gene (Weber et al., 2002) and *Kcnq4* (present study) were identified as simultaneously expressed T3-dependent genes that are, however, differentially regulated by either TRβ or TRα1. A role for TRα1 in inner-ear development and specifically final differentiation of OHCs was demonstrated. Furthermore, the data show for the first time that apo-TRα1 can repress a T3 target gene independently but in parallel to TRβ acting on a different T3 target gene within the same cell.

Results

KCNQ4 and prestin protein expression during early stages of auditory function

Before the onset of hearing, the subcellular localization of the two outer hair cell proteins KCNQ4 and prestin overlapped between P4 and P7, occupying the entire basolateral membrane area of the OHCs (Fig. 1A, P8). Between P8 and P12, however, the distribution of KCNQ4 gradually shifted to the basal pole of the OHC membrane, whereas prestin shifted to the lateral membrane (Fig. 1A, P12), resulting in a completely separated but neighboring distribution from P12 onwards (Fig. 1B).

KCNQ4 and prestin protein expression are differentially affected by hypothyroidism

In the absence of thyroid hormone, the expression of prestin was reduced and the distribution pattern remained immature (Fig. 2A, hypo, red) (Weber et al., 2002). By contrast, KCNQ4 expression was completely lacking in the OHCs of hypothyroid rats (Fig. 2A, hypo, green). Using northern blot analysis both the ~3.9 kb and the ~3.8 kb *Kcnq4* transcripts were detected (Fig. 2B, con as) (Kubisch et al., 1999), which were significantly reduced in the cochleae of hypothyroid animals (Fig. 2B, hypo, as). The corresponding sense probes showed no signal (Fig. 2B, con, sense). Reduced *Kcnq4* mRNA levels were also noted in the cochleae of P12 hypothyroid rats (Fig. 2C, hypo) compared with control animals (Fig. 2C, con) using semi-quantitative RT-PCR. The housekeeping gene cyclophilin was used as an internal control. In addition, *Kcnq4* mRNA was detected by in situ hybridization in both outer and inner hair cells of control animals (Fig. 2D, control) (see also Oliver et al., 2003) and was absent in age-matched hypothyroid rats (Fig. 2D, hypo).

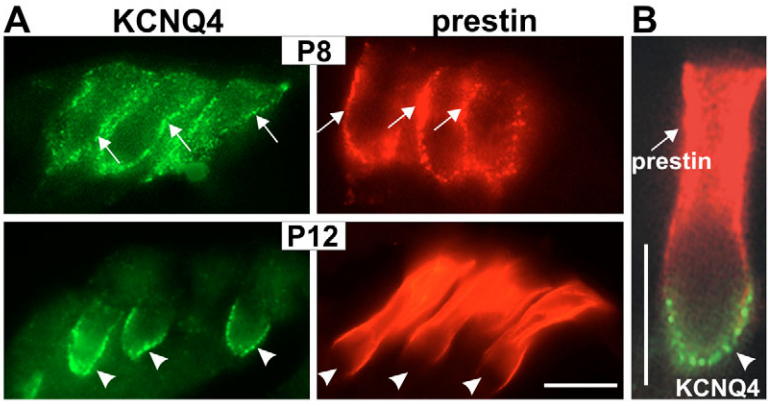

Fig. 1. Coincident redistribution of KCNQ4 and prestin in rat OHCs. (A) Immunohistochemistry shows KCNQ4 (green) and prestin (red) in OHCs before (P8) and at onset of hearing (P12). (B) Double immunohistochemistry of prestin (red) and KCNQ4 (green) in a mature (P21) OHC. Arrows, KCNQ4 and prestin protein; arrowheads, basal pole of OHCs. Bars, 20 μm.

In two commonly used mouse models of hypothyroidism, i.e. *Tshr*^{hyt} mutants (*Hyt/Hyt*) which have a point mutation in the thyrotropin receptor, and *Pax8*$^{-/-}$ mutants which lack thyroid follicular cells, similar KCNQ4 and prestin expression patterns were observed (data not shown). This verifies the uniformity of TH influence, independent of the mode of induction of hypothyroidism.

KCNQ4 but not prestin protein expression differs depending on the absence of thyroid hormone or its receptors

KCNQ4 and prestin protein expression profiles in the absence of TH were compared with those in *TRα1*$^{-/-}$/*β*$^{-/-}$ mutant mice, which carry deletions of both TRα and TRβ. Immunohistochemical analyses were carried out with antibodies against KCNQ4 or prestin, along with the efferent-specific marker synaptophysin (Gil-Loyzaga and Pujol, 1988; Knipper et al., 1995), shown for P10 animals in Fig. 3.

Similar to prestin expression profiles in hypothyroid animals (Fig. 3A, red) prestin was expressed in *TRα1*$^{-/-}$/*β*$^{-/-}$ mutants (Fig. 3C, red), however its distribution remained immature. Surprisingly, in contrast to absent KCNQ4 expression in hypothyroid animals (Fig. 3B, red) KCNQ4 was expressed when both TRα and TRβ were deleted (Fig. 3D, red), although its subcellular distribution was still immature. The results suggest that *Kcnq4* gene expression is regulated by a TH aporeceptor that may repress transcription (Perissi et al., 1999), and when deleted, might cause the less-severe KCNQ4 phenotype observed in the *TRα1*$^{-/-}$/*β*$^{-/-}$ mutants.

KCNQ4 and prestin protein expression is differentially regulated by either TRα1 or TRβ

To test this inference, the effect of hypothyroidism in animals with single TR deletions was considered. The TR subtype responsible for repression of *Kcnq4* should be identifiable, as its deletion should render the *Kcnq4* gene independent of the influence of TH.

KCNQ4 and prestin protein expression was analyzed in both *TRα1*$^{-/-}$ and *TRβ*$^{-/-}$ mutants in which hypothyroidism had been induced by treating neonates with the goitrogen MMI (methyl-mercapto-imidazol). A subset of goitrogen-treated mutant mice were rescued from hypothyroidism by daily injections of T4 from birth onwards to serve as a control condition. When hypothyroidism was induced in neonatal *TRβ*$^{-/-}$ mutants, KCNQ4 protein was not expressed in OHCs (Fig. 4A, red), whereas prestin was expressed but the immature subcellular distribution persisted (Fig. 4C, red). By contrast, KCNQ4 expression was normal in *TRα1*$^{-/-}$ mutants even under conditions of induced hypothyroidism (Fig. 4B, red), confirming that the TRα1 deletion promoted the expression of

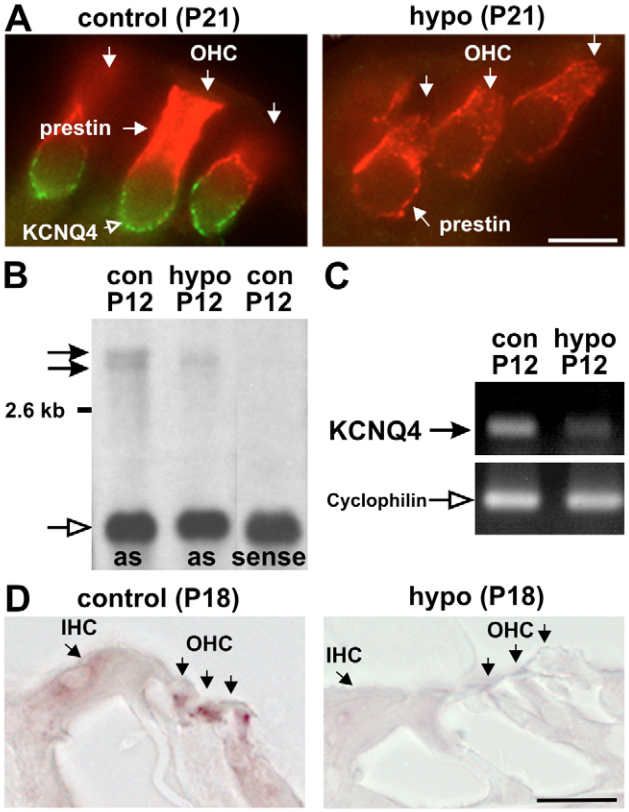

Fig. 2. KCNQ4 expression in control and hypothyroid rats. (A) Double immunohistochemistry of prestin (red) and KCNQ4 (green) in OHCs of control and hypothyroid (hypo) rats at P21. (B) Using northern blotting, both, the ~3.9 kb and ~3.8 kb *Kcnq4* transcripts (filled arrows), are reduced in the cochleae of P12 hypothyroid rats (hypo) compared with controls (con), detected with a *Kcnq4*-specific antisense riboprobe (as). Sense control shows no signal. Blots were probed with a cyclophilin-antisense probe (open arrow) as a housekeeping gene. (C) Reduced *Kcnq4* mRNA levels in the cochlea of P12 hypothyroid rats (hypo) compared with controls (con) is also noted using semi-quantitative RT-PCR. The housekeeping gene cyclophilin was used as a control. (D) Localization of *Kcnq4* mRNA in control and hypothyroid (hypo) rat cochleae at P18 using in situ hybridization. Note the strong signals in OHCs and a weaker signal in IHC of controls, whereas no signals are detectable in hair cells of hypothyroid rats. Bars, 10 μm (A); 20 μm (D).

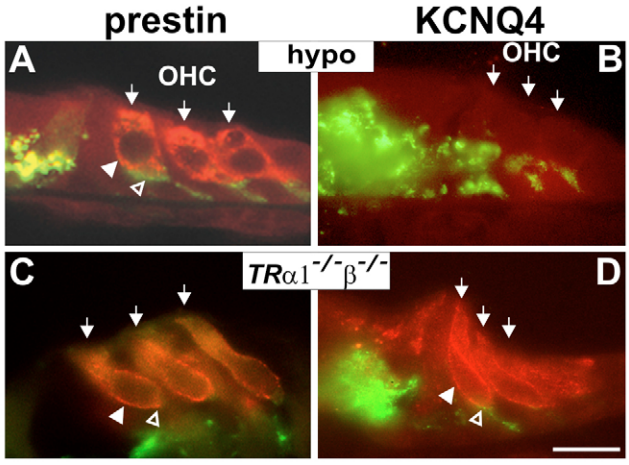

Fig. 3. KCNQ4 and prestin expression in OHCs of *TRα1*$^{-/-}$*β*$^{-/-}$ mutants compared with hypothyroidism at P10. (A,B) In OHCs of hypothyroid rats (hypo), prestin is expressed but its immature distribution persists (A, red) whereas KCNQ4 expression is absent (B, red). (C,D) In *TRα1*$^{-/-}$/*β*$^{-/-}$ mutants, both prestin (C, red) and KCNQ4 (D, red) are expressed but their immature distribution persists. Double immunohistochemistry was performed with synaptophysin (green). Open arrowheads, basal pole of OHCs; filled arrowheads, KCNQ4 and prestin protein. Bar, 20 μm.

KCNQ4. In the case of the prestin gene, the deletion of TRα1 did not alter the typical expression profile observed in hypothyroidism; i.e. an immature subcellular distribution pattern persisted (Fig. 4D, red). These findings collectively validate the view that apo-TRα1 represses KCNQ4 expression, but does not affect the expression of prestin.

Treatment with T4 rescued the KCNQ4 phenotype from neonatally induced hypothyroidism in *TRβ*$^{-/-}$ mutants (Fig. 4E, red), while not affecting the normal phenotype in *TRα1*$^{-/-}$ mutants (Fig. 4F, red), a finding that is consistent with the idea that TH mediates KCNQ4 expression independently of TRβ activity by releasing the *Kcnq4* gene from the repressive action of TRα1. Consistent with the idea that TRβ rather than TRα1 controls prestin expression and distribution, prestin expression is insensitive to TH when TRβ is deleted (Fig. 4G, red) whereas its expression remains dependent on TH in *TRα1*$^{-/-}$ mutants (Fig. 4H, red). Thus, OHCs express two genes that are simultaneously but differentially regulated by either TRα1 or TRβ.

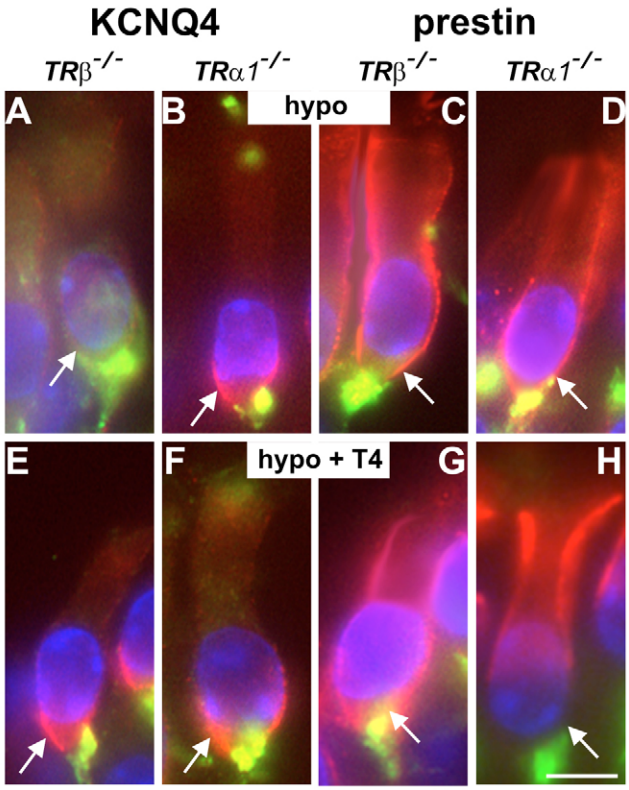

Fig. 4. Deletion of TRα1 but not TRβ exclusively restores KCNQ4 expression in OHCs despite hypothyroidism. (A,C) At P12, in hypothyroid *TRβ*$^{-/-}$ mutants (hypo), KCNQ4 protein was not observed (A), prestin was expressed but its distribution remained immature (C). (B,D) In hypothyroid *TRα1*$^{-/-}$ mutants (hypo), KCNQ4 appears normal (B), whereas prestin persists in an immature distribution (D). (E,F) T4-mediated rescue leads to a normal adult expression of KCNQ4 in hypothyroid *TRβ*$^{-/-}$ mutants (E), like in T4-treated *TRα1*$^{-/-}$ mutants (F). (G,H) T4 does not rescue prestin from its immature pattern in hypothyroid *TRβ*$^{-/-}$ mutants (G), but does so in hypothyroid *TRα1*$^{-/-}$ mutants (H). Sections were co-immunolabeled with synaptophysin (green) and DAPI (blue). Arrows, basal pole of OHC. Bar, 10 μm.

A TRE in the *KCNQ4* gene mediates repressive apo-TRα1 activity on the *Kcnq4* promoter in the absence of TH in vitro

The data so far clearly indicate a difference in the regulation of prestin and KCNQ4 expression by TRβ or TRα1, respectively. Although TREs in the prestin gene have been described and one, TRE^{Prest}, has been functionally characterized (Weber et al., 2002), information about TREs associated with the *Kcnq4* gene is currently unavailable. Using MatInspector professional software (release 2.0; http://www.genomatix.de) (Quandt et al., 1995), putative response elements, TRE^{KCNQ4}, were identified 3-10 kb upstream of the transcription start site, as deduced by Kubisch et al. (Kubisch et al., 1999) for the human *KCNQ4* gene (Fig. 5A). We focused on three identified putative TRE^{KCNQ4} because they contained two hexamers that are almost identical to the consensus sequence AGGTCA. The hexamers are organized as either a direct repeat (TRE-1^{KCNQ4}), a palindrome (TRE-2^{KCNQ4}), or an inverted palindrome (TRE-3^{KCNQ4}) with a 4, 8 and 8 bp spacer, respectively (Fig. 5A).

The protein binding properties of the three TRE^{KCNQ4} were studied using electromobility shift assay (EMSA). Recombinant chicken TRα (cTRα) shifted [^{32}P]TRE-3^{KCNQ4} (Fig. 5B, lane 4) to similar positions as a ^{32}P-labeled control TRE (DR4; Fig. 5B, lane 1), which correspond to a homodimer complex with a lower electromobility and a monomer complex with a higher electromobility. Neither [^{32}P]TRE-1^{KCNQ4} (Fig. 5B, lane 2) nor [^{32}P]TRE-2^{KCNQ4} (Fig. 5B, lane 3) was able to bind cTRα. The shift of [^{32}P]TRE-3^{KCNQ4} (Fig. 5B, lane 4) was equally diminished whether unlabeled TRE-3^{KCNQ4} (Fig. 5B, lane 5) or DR4 (Fig. 5B, lane 6) oligomers were added as a competitor. Unlabeled oligomers specific for the transcription factor TFIID had no effect on this interaction (Fig. 5B, lane 7). Nucleotide exchanges introduced in the 5′ half-site of the unlabeled TRE-3^{KCNQ4} (Fig. 5C, TRE-3mut1) used as a competitor had no influence on the capability of cTRα to shift the [^{32}P]TRE-3^{KCNQ4} (Fig. 5B, lane 8), indicating that competitor function is lost upon mutation of the 5′ half-site. Nucleotide exchanges introduced in the 3′ half-site of TRE-3^{KCNQ4} (Fig. 5C, TRE-3mut2) still abolished the interaction of cTRα and [^{32}P]TRE-3^{KCNQ4} (Fig. 5B, lane 9), indicating that this oligomer still functions as a competitor. The results indicate that a TRE upstream of the *KCNQ4* promoter is capable of binding TRα.

Using PromoterInspector software (release 2.0; http://www.genomatix.de) (Scherf et al., 2000), a minimal promoter sequence in the 5′ upstream region starting at position –495 bp to +45 bp relative to the start point of transcription of the *KCNQ4* gene was identified. The introduction of this sequence into a promoterless reporter gene vector led to a significant 4.3-fold (±0.3 s.d.; n=4) increase in reporter gene activation (Fig. 5D, white column) when compared with the promoterless vector, specifying the minimal promoter of *KCNQ4*.

A series of transfections were performed to examine the TH responsiveness of the reporter gene expression upon the subcloning of the TRE-3^{KCNQ4} upstream of the *KCNQ4* promoter sequence. The introduction of the TRE-3^{KCNQ4} upstream of the identified promoter sequence led to a significant decline of the reporter gene activity to 2.1±0.5 s.d. (P<0.05; n=4) with co-transfected TRα in the absence of

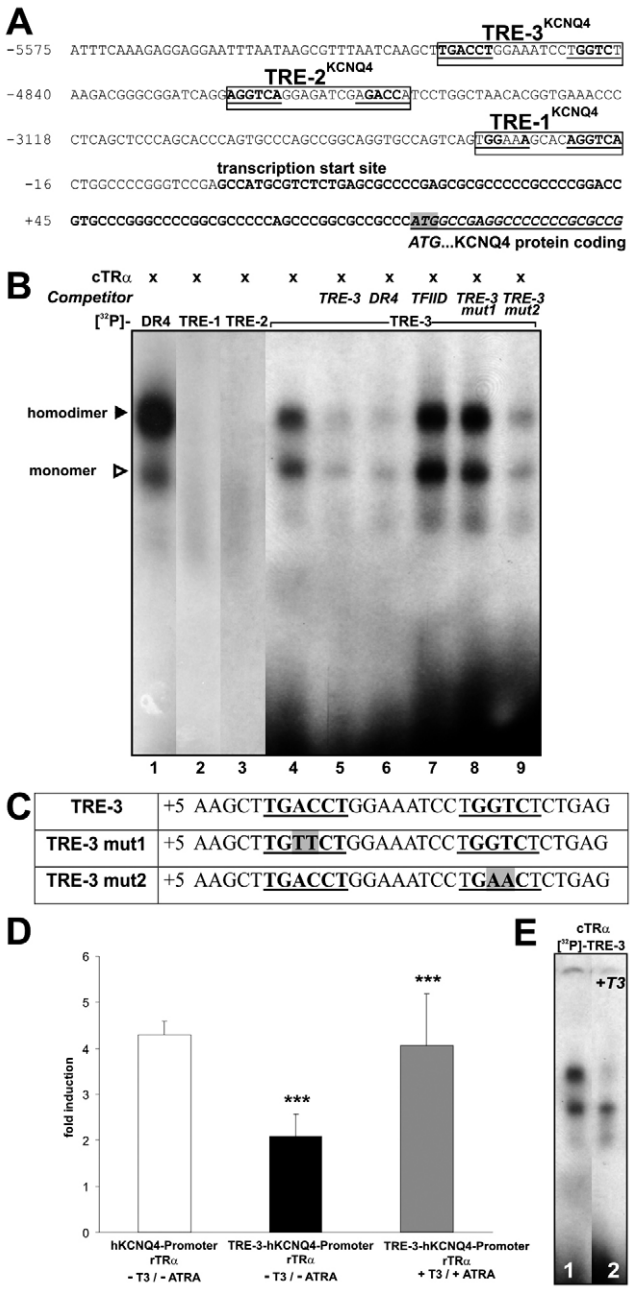

Fig. 5. Binding of TR to TRE^{KCNQ4} analyzed by EMSA. (A) Sequence and localization of TRE-1^{KCNQ4}, TRE-2^{KCNQ4}, and TRE-3^{KCNQ4} relative to the transcriptional start site. Hexamers are underlined and nucleotides matching consensus sequence are in bold. (B) EMSA of the three TRE^{KCNQ4}. Recombinant chicken TRα (cTRα) shifts [^{32}P]TRE-3 (lane 4), but not [^{32}P]TRE-1 (lane 2) or [^{32}P]TRE-2 (lane 3) to positions similar to [^{32}P]DR4 (lane 1), which correspond to a higher cTRα homodimer and a lower cTRα monomer complex. In competitor experiments, the interaction between cTRα and [^{32}P]TRE-3 (lane 4) is significantly reduced in the presence of an excess of unlabeled competitor oligomer TRE-3^{KCNQ4} (lane 5) and DR4 (lane 6) but not in the presence of unlabeled TFIID-specific oligomer (lane 7). Mutations in the 5′ half of unlabeled TRE-3^{KCNQ4} (TRE-3mut1) have no influence on the capability of cTRα to shift the [^{32}P]TRE-3 (lane 8). Mutations in the 3′ half of TRE-3^{KCNQ4} (TRE-3mut2) still abolish the interaction (lane 9) to a similar degree as unlabeled TRE-3^{KCNQ4} or DR4 competitor oligomers (lanes 5, 6). (C) Sequence annotation of the mutated TRE-3^{KCNQ4} oligomers used as competitors. Mutated residues are highlighted. (D) Functional analysis of the putative human *KCNQ4* promoter and the TRE-3^{KCNQ4} in reporter gene assays. Introduction of the human *KCNQ4* promoter into a promoterless vector leads to 4.3-fold induction of reporter gene expression compared with the promoterless vector (4.3-fold ±0.3 s.d.; n=4; white column). Insertion of the TRE-3^{KCNQ4} upstream of the human *KCNQ4* promoter leads to a decreased 2.1-fold induction of reporter gene expression (2.1±0.5 s.d., ***P<0.05; n=4) in the absence of ligands (black column) which could be overcome upon addition of 150 nM T3 and 1.5 μM ATRA restoring promoter activity to 4.1-fold induction of reporter gene expression (4.1±1.1 s.d., ***P<0.05; n=3; gray column). (E) Compared with control conditions (lane 1) addition of 600 nM T3 (lane 2) impairs the binding of cTRα homodimers (higher shift band) to [^{32}P]TRE-3^{KCNQ4} whereas monomer binding is unaffected.

ligands (Fig. 5D, black column). The addition of T3 had only a subtle effect on the reduction of the reporter gene activity (data not shown). Only recently, Lee and Privalsky (Lee and Privalsky, 2005) showed that TR-RAR (retinoic acid receptor) heterodimers can also form on different TREs, including inverted palindromes. In contrast to TR-RXR (retinoid X receptor) heterodimers which were shown to recruit only co-activators, TR-RAR dimers can also recruit co-repressors, which are, however, only efficiently released from TR-RAR heterodimers upon the addition of both ligands, T3 and all-trans retinoic acid (ATRA) (Lee and Privalsky, 2005). Indeed, in contrast to adding only T3, the addition of T3 and ATRA led to a significant increase of reporter gene activity (4.1±1.1 s.d.; P<0.05; n=3) to similar levels seen with the *KCNQ4* promoter alone (Fig. 5D, compare gray column with white column), indicating an involvement of endogenous RARs.

In line with previous experiments (Yen et al., 1992), we noted that in EMSAs T3 decreased TRα homodimer binding to TRE-3^{KCNQ4} whereas monomer binding remained unaffected (Fig. 5E).

Both, TRα and TRβ bind to TRE-3^{KCNQ4} and to TRE^{Prest}

To gain further insight into the mechanism of how differential TR activity on two genes is achieved in a single cell, we verified in a first approach the parallel expression of TRα1 and TRβ in OHCs at the time of final hair cell differentiation. To date, evidence of TR expression in OHCs has come from separate studies done at the protein level, for TRα1 (Lautermann and ten Cate, 1997) and TRβ (Knipper et al., 2001). Using a novel technique (Michna et al., 2003) to specifically dissect OHCs from rodents, both TRα1 and TRβ transcripts were detected in isolated OHCs around the time of onset of hearing by RT-PCR (data not shown). In a next step we questioned a preference of TRβ or TRα for either TRE^{Prest} or TRE-3^{KCNQ4}. Using EMSA, in vitro translated rat TRα (rTRα) and TRβ (rTRβ) shifted the [^{32}P]TRE-3^{KCNQ4} (Fig. 6, lane 1 and 3) and [^{32}P]TRE^{Prest} (Fig. 6, lane 5 and 7) to positions comparable to those of cTRα. Unlabeled TRE-3^{KCNQ4} and TRE^{Prest} competitor oligonucleotides specifically blocked the binding of rTRα and rTRβ to [^{32}P]TRE-3^{KCNQ4} (Fig. 6, lane 2 and 4) and [^{32}P]TRE^{Prest} (Fig. 6, lane 6 and 8), respectively, indicating

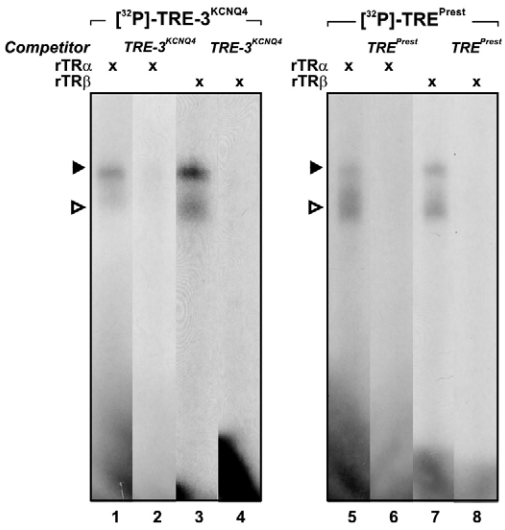

Fig. 6. Comparison of binding properties of TRE-3^{KCNQ4} and TRE^{Prest} by EMSA. In vitro translated rat TRα (rTRα) and rTRβ shifts [^{32}P]TRE-3^{KCNQ4} (lanes 1,3) as well as [^{32}P]TRE^{Prest} (lane 5,7) to two complexes with different electromobility (filled and open arrowheads). The interaction of rTRα or rTRβ with [^{32}P]TRE-3^{KCNQ4} is significantly reduced in the presence of an excess of unlabeled TRE-3^{KCNQ4} competitor oligomers (lanes 2,4). The same is true for rTRα or rTRβ with [^{32}P]TRE^{Prest} by using TRE^{Prest} as a competitor (lanes 6,8).

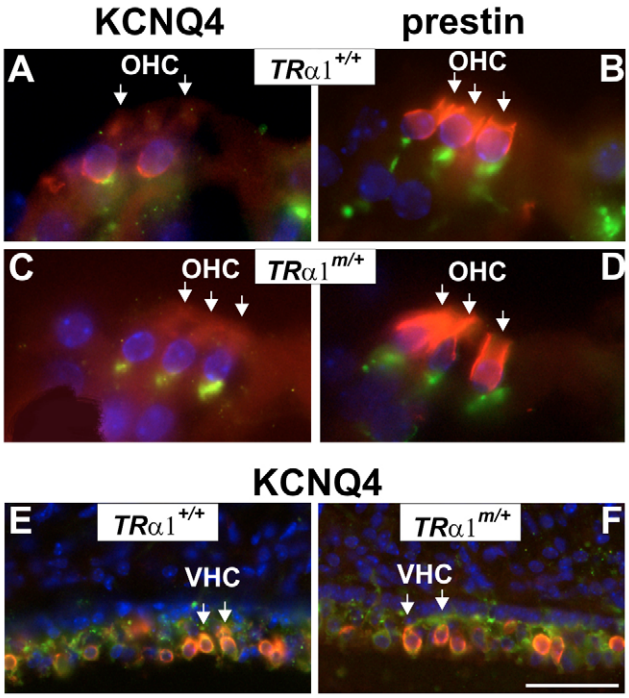

Fig. 7. KCNQ4 and prestin expression in OHCs of heterozygous $TR\alpha1^{m/+}$ mutants at P13. (A,B) In OHCs of $TR\alpha1^{+/+}$ wild type, expression and distribution of KCNQ4 (A, red) and prestin (B, red) is normal. (C,D) In OHCs of $TR\alpha1^{m/+}$ mutants KCNQ4 is completely absent (C) whereas prestin expression and distribution is normal (D). (E,F) By contrast, KCNQ4 expression in vestibular hair cells (VHC) is normal within the same sections of both the wild type (E) and $TR\alpha1^{m/+}$ mutants (F). Sections were co-immunolabeled with synaptophysin (green) and DAPI (blue). Bar, 20 μm.

that both receptors, TRα and TRβ, bind to both TRE-3^{KCNQ4} and TRE^{Prest}.

A dominant-negative mutation in the TRα1 receptor causes repression of *Kcnq4* gene expression but has no effect on prestin

The repressive activity of TRα1 on *Kcnq4* but not on the prestin gene was confirmed by analyzing a mouse model in which the *TRα1* gene expressed a dominant-negative point mutation (R384C) that renders the receptor insensitive to TH (Tinnikov et al., 2002). Genes that are repressed by *TRα1* can be identified by a persistence of gene repression. The expression profiles of KCNQ4 and prestin were analyzed in wild-type ($TR\alpha1^{+/+}$) and heterozygous $TR\alpha1^{m/+}$ mutants at P13 (Fig. 7). Unlike wild-type animals, KCNQ4 was absent in OHCs of all cochlear turns of $TR\alpha1^{m/+}$ mutants (Fig. 7A,C, KCNQ4, red), whereas prestin expression and distribution was normal in OHCs of both wild-type and mutant mice (Fig. 7B,D, prestin, red). Interestingly, in the same sections where KCNQ4 was absent in OHCs, KCNQ4 expression in vestibular hair cells (VHC) (Kharkovets et al., 2000) of wild-type and $TR\alpha1^{m/+}$ mutants was normal (Fig. 7E,F, KCNQ4, red). This clearly demonstrates the selective nature of the apo-TRα1 repressive influence on KCNQ4 expression in OHCs but not in VHCs. Furthermore, the normal expression of prestin in OHCs of $TR\alpha1^{m/+}$ mutants provides additional support for the view that TR aporeceptors act independently on the two simultaneously expressed T3 target genes in OHCs.

Conclusion

The data presented here reveal that the two genes *Kcnq4* and prestin, both expressed in OHCs, are differentially regulated by one of the two TH receptors, TRα1 and TRβ, respectively. A theoretical mechanism for this TR-specific regulation of KCNQ4 and prestin expression is shown in Fig. 8.

In this model, prestin expression is reduced under the condition of hypothyroidism, while it is enhanced through the actions of TRβ in the presence of TH (Weber et al., 2002). Because prestin is normally expressed in $TR\alpha1^{m/+}$ mutant mice (Fig. 7), but its immature distribution is observed in both TH-deficient and $TR\beta^{-/-}$ mice (Fig. 3), it is reasonable to characterize the apo-TRβ as a 'silent' TH-receptor (Chassande, 2003) in the case of prestin (Fig. 8A). The fact that prestin is expressed in the absence of TH or TRβ (Fig. 3) indicates that a baseline level of transcription is independent of TH-TR complexes (Fig. 8A). In this regard, it has been suggested that recently identified cis-active elements in the upstream region of exon 1 of the prestin gene (Knipper et al., 2005) may recruit a pre-initiation complex mediating TH-independent basal levels of prestin transcription. Furthermore, prestin expression is transcriptionally enhanced when TH is bound to TRβ (Fig. 8B), a finding that confirms the role played by TRβ.

Likewise, findings from this investigation support a model in which apo-TRα1 represses KCNQ4 expression (Fig. 8C). This conclusion is based on several independent findings. First, it is notable that KCNQ4 is not expressed under hypothyroid conditions (Fig. 2), nor is the protein expressed in $TR\alpha1^{m/+}$

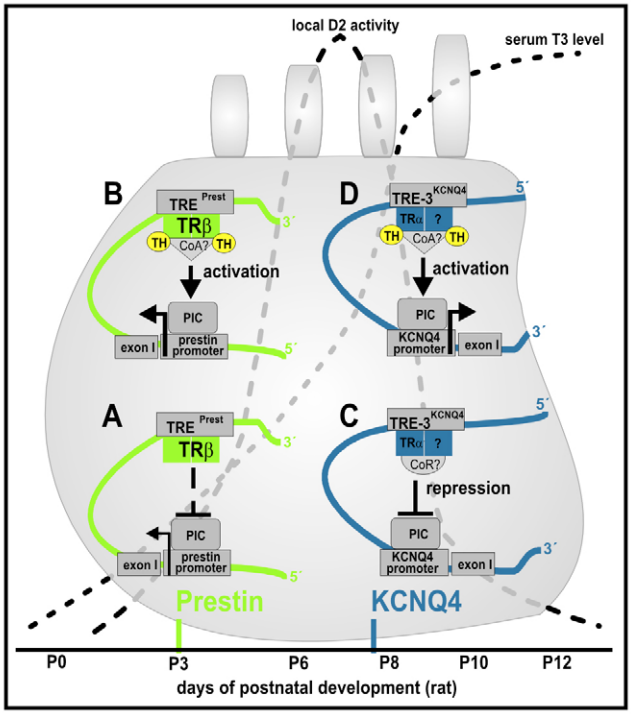

Fig. 8. Model of the regulation of prestin and *Kcnq4* gene expression by an interplay of TH and distinct TR isoforms in a single OHC. (A) In the absence of TH, apo-TRβ exerts only a weak repression of prestin, allowing basal transcription. (B) Transcription is activated in the presence of TH by TRβ. (C) In the absence of TH, apo-TRα1 represses *Kcnq4*. (D) In the presence of TH, TRα1 activates *Kcnq4*. Putative co-repressors (CoR) and co-activators (CoA) and transient local peak of cochlear D2 activity may be involved. D2 activity is depicted according to data from Campos-Barros et al. (Campos-Barros et al., 2000). D2, 5′-deiodinase type 2; PIC, pre-initiation complex.

mutant mice (Fig. 7). When these observations are taken together with the observation that the deletion of TRα1, but not TRβ, rescues KCNQ4 expression from the consequences of hypothyroidism (Fig. 4), as does T4 treatment of hypothyroid *TRβ*$^{-/-}$ mice (Fig. 4), one is drawn to the conclusion that the repressive action of apo-TRα1 is overcome in the presence of TH, leading to activation of *Kcnq4* transcription by the dynamics of derepression (Fig. 8D).

Taking into account that the identified regulatory elements TRE^{Prest} (Weber et al., 2002) and TRE-3^{KCNQ4} (Fig. 5) did not display TR isoform-specific binding properties (Fig. 6), the presence of both receptors in isolated OHCs suggests that distinct sequence characteristics in the regulatory region of both individual genes are required to define the differential recruitment of proteins (co-factors), influencing the assembly and activity of the pre-initiation complex.

Discussion

In this study we provide evidence that OHCs express at least two T3 target genes, *Kcnq4* and prestin, which are differentially regulated by TRα1 or TRβ, respectively. The findings reported here reveal for the first time a role of TRα1 in inner-ear development, and suggest a molecular mechanism for the occurrence of gene repression parallel to the gene enhancement mediated by specific TR-isoforms in a single cell. Moreover, a crucial time period is defined during which a switch from apo-TRα1 to holo-TRα1 occurs independently of the presence of TRβ activity, an event that is presumably defined by a local peak of TH level.

TRα1 activity during final differentiation of the inner ear

The abnormal regulation of genes controlled by TRβ has long been presumed to be the cause of profound neurosensory deafness associated with congenital hypothyroidism (Forrest et al., 1996a; Forrest et al., 1996b). Resistance to TH owing to a point mutation within the TRβ gene (Adams et al., 1994; Refetoff et al., 1967; Weiss and Refetoff, 2000) has suggested a crucial role of apo-TRβ in normal developmental processes, including development of hearing. However, genes that are affected by TRβ have not been identified so far. Until now no evidence for a role of TRα in hearing existed (Ng et al., 2001). This may be due to the fact that to date most terminal differentiation processes in the organ of Corti have not been regarded in the context of hypothyroidism.

Apo-TRα1 has recently been shown to exert a repressive influence on gene regulation (Flamant et al., 2002). The unlocking from TRα1 aporeceptor-mediated gene repression appears to become necessary towards the second to third postnatal week in rodents. T3-based compensatory treatments indicate that the brain (Eayrs, 1971; Morte et al., 2002), bone, spleen, intestine (Flamant et al., 2002), heart (Mai et al., 2004) and cochlea (Christ et al., 2004; Knipper et al., 2001) depend on T3 during the second postnatal week. If, however, T3 replacement occurs beyond the second postnatal week, the capacity to rescue hypothyroidism-induced pathology is significantly reduced (Christ et al., 2004; Flamant et al., 2002; O'Shea and Williams, 2002; Tinnikov et al., 2002).

A life-threatening situation resulting from a failure of timely activation of distinct genes repressed by the apo-TRα1 was demonstrated by the early postnatal death of *Pax8*$^{-/-}$ mice, which could be prevented by the deletion of TRα (Flamant et al., 2002). Although recent findings suggest that in addition to TRα1, TRα2 has to be deleted to rescue *Pax8*$^{-/-}$ mice (Mittag et al., 2005), data in the present study (Figs 4, 7) point to a restricted role of apo-TRα1 on *Kcnq4*. Considering the detrimental influence of a persistent gene repression mediated by TH aporeceptors (Flamant and Samarut, 2003), it will be challenging to investigate whether postnatal death of OHCs in *Pax8*$^{-/-}$ mice (Christ et al., 2004) is due to persistent repressive activity of apo-TRα1 on *Kcnq4*. This is important in light of the previously reported death of OHCs caused by either a decreased KCNQ4 expression (Rüttiger et al., 2004), a pharmacological blockade of KCNQ4 channels (Nouvian et al., 2003) or deletion of *Kcnq4* (Kharkovets et al., 2006).

Specificity of TR-mediated transcriptional control of two concomitantly expressed T3 target genes in a single cell

Based on recent efforts to classify TRs (Chassande, 2003), we suggest that TRβ acts on the prestin gene as a 'silent' receptor in the absence of TH (Fig. 8A), whereas TRα1 acts as apo-receptor on *Kcnq4*. Previously acquired data (Knipper et al., 1999; Lautermann and ten Cate, 1997) and data resulting from the present study showing that both TRα and TRβ are expressed in OHCs at the time of KCNQ4 and prestin expression, exclude the possibility that tissue-specific differences in TR levels define

TR specificity, as previously suggested (Dillmann, 2002). We cannot, however, rule out the fact that differences in the level of either TRα or TRβ in OHCs may play a role in TR specificity. As both TRs were able to bind to both TRE^{Prest} and TRE^{KCNQ4} (Fig. 6), we can be certain that the TR specificity seen in the case of *Kcnq4* and prestin is not determined by differences in sequence-specific binding properties of the TREs, as previously described (Olson et al., 1998).

Local changes in T3 level should instead be considered to play a crucial role in influencing TR specificity. Indeed, in humans and rodents the critical developmental time period of the inner ear occurs in parallel to the natural rise of TH blood plasma levels (Deol, 1973; Knipper et al., 2000; Uziel et al., 1985).

The importance of T3 availability as a factor controlling TR-regulated transcription was clearly demonstrated in a recent study of *TRα1*$^{m/m}$ mutant mice. In this strain, the affinity of TRα1 for TH is reduced as a consequence of a point mutation (Tinnikov et al., 2002) leading to mortality within the first 3 postnatal weeks, as in *Pax8*$^{-/-}$ mice. The pathological consequences of the condition were reversible upon compensatory T3 levels (Tinnikov et al., 2002). The importance of circulating T3 for the conversion of apo-TRα1 to the holoreceptor configuration was also emphasized in studies analyzing TRα1 function in heart tissue, intestine, bone and brain (Flamant et al., 2002; Mai et al., 2004).

The role of local T3 levels, which are determined by deiodinase activity, has been shown in target tissues during amphibian metamorphosis (Becker et al., 1997; Huang et al., 1999) and rat brain development (Kaplan and Yaskoski, 1981; Obregon et al., 1991). Hearing loss in 5′-deiodinase type 2 (D2)-deficient mice (Ng et al., 2004) was only recently discussed in the context of a striking peak of D2 activity in the cochlea between P5 and P10 (Campos-Barros et al., 2000). Although the role of deiodinase in the switch from apo-TR to holo-TR has been hypothesized (Chassande, 2003), supporting data have been unavailable until now.

The data in the present study show that the time period over which the two T3 target genes in OHCs are activated and/or modulated is brief and occurs between P6 and P10, when there is a peak in 5′-deiodinase activity (Campos-Barros et al., 2000) and a steep rise in the TH blood plasma level (Knipper et al., 2000). Local T3 levels thus may split the genes into TRα1-affected or -unaffected categories. T3 may induce a conformational change in the receptor that destabilizes its bond with co-repressors and facilitates the binding of co-activators (Lazar, 2003).

Retinoic acid, shown to be required as an additional ligand to overcome the repressive effect on *Kcnq4* promoter activity in vitro (Fig. 6) should be considered as a further ligand needed for derepression. Lee and Privalsky (Lee and Privalsky, 2005) reported that TRα1 forms heterodimers with RAR, which in contrast to TR-RXR heterodimer recruit both co-activators and co-repressors. TRα1-RAR heterodimers bound to the inverted palindrome TRE-3^{KCNQ4} in OHCs may thus recruit co-repressors until TH levels rise during early postnatal development. Although several studies underscore the importance of RAR for inner-ear development (Raz and Kelley, 1999; Romand et al., 2002), changes in retinoic acid levels during postnatal development have not been reported (Romand, 2003).

TRβ activity in the maturation of OHCs

As higher T3 levels seem to be required to release apo-TRα1 from TRE-3^{KCNQ4}, lower T3 levels may be sufficient for the recruitment of the TRβ complex to TRE^{Prest} (Fig. 8). Prestin expression and distribution are known to be diminished and immature in hypothyroidism (Weber et al., 2002), as well as in *TRβ*$^{-/-}$ and *TRα1*$^{-/-}$/*β*$^{-/-}$ mutants as shown in the present study. Although we cannot rule out the possibility that the influence of TH on the redistribution of prestin is indirect, this condition may be causally linked to the reduced nonlinear capacitance of OHCs observed in *TRβ*$^{-/-}$ and *TRα1*$^{-/-}$/*β*$^{-/-}$ mutants (Rusch et al., 2001).

In conclusion, the characterization of differentially regulated T3 target genes in the same cell may provide the basis for novel insight into the mechanism of subcellular TR specificity. Given the fact that TRα1 and TRβ show no preference in binding to TRE^{Prest} or TRE-3^{KCNQ4}, the specificity of TR binding in postnatal OHCs must be achieved by additional gene-specific differences in the upstream regions of the prestin and *Kcnq4* genes. These differences influence the level of TR-associated DNA-bound transcription factors, as previously proposed (Glass and Rosenfeld, 2000). The composition of regulatory elements may then act in concert with specific local T3 levels to alter dissociation or recruitment rates of co-repressor or co-activator complexes (Fig. 8) (Hermanson et al., 2002; Kamei et al., 1996; Onate et al., 1995).

Until now human patients with mutant TRα1 have not been identified; as either the effects on a human phenotype are too mild, spontaneous miscarriages occur at embryonic or fetal stages of development, and/or the symptoms have not been attributed to defects in TRα1 (Tinnikov et al., 2002). Based on the findings reported here, the latter explanation may apply most appropriately in the case of the auditory system.

Materials and Methods

Animals and drug administration

Wistar rats, *Pax8*$^{-/-}$ (Mansouri et al., 1998), *Tshr*^{hyt} (*hyt/hyt*) (Walsh and McGee, 2001), *TRα1*$^{-/-}$ (Wikstrom et al., 1998), *TRβ*$^{-/-}$ (Forrest et al., 1996a), *TRα1*$^{-/-}$/*β*$^{-/-}$ (Gothe et al., 1999) and *TRα1*$^{m/+}$ (Tinnikov et al., 2002) mice were used (Table 1). MMI treatment of mice, thyroxin (T4) administration, and T4 and T3 plasma titer determinations were performed as described (Knipper et al., 2000; Weber et al., 2002). Animal experiments were approved and complied with all protocol requirements at the University of Tübingen.

Tissue preparation

Cochleae of untreated (control), MMI-treated (hypothyroid) and T4-treated (hypothyroid+T4) animals were prepared and cryosectioned as described (Knipper et al., 1998).

Riboprobe synthesis and in situ hybridization

Using the *KCNQ4*-specific oligonucleotide primers rKCNQ4-USP3 and rKCNQ4-DSP9 (supplementary material, Table S1) a PCR fragment was amplified from rat cochlear cDNA, cloned into pCR®II TOPO Vector (Invitrogen) and sequenced. *Kcnq4*-specific riboprobes were synthesized and in situ hybridization was performed as described (Knipper et al., 1999; Knipper et al., 2000; Weber et al., 2002).

Northern blot

Northern blots were performed as described (Knipper et al., 1998; Knipper et al., 1999). The effect of TH on mRNA levels was semi-quantitatively evaluated using mRNAs isolated from a similar number of cochleae as described (Knipper et al., 1998). To ensure the loading of similar amounts of mRNA per lane blots were probed with the housekeeping gene cyclophilin (Thellin et al., 1999).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed as described (Rüttiger et al., 2006). In brief, cochlear mRNA from P12 hypothyroid and control rats was isolated using the Dynabeads mRNA Direct Kit (Dynal) and reverse-transcribed with Superscript II

(Invitrogen). Using the oligonucleotides rKCNQ4-USP3 and rKCNQ4-DSP3 and cyclophilin-f and cyclophilin-r (supplementary material Table S1) as internal controls semi-quantitative RT-PCR was performed on equivalent amounts of mRNA isolated from four cochleae of control and hypothyroid rats. PCR was repeated three times using different animals.

Generation of KCNQ4 antibody

An antibody against a conserved part of the C-terminus of KCNQ4 (CQTLSISRSVSTNMD-COOH) was raised in rabbits, purified by affinity chromatography and tested by immunoblotting and immunohistochemistry by preincubation with the synthetic peptide (data not shown).

Fluorescence immunohistochemistry

Cochlear sections of rats and mice were stained and imaged as described (Knipper et al., 1998; Knipper et al., 2000). The rabbit and goat (Santa Cruz) polyclonal antibody against KCNQ4, rabbit polyclonal antibody against prestin (Weber et al., 2002), sheep polyclonal antibody against synaptophysin (The Binding Site) were visualized with Cy3- (Jackson ImmunoResearch Laboratories) or Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes) and counter-stained with DAPI (Vector Laboratories).

Oligonucleotides and labeling

In EMSA, 3.85 pmol of the double-stranded synthetic oligonucleotides TRE-1^{KCNQ4}, TRE-2^{KCNQ4}, TRE-3^{KCNQ4}, TRE-3mut1, TRE-3mut2, DR4, TRE^{Prest} and TFIID (supplementary material, Table S1) were labeled with [γ-^{32}P]ATP (5000 Ci/mmol; Amersham Biosciences) by phosphorylation with T4 polynucleotide kinase (Promega) and purified with the QIAquick Nucleotide Removal Kit (Qiagen).

Cloning of the human KCNQ4 promoter

Genomic DNA was isolated from human blood with the QIAmp Blood Kit (Qiagen). PCR was performed with the oligonucleotide primers PromK4USP and PromK4DSP and nested primers sPromK4USP(*Bgl*II) and sPromK4DSP(*Hin*dIII) (supplementary material Table S1). A 739 bp PCR fragment was amplified, cloned into the pGL3basic vector (Promega) and sequenced.

Cloning of the reporter gene constructs

The synthetic double-stranded oligonucleotide TRE-3^{KCNQ4} (supplementary material Table S1) was cloned into the *Sac*I and *Xho*I sites of the pGL3 vector containing the putative human KCNQ4 promoter. The previously described rat TRα expression vector was used (Weber et al., 2002).

Transfection and reporter gene assays

HEK293 cells were cultured and transfected with 500 ng of each reporter construct, 500 ng of the control plasmid RSV-LacZ, and 500 ng of the TRα expression vector for 12 hours as described (Weber et al., 2002). Transfected cells were incubated for 48 hours in fresh medium containing serum either depleted of T3 and retinoic acid (Weber et al., 2002) or containing 150 nM T3 (Sigma) or 1.5 μM all-trans-retinoic acid (ATRA; Sigma). The luciferase assay system (Promega) and the β-galactosidase enzyme system (Promega) were used to determine luciferase activity and transfection efficiency as described (Weber et al., 2002). Four independent experiments were performed, each containing three to six independently prepared transfection mixtures.

In vitro translation

Rat TRα and TRβ were translated in vitro from plasmids (Weber et al., 2002) using the TNT® T7 coupled reticulocyte lysate system (Promega).

Electromobility-shift assay (EMSA)

EMSAs were performed as described (Weber et al., 2002). Unlabeled competitor oligonucleotides (200-fold excess) or T3 (150 nM) were preincubated with recombinant chicken TRα (Santa Cruz) for 30 minutes before adding the radiolabeled oligonucleotides.

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