Molecular Aspects of Thyroid Hormone Actions

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Cellular actions of thyroid hormone may be initiated within the cell nucleus, at the plasma membrane, in cytoplasm, and at the mitochondrion. Thyroid hormone nuclear receptors (TRs) mediate the biological activities of T_3 via transcriptional regulation. Two TR genes, α and β, encode four T_3-binding receptor isoforms (α1, β1, β2, and β3). The transcriptional activity of TRs is regulated at multiple levels. Besides being regulated by T_3, transcriptional activity is regulated by the type of thyroid hormone response elements located on the promoters of T_3 target genes, by the developmental- and tissue-dependent expression of TR isoforms, and by a host of nuclear coregulatory proteins. These nuclear coregulatory proteins modulate the transcription activity of TRs in a T_3-dependent manner. In the absence of T_3, corepressors act to repress the basal transcriptional activity, whereas in the presence of T_3, coactivators function to activate transcription. The critical role of TRs is evident in that mutations of the TRβ gene cause resistance to thyroid hormones to exhibit an array of symptoms due to decreasing the sensitivity of target tissues to T_3. Genetically engineered knockin mouse models also reveal that mutations of the TRs could lead to other abnormalities beyond resistance to thyroid hormones, including thyroid cancer, pituitary tumors, dwarfism, and metabolic abnormalities. Thus, the deleterious effects of mutations of TRs are more severe than previously envisioned. These genetic-engineered mouse models provide valuable tools to ascertain further the molecular actions of unliganded TRs *in vivo* that could underlie the pathogenesis of hypothyroidism.

Actions of thyroid hormone that are not initiated by liganding of the hormone to intranuclear TR are termed nongenomic. They may begin at the plasma membrane or in cytoplasm. Plasma membrane-initiated actions begin at a receptor on integrin $\alpha v\beta 3$ that activates ERK1/2 and culminate in local membrane actions on ion transport systems, such as the Na^+/H^+ exchanger, or complex cellular events such as cell proliferation. Concentration of the integrin on cells of the vasculature and on tumor cells explains recently described proangiogenic effects of iodothyronines and proliferative actions of thyroid hormone on certain cancer cells, including gliomas. Thus, hormonal events that begin nongenomically result in effects in DNA-dependent effects. L-T_4 is an agonist at the plasma membrane without conversion to T_3. Tetraiodothyroacetic acid is a T_4 analog that inhibits the actions of T_4 and T_3 at the integrin, including angiogenesis and tumor cell proliferation. T_3 can activate phosphatidylinositol 3-kinase by a mechanism that may be cytoplasmic in origin or may begin at integrin $\alpha v\beta 3$. Downstream consequences of phosphatidylinositol 3-kinase activation by T_3 include specific gene transcription and insertion of Na, K-ATPase in the plasma membrane and modulation of the activity of the ATPase.

Thyroid hormone, chiefly T_3 and diiodothyronine, has important effects on mitochondrial energetics and on the cytoskeleton. Modulation by the hormone of the basal proton leak in mitochondria accounts for heat production caused by iodothyronines and a substantial component of cellular oxygen consumption. Thyroid hormone also acts on the mitochondrial genome via imported isoforms of nuclear TRs to affect several mitochondrial transcription factors. Regulation of actin polymerization by T_4 and rT_3, but not T_3, is critical to cell migration. This effect has been prominently demonstrated in neurons and glial cells and is important to brain development. The actin-related effects in neurons include fostering neurite outgrowth. A truncated TRα1 isoform that resides in the extranuclear compartment mediates the action of thyroid hormone on the cytoskeleton. **(*Endocrine Reviews* 31: 139–170, 2010)**

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Abbreviations: Ang, Angiopoietin; bFGF, basic fibroblast growth factor; bHLH, basic helix loop helix; CAM, chorioallantoic membrane; CoA, coactivator; CoR, corepressor; CTBP, cytosol thyronine-binding protein; DR, direct repeat; DRIP, vitamin D receptor interacting protein; EGF, epidermal growth factor; EGFR, EGF receptor; GFP, green fluorescent protein; HDAC, histone deacetylase; HIF-1α, hypoxia-inducible factor-1α; IFN, interferon; LBD, ligand-binding domain; LXR, liver X receptor; NCoR, nuclear receptor CoR; PA28γ, proteasome activator 28γ; PI3K, phosphatidylinositol 3-kinase; PKM, pyruvate kinase monomer; PPAR, peroxisome proliferator-activated receptor; PTTG, pituitary tumor-transforming gene; RGD, Arg-Gly-Asp; RTH, resistance to thyroid hormone; RXR, retinoid X receptor; SMRT, silencing mediator of retinoid and thyroid hormone receptor; SR, sarcoplasmic reticulum; SRC, steroid hormone receptor CoA; tetrac, tetraiodothyroacetic acid; TR, thyroid hormone receptor; TRAP, TR-associated protein(s); TRE, thyroid hormone response element; Trip, TR-interacting protein; TSH-omas, TSH-secreting pituitary adenomas; UCP, uncoupling protein.

I. Genomic Actions of Thyroid Hormone

A. Multiple forms of thyroid hormone nuclear receptors

Using isolated rat nuclei, Tata and Widnell first showed that T_3 stimulates DNA-dependent RNA-polymerase activity to increase synthesis of new RNAs (1). Subsequently, Oppenheimer *et al.* (2) and Samuels *et al.* (3) demonstrated high-affinity, low-capacity binding sites in the nuclei of rat tissues and cultured GH cells, respectively. These studies, whereas considered correlative at the time, strongly suggested that thyroid hormone nuclear receptors (TRs) mediated the transcriptional activities of T_3. In the ensuing years, efforts were made to purify TRs from rat liver (4–6) but met with only limited success. It was not until the cloning of the TRs in 1986 that it became possible to characterize their molecular properties and to study directly how TRs regulate the transcriptional activities of T_3 target genes (7, 8).

TRs are members of the nuclear receptor superfamily and function as T_3-inducible transcription factors. TRs are derived from two genes located on two different chromosomes (9) (Fig. 1A). The TRβ gene, located on chromosome 3, encodes three T_3-binding TRβ isoforms (β1, β2, and β3) (10). These TRβ isoforms share high sequence homology in the DNA and T_3-binding domains but differ in the length and amino acid sequences in the amino terminal A/B domain. Internal usage of ATG leads to the TRΔβ3 that lacks the amino A/B and DNA-binding domains but retains T_3-binding activity (10). The TRα gene, located on chromosome 17, encodes one T_3-binding TRα1 and two splicing variants (TRα2 and TRα3). These TRα1 variants differ from TRα1 in the length and amino acid sequences in the C-terminal region, beginning at amino acid 370. These TRα1 variants have no T_3-binding activity (11). Truncated TRs, transcribed from an internal promoter located in intron 7, give rise to TRΔα1 and TRΔα2 that lack amino-terminal A/B and DNA domains but retain most of the T_3-binding domain (12).

Similar to other members of the receptor superfamily, TRs consist of single polypeptide chains with modular functional domains (13, 14) (Fig. 1A). The amino-terminal A/B domains vary among TR isoforms that are involved in transcription regulation. Located centrally in the molecules is the highly conserved DNA-binding domain that interacts with thyroid hormone response elements (TREs) of T_3 target genes. The carboxyl-terminal ligand-binding domain (LBD) shares high sequence homology among TR isoforms that assume multifunctions. LBD not only binds thyroid hormones but also interacts with a host of corepressors (CoRs) and coactivators (CoAs), collectively known as receptor coregulators (15). The LBD is also involved in homodimerization of DNA-bound TRs and their heterodimerization with other members of the receptor superfamily, notably with the retinoid X receptors (RXRs). Analysis of the structure of LBD indicates that ligand binding induces dramatic structural changes that facilitate dissociation of repressors and association of activators (16, 17).

The expression of TR isoforms is tissue-dependent and developmentally regulated (9). TRα1 is constitutively expressed at embryonic development, and TRβ is expressed toward the later stage of development (18). TRβ1 is expressed predominantly in the kidneys, liver, brain, heart, and thyroid; at lower levels in the skeletal muscle, lungs, and spleen; but not at all in the testes (9, 10, 19). TRβ2 is mainly expressed in the brain, retina, and inner ears; at low levels in the lungs and heart; but not in other tissues (10, 20). TRβ3 is predominantly expressed in the kidneys, liver, and lungs; at low levels in the skeletal muscle, spleen, brain, and heart; but not at all in the testis (10). TRα1 and TRα2 are expressed at the highest levels in the brain; at lower levels in the kidneys, skeletal muscle, lungs, heart, testes, and liver; but not in the testes (10).

B. Isoform-dependent functions of TRs

The molecular diversity of TRs raises the question as to whether the TR isoforms have distinct functions or simply serve a redundant role for each other. The tissue-dependent and developmentally regulated differential expression of TR isoforms suggests that TRs may mediate subtype-specific functions. This possibility is supported by distinct patterns of spatiotemporal TR isoform expression in the embryonic and postnatal nervous system (18). TRα1- and TRα2 mRNAs are similarly and widely expressed in the fetal neocortical plate, site of cortical neu-

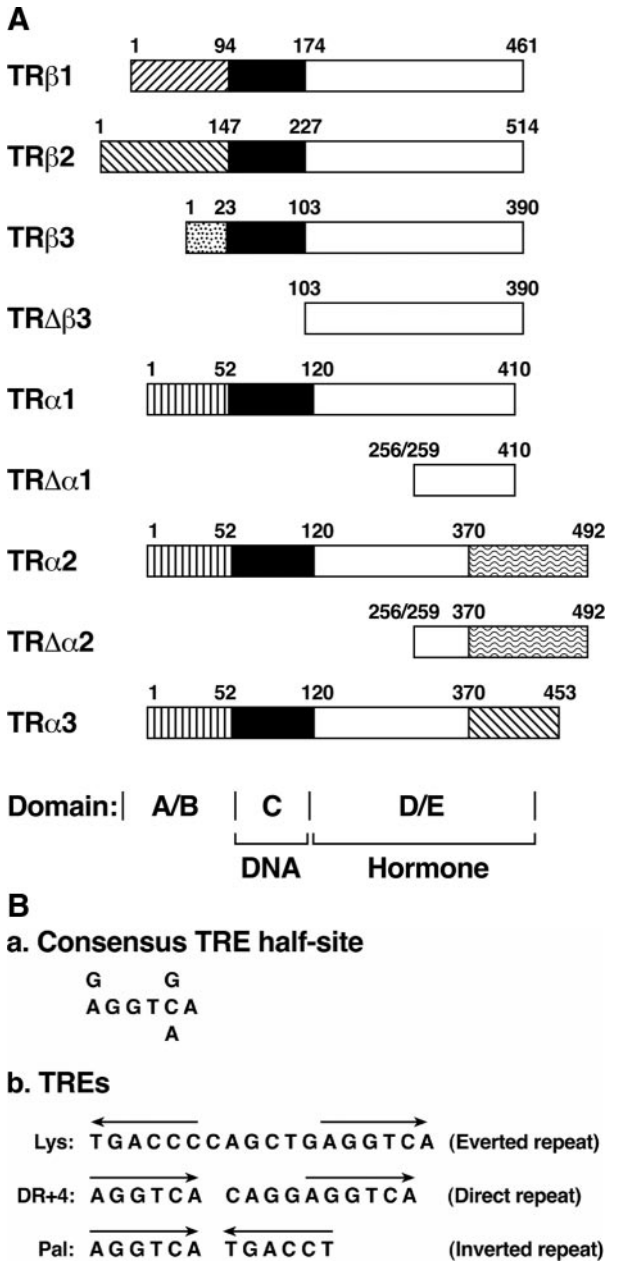

FIG. 1. A, Schematic representation of TR isoforms. TRs are encoded by the TRβ and TRα genes located on different chromosomes. Alternative splicing of primary transcripts gives rise to four thyroid hormone binding isoforms. TRs share high sequence homology in the DNA binding domain C (*solid bar*) and the hormone binding domain D/E (*open bar*). The amino-terminal A/B domains are variable in length and amino acid sequence as indicated by *different symbols*. The amino acids of the truncated TRs (TR$\Delta\beta$3, TR$\Delta\alpha$1, and TR$\Delta\alpha$2) at the amino and carboxy termini are indicated by *numbers*. B, The DNA sequence (a) and the arrangement (b) of the TRE half-site binding motifs. The Lys TRE was identified in the promoter of lysozyme gene. DR+4 TRE represents the direct repeat separated by four nucleotides. Pal is TRE half-site in palindromic arrangement.

ronal differentiation. In contrast, TRβ1 transcripts are restricted in distribution, with prominent expression in zones of neuroblast proliferation such as the germinal trigone and the cortical ventricular layer. TRβ2 transcripts are expressed in the developing hippocampus and striatum (20). These differential spatiotemporal expression patterns suggest that TR isoforms could play distinct functional roles during development. Gene inactivation studies, however, provide *in vivo* evidence to indicate that the TR isoforms can have both subtype-specific and overlapping functional roles (21–25). Mice lacking TRα1 have a lower heart rate, abnormal heart contractility, and decreased body temperature (23). In contrast, mice in which the TRβ gene is selectively inactivated have a mild dysfunction of the pituitary-thyroid axis and a deficit in auditory function and eye development (20–22). Moreover, mice in which both TRα1 and TRα2 are deleted have impaired postnatal development and decreased postnatal survival (24). These distinct phenotypes exhibited by mice in which individual TR genes are selectively deleted indicate that TR isoforms mediate specific functions. When both TRα and TRβ genes are inactivated, an array of phenotypes are detected, including severe dysfunction of the pituitary-thyroid axis, retarded growth, and delayed bone maturation, which are not found in the single receptor-deficient mice (25). These findings indicate that TRα1 and TRβ can substitute for each other to mediate some actions of T_3 and also mediate isoform-specific functions.

The TR isoform-dependent phenotypes exhibited by mice deficient in TR genes prompted the question as to whether TR isoforms specifically regulate certain target genes that mediate the TR isoform-dependent phenotypic expression. The question was addressed by using the cDNA microarray approach to compare the gene expression profiles in the livers of TRα knockout, TRβ knockout, and wild-type mice (26). Yet, hierarchical clustering analyses indicate that no clusters of target genes that are selectively activated by either TR isoform were identified (26), suggesting that TR isoform-specific regulation of target genes is rare in the liver. However, using a similar approach, Flores-Morales *et al.* (27) found 155 hepatic genes that are regulated after treatment with T_3 for 2 h in wild-type mice, whereas only 84 hepatic genes are regulated in TRβ knockout mice under the same conditions. These findings raised the possibility that some T_3 target genes in the liver could be specifically regulated by TRβ1. It is currently unclear what underlies the differences between these two studies. It is possible that the mouse strains and the different experimental conditions could account for the discrepancy. Moreover, it has been shown that TRα1 and TRα2 isoforms are zonally expressed around the central vein in rat liver and that the portal to central gradient of TRα1 is broader than that of TRβ1. Moreover, the expression of the TRα2 protein showed a diurnal variation with a peak in the afternoon when the animals are least active, whereas no such variation was

found for the TRα1 protein (28). Although it is not clear whether such zonal distribution and diurnal changes of TR isoforms in rats also occur in mice, the possibility exists that sampling of livers for array analyses could also contribute to the discrepancy observed by these two studies.

However, it is important to understand the underlying mechanisms resulting in distinct phenotypic expression in mice deficient in one TR isoform or the other, or both. The availability of various genetically engineered mice would allow the use of high throughput array approach to ascertain whether there are clusters of genes that are preferentially regulated by one TR isoform that could impact the biological functions of other target tissues, such as brain, heart, bone, kidney, and thyroid. The major TR isoform in the brain, heart, and bone is TRα, and that in the kidney and thyroid is TRβ. Consistent results obtained under defined conditions could reveal whether tissue-dependent abundance of TR isoform proteins might be one of the mechanisms underlying the phenotypic expression of mice deficient in one TR isoform or the other. This question warrants additional studies in the future.

C. Multilevel regulation of TR transcription activity

1. Diversity of TREs

TRs are DNA-binding transcription factors that recognize specific DNA sequences on the promoters of T_3 target genes. In the years after the cloning of TR cDNAs, flurries of activity to map and characterize TREs on T_3 target genes led to identification of TREs with a core consensus sequence of the hexanucleotide "half-site" (A/G)GGT(C/A/G)A (Fig. 1B). Analyses of the TREs in the promoters of many T_3 target genes have shown that the TRE half-sites exist in pairs (Fig. 1B). The half-site binding motif can be arranged as an everted repeat (as in the chicken lysozyme gene, Lys; Fig. 1B), as a direct repeat (as in the malic enzyme gene, DR+4; Fig. 1B), or as an inverted repeat (palindrome, Pal, as in the GH gene; Fig. 1B). The spacing between the two half-sites also varies, depending on the orientation of the half-sites. For the everted repeat, there is a spacing of six nucleotides. For the direct repeat, the spacing is four nucleotides. For the palindromic arrangement, no spacing separates the two half-sites. Analyses of T_3 target genes showed that TRE direct repeats are more common than TRE inverted repeats (29). Furthermore, in the promoters of some genes, clusters of mixed types of TREs are present to interact with TR for maximal T_3 responses. The GH gene is an example in that one hexamer is common for a direct repeat TRE, and a palindromic TRE is common for cooperative binding of the TR dimer-T_3 complex (30).

2. Crosstalk with other signaling pathways via heterodimeric partners of TR

TRs bind to TREs not only as homodimers but also as heterodimers with other members of the receptor superfamily, such as RXRs, vitamin D receptor, and all subtypes of the retinoic acid receptors. Heterodimerization with RXR dramatically increases the binding of TRs to TREs, the responsiveness of TR to T_3, and the transcriptional activation (31). Thus, heterodimerization provides an important means to modulate the functions of TR. Due to ubiquitous distribution of RXR and its promiscuity in heterodimerization with many members of the receptor superfamily, heterodimerization with RXR provides a means for TR to crosstalk with other receptors. Crosstalk with peroxisome proliferator-activated receptor (PPAR) signaling via heterodimerization with RXR by TR is a prominent example. PPARγ regulates the expression of its target genes by binding to the PPAR response element (direct repeat+1; DR1) as a heterodimer with RXR. Recently, it was shown that TRβ competes with PPARγ for binding to DR1 as a heterodimer with RXR *in vitro* and *in vivo* to repress the transcriptional activity of PPARγ (32). Because PPARγ plays a key role in lipid metabolism, carcinogenesis, and cardiovascular diseases (33–35), this mode of TR action via crosstalk with other receptors expands TR effects via direct binding to TREs on the promoter of target genes.

The gene regulatory activity of TR could also be affected by other receptors that heterodimerize with RXR. This is shown by the regulation of 7α-hydroxylase (CYP7A1) that is the rate-limiting enzyme in cholesterol metabolism. TRβ/RXR binds to the TRE (DR+4) on the promoter of the CYP7A1 gene to positively regulate its expression. However, the liver X receptor (LXR)/RXR heterodimer also binds to the same TRE site to activate the expression of the CYP7A1 gene. Cell-based studies indicate that cotransfection of TRβ with LXR-α inhibits the activity of LXR-α transcription activity of the CYP7A1 promoter (36). This inhibition is due to competition of LXR with TRβ for heterodimerizing with RXR in binding to DR+4 (36). These findings show that LXR crosstalks with TRβ via heterodimerization with RXR, and via this network the activity of TRβ is modulated by other receptors.

3. Modulation of TR functions by other cellular proteins

Central to the understanding of TR action are the mechanisms by which the diverse effects of T_3 are achieved. Recent studies suggest that the diverse effect of TR could be mediated by interaction with a host of cellular proteins. These cellular proteins could be expressed in a tissue-dependent and developmentally regulated manner. This, together with the known differential expression of TR iso-

forms, diversity of TREs, and various heterodimeric partners, could further fine-tune the transcriptional activity of TR in different tissues. Thus, the diverse effects of TR could be achieved via combinatorial complexes of TR with various cellular proteins. Many TR-interacting proteins have recently been identified and are briefly reviewed in *Section I.C.3.a.*

a. Nuclear receptor coregulators. The search for bridging factors that could communicate the signals of TRE-bound TR with the basal transcription machinery for efficient transcription led to the identification of the first transcriptional mediator that interacts with both TRβ1 and RXR in a ligand-dependent fashion. This protein, Trip1 (for TR-interacting protein), shares striking sequence conservation with the yeast transcriptional mediator Sug1 (37). Soon after the discovery of Trip1, many coregulatory proteins (CoR and CoA) for TRs have also been discovered via various screening strategies. Structures and functions of these coregulators have been intensively studied in the past decade, and much has been learned about how TRs interact with the coregulators. A simplified molecular model for the regulation of T_3-dependent positively regulated genes is shown in Fig. 2. In the absence of T_3, the unliganded TR binds to TRE as a heterodimer with RXR. The association of unliganded TR/RXR with CoA results in the repression of basal transcriptional activity (Fig. 2A). Binding of T_3 to TR induces structural changes (16), allowing the liganded TR to recruit CoA and other associated proteins to modify chromatin structures to facilitate transcriptional activation (Fig. 2B).

Coactivators (CoAs). The steroid hormone receptor CoA (SRC-1) is the first CoA to be cloned that binds to the liganded TR in addition to other members of the receptor superfamily (38, 39). Subsequently identified were other related CoAs, known as SRC-2/TIF2 (transcription intermediary factor 2)/GRIP1 (GR-interacting protein 1) and SRC-3/TRAM-1 (thyroid hormone receptor activator molecule 1)/RAC3 (receptor-associated CoA 3)/ACTR (activator of thyroid receptor)/pCIP (p300/CBP cointegrator-associated protein) (40). These CoAs share considerable sequence homology (~40%), have functional properties similar to SRC-1, and are now designated as the SRC/p160 family members (40). These SRCs share a highly conserved basic helix loop helix (bHLH) and Per-ARNT-Sim (PAS) A/B domain (bHLH-PAS; Fig. 3) in the amino-terminal region that functions as a DNA-binding domain or dimerization interaction region for other transcription factors (41). In the center of the molecule, three copies of signature motifs LXXLL (in the one letter code of amino acids, X indicates any amino acid) termed the NR box are located (41–44). Mutational analysis indicates that distinct NR boxes interact differentially with different nuclear receptors (receptor-specific codes), and the selectivity may arise from the amino acids located adjacent to the different LXXLL motifs (42). X-ray crystallographic analysis of TRβ-LBD complexed with T_3, and a 13-amino acid peptide NR box of GRIP1 revealed the details in the molecular interaction of receptor and CoA (45). The leucines of the α-helical NR box make contacts with a hydrophobic groove consisting of residues from helices 3, 4, 5, and 12 of TRβ. A single LXXLL peptide interacts with each monomer of TRβ dimer (42).

Near the C-terminal region, SRC-1 contains two activation domains, AD1 and AD2, responsible for its coactivation function. Both AD domains are required for optimal coactivation although they act independently. AD also contains three additional LXXLL motifs that interact with general transcriptional activators CBP/p300 and P/CAF (41, 44). Like CBP/p300, SRC-1 also functions as a histone acetyltransferase, and this activity is localized in the C-terminal region of the protein (Fig. 3). The histone acetyltransferase functions to modify chromatin structures to facilitate transcriptional activity of nuclear receptors.

In addition to SRC/p300 complexes, the transcriptional activity of TRs is also regulated by other large complexes. Using epitope-tagged TR, TR-associated proteins (TRAP) were affinity-purified from a cell line stably expressing TR (46). The TRAP complex, which does not exhibit intrinsic histone acetyltransferase activity, was shown to enhance the transcription activity of TR in a chromatin-free system (47). This complex contains at least

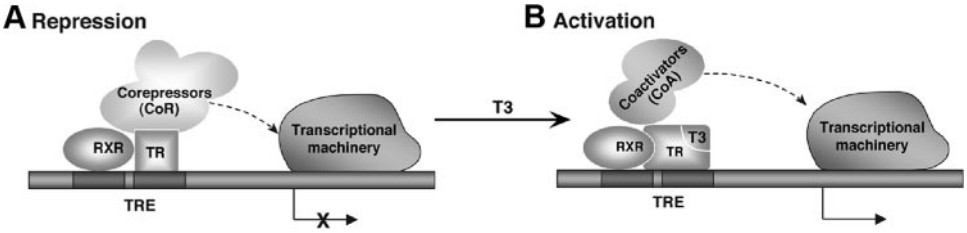

FIG. 2. A simplified molecular model for transcriptional repression by unliganded TR (A) and activation by liganded TR (B). Interaction of the unliganded TR with the complex of CoRs and its associated proteins leads to repression of transcription (A). Interaction of the liganded TR with CoAs (*e.g.*, SRC/p160 or TRAP/DRIP complex) leads to transcriptional activation (B).

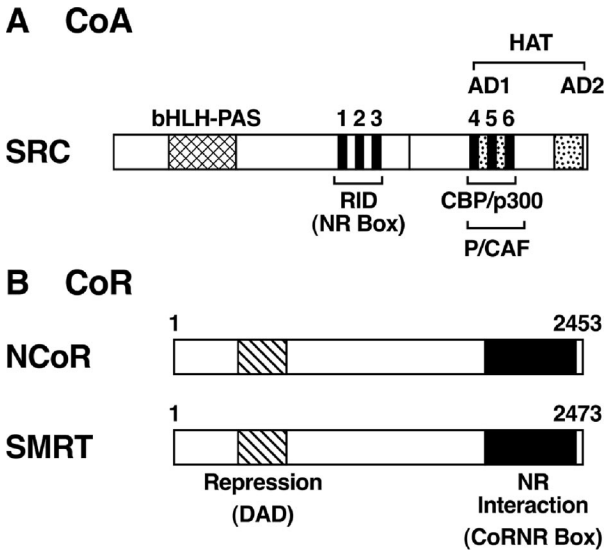

FIG. 3. Schematic representation of SRC/p160 CoA family (A) and NCoR/SMRT CoRs (B). A, The location of the receptor interaction domain (RID) in the SRC is indicated. RID and activation domain 1 (AD1) each contains three LXXLL motifs. The specific domains for interaction with P/CAF, CBP/p300, as well as the histone acetyltransferase domain, are indicated. Located near the amino-terminal region is the highly conserved bHLH–PAS domain that functions as a DNA-binding or dimerization surface in many transcription factors. B, The nuclear receptor (NR) interaction region that contains the "CoRNR box" motifs near the C-terminal region is indicated. Near the amino-terminal region is the deacetylase activation domain (DAD) that interacts with and activates HDAC3, required for repression by TR.

nine proteins, with molecular weight ranging from approximately 70 to approximately 230 kDa. One of these proteins, TRAP220, was found to interact with TR in response to T_3 (47, 48). A complex containing similar components was found to interact with vitamin D receptor (vitamin D receptor interacting proteins; DRIPs) and to enhance its transcriptional activity (49). Thus, different receptors could be mediated by the TRAP/DRIP complex for the regulation of transcriptional activity.

That TR is associated with multiple complexes prompted the question as to how different complexes collaborate to activate ligand-dependent transcription activity of TR. Several interesting models have been proposed. These different complexes could perform different tasks in a sequential order, in a combinatorial overlapping utilization of the complexes, or in a target gene-specific utilization of complexes in responding to different signals (50). It is possible that the mode of actions could depend on the target genes and the cellular context. The elucidation of these possibilities will require additional studies.

Corepressors (CoRs). One of the functional characteristics of TR is its ability to repress, or silence, the basal transcription in the absence of ligand. Intensive studies in the past decade have indicated that this repression occurs via interaction of TR with CoR proteins. The first CoRs cloned were NCoR (nuclear receptor CoR) (51) and its homolog, SMRT (silencing mediator of retinoid and thyroid hormone receptor) (52). Subsequently, other nuclear proteins such as Hairless (53), Alien (54), RIP-140 (55, 56), and SUN-CoR (57) were reported to function as CoRs for TRs. Our current understanding of how CoRs mediate the basal repression of TRs is mostly learned from the studies using NCoR and SMRT because they are the best characterized.

NCoR or SMRT binds to the surface of the TR molecule consisting of helices 3, 4, and 5 via its "CoRNR" box with repeated motifs of (I/L)XX(I/V)I in the C-terminal region (58, 59) (Fig. 3B). NCoR and SMRT are associated with other proteins such as transducin-like protein (TBL1, or TBL1R) and histone deacetylase 3 (HDAC3) to form large repression complexes (60). NCoR and SMRT interact with HDAC3 via a region denoted the deactylase activation domain that contains the SANT1 motif (59, 60) (Fig. 3B). HDAC3 is required for repression by TR (59). However, the repression by TR could also be mediated by HDAC3-independent mechanisms via TBL1, which interacts with histones (60, 61).

Although NCoR and SMRT are highly homologous and the mode of action appears to be similar, the gene inactivation in mice suggests that these two CoRs have nonredundant biological functions (62). Mice with NCoR knockout are embryonic lethal, suggesting the SMRT cannot compensate for the functions of NCoR involved in development and survival (63). Although NCoR has been implicated in human diseases such as acute promyelocytic leukemia (retinoic acid receptor translocation) (64), acute myeloid leukemia (AML1-ETO translocation) (65), and thyroid hormone resistance (66), and more recently in the regulation of oncogenic functions of a mutated TRβ (denoted TRβPV) in thyroid carcinogenesis (67) (see *Section I.D.1.b.*), it is not yet known whether SMRT is involved in human diseases. Thus, these observations suggest that these two CoRs could mediate distinct biological functions. With the increasing numbers of CoRs that are being discovered and being characterized, novel mechanisms in the regulation of TR functions should soon be forthcoming.

b. Other TR interacting proteins. In addition to nuclear receptor coregulators that modulate the transcriptional activity of TR in a hormone-dependent manner, the activity of TRs can also be regulated by other cellular proteins. These TR-interacting proteins are functionally diverse, ranging from transcription regulators, to cytoskeletal architecture modulators, to tumor suppressors and promoters. In their own right, these TR-interacting proteins occupy a critical position in regulating cellular functions.

The mode of interaction of TRs with each protein varies, and the mechanisms by which these proteins alter the functions of TR also differ. The interaction with TR also alters the functions of these proteins.

Cyclin D1. Cyclin D1 regulates cell cycle progression, and its overexpression is associated with tumorigenesis (68). Lin *et al*. (69) found that cyclin D1 physically interacts with the C-terminal region of the LBD of TRβ1 in a T_3-independent manner. Cyclin D1 acts to repress both the silencing activity of the unliganded TRβ1 and the transcriptional activity of the liganded TRs. The binding of TR to TRE is not affected by its association with cyclin D1. Interestingly, cyclin D1 acts as a bridging factor to recruit HDAC3 to augment the silencing activity of the unliganded TRβ1 and to mediate the repression of the T_3-dependent transcriptional activity. Thus, the interaction of TR with cyclin D1 raises the possibility that TR could mediate its functions via the cyclin D1 regulatory network (70).

EAR-2. EAR-2, an orphan nuclear receptor, was identified as an interacting protein for TRβ in human colon carcinoma RKO cells (71). EAR-2 is a distant member of the chick ovalbumin upstream promoter-transcription factor of the orphan nuclear receptor family. TRβ1 physically interacts with EAR-2 *in vitro* and in cells independent of T_3. The binding site was mapped to the C-terminal region of TRβ. Binding of TRβ1 to TREs is competitively inhibited by EAR-2. In several cultured cell lines, both the T_3-dependent and T_3-independent TRβ1 transcriptional activities are repressed by EAR-2. However, the sensitivity of repression depends on the cell type, thereby suggesting that the cellular context plays an important role in the repression effect of EAR-2 (71). One of the possible cellular factors that could affect the cell-type dependent repression effect of EAR-2 is SRC-1 because the repression effect of EAR-2 on the T_3-dependent transcriptional activity is reversed by the transfected SRC-1 in cells (71). Because the expression of SRC-1 is cell-type dependent (38), the extent of negative regulation most likely will depend on the expression levels of EAR-2, TR, and CoAs in cells. This dependence suggests that the diverse functions of TR are likely mediated by a large network of regulatory proteins.

Tumor suppressor p53. The tumor suppressor p53 is a critical transcription factor in the regulation of the cell cycle and in tumorigenesis (72). Yap *et al.* (73) found that TRβ1 physically interacts with p53 through its DNA-binding domain. The regions of p53 responsible for its interaction with TRβ1 are located in the DNA-binding domain and at the carboxyl-terminal polymerization domain (74). The TR DNA-binding domain is the interaction site with p53 (74, 75). This physical interaction leads to the inhibition of the binding of TRβ1 to TREs. In transfected cells, p53 represses the T_3-dependent transcription of TRβ1 (73). In rat pituitary GH-producing GC cells that endogenously express TR, expression of p53 leads to a repression of a TR-target gene, the GH gene (76). Thus, p53 is a negative regulator of the transcriptional activity of TRβ1.

That the DNA-binding and the carboxyl-terminal polymerization domains of p53 are the binding sites with TRβ1 raised the possibility that the activity of p53 could be affected by binding to TRβ1. Indeed, the association of p53 with TRβ1 increases the binding of p53 to p53 DNA-binding elements (74). This increase in DNA-binding, however, results in repression of p53-dependent transcription activation in transfected cells. Furthermore, this association leads to an inhibition of the p53-mediated induction of bax and gadd45 expression (74). Because the expression of bax and gadd45 is directly regulated by p53, these results indicate that TRβ1 can modulate p53-regulated gene expression. Taken together, the crosstalk between these two important transcription factors could play an important role in the biology of normal and cancer cells.

Gelsolin. Gelsolin is an actin-binding protein, and in the presence of calcium it is able to sever actin and cap the growing end of the released filament (77). It is involved in controlling cell morphology, motility, growth, and apoptosis (78). Phosphoinositides can bind to gelsolin and regulate its interaction with actin (79). Its important role in phospholipid signaling pathways was recently demonstrated by the observation that gelsolin-induced epithelial cell invasion is dependent on phosphatidylinositol 3-kinase (PI3K)-Rac pathways (79). During thyroid carcinogenesis, the expression of gelsolin was found down-regulated (80). Additional biochemical analyses demonstrated that gelsolin physically interacts with TRβ and its mutant TRβPV *in vivo* and *in vitro*. The interaction regions were mapped to the C terminus of gelsolin and the DNA-binding domain of TR. Interestingly, the interaction of gelsolin is weakened by the mutation of TR, resulting in perturbation of cytoskeletal architectures. These results revealed a novel function of TR in maintaining the integrity of cellular cytoskeletal structure and functions via physical interaction with gelsolin (80).

Pituitary tumor-transforming gene (PTTG). PTTG, also known as securin, is a critical mitotic checkpoint protein that helps hold sister chromatids together before entering anaphase (81). It was originally isolated from GH4 pituitary cells and shown to cause *in vitro* cell transformation and to induce tumor formation *in vivo* (82). Overexpression of PTTG has been detected in human thyroid carcinomas (83, 84), colorectal carcinoma (85), pituitary adenomas (86), and hematopoietic neoplasms (87). Im-

portantly, PTTG was found to induce genetic instability in a variety of cells including thyroid cells (88, 89). The overexpression of PTTG in thyroid tumors of a mouse model of thyroid cancer that harbors a TRβ mutant ($TR\beta^{PV/PV}$ mice), TRβPV, led to the discovery that PTTG is a TR-interacting protein (89). *In vitro* and cell-based studies showed that the PTTG protein is physically associated with TRβ as well as the mutated TRβPV. The DNA-binding domain of TR is the site that interacts with the amino-terminal region of PTTG. Concomitant with T_3-induced degradation of TRβ (90), PTTG proteins are degraded by the proteasome machinery, but no such degradation occurs when PTTG is associated with the mutant TRβPV (91). A recent study has demonstrated the interaction of SRC-3 with proteasome activator 28γ (PA28γ) and that the degradation of SRC-3 is mediated by the PA28γ proteasome (92). The direct interaction of TRβ with SRC-3 upon T_3-binding activates the PA28γ proteasome-mediated degradation of PTTG. In contrast, TRβPV, which does not bind T_3, could not form active complexes via direct interaction with SRC-3/PA28γ. The discovery that the liganded TRβ, via physical interaction with PTTG, regulates cellular levels of PTTG protein has important implications in cancer biology in that aberrant accumulation of PTTG is known to cause genetic instability that could underlie cancer development.

β-Catenin. β-Catenin, a structural component of cell adhesion complexes, interacts with the transmembrane E-cadherin to regulate actin filament assembly to regulate cellular functions (93). In addition, β-catenin also functions as a CoA for a family of transcription factors known as the T-cell factor/lymphoid enhancer factor (TCF/LEF). Upon increased cellular levels and nuclear accumulation, β-catenin/TCF complexes bind to the promoters of downstream target genes involved in cell proliferation, survival, and migration (94). The cellular abundance of β-catenin was found highly elevated in the thyroid tumors of $TR\beta^{PV/PV}$ mice (95). Studies to understand how TRβ and its mutants regulate the cellular levels of β-catenin *in vivo* led to the discovery that TRβ interacts with β-catenin *in vitro* as well as *in vivo* (95). The interaction region of TR was mapped to the DNA-binding domain, and the interaction of β-catenin with TR favors the unliganded state of TRβ. Via physical interaction in a T_3-dependent manner, TRβ could regulate the β-catenin protein levels through Adenomatous Polyposis Coli-independent proteasome pathways. The functional significance of the regulatory mechanism is evident in that $TR\beta^{PV/PV}$ mice, which harbor a mutated TR (TRβPV) that has lost T_3-binding activity, have elevated β-catenin protein levels. This aberrantly increased β-catenin protein level could lead to activation of β-catenin-regulated downstream target genes to contribute to thyroid carcinogenesis in these $TR\beta^{PV/PV}$ mice.

D. TR mutations and disease

Given the critical role of TRs in cellular functions, it is reasonable to expect that mutations of TRs could have deleterious effects. Indeed, shortly after the cloning of the TR genes (7, 8), a tight linkage was discovered between the affected family members with resistance to thyroid hormone (RTH) and the TRβ gene (96). The identification of a Pro453 His mutation in the TRβ gene of one kindred established that RTH is caused by mutations of the TRβ gene (97). To date, about 124 different mutations in the TRβ gene have been reported in more than 374 families and 532 affected individuals (98). In addition to RTH, other abnormalities associated with mutations of the TRβ gene have been uncovered from the mouse models harboring TRβ mutations (99). So far, mutations of the TRα gene have not been reported in humans. However, abnormalities from mice harboring mutations of the TRα gene have been described (99). Significant advances have been made in understanding the *in vivo* actions of TR mutants by using the genetically engineered mice that will be highlighted in *Section I.D.1.*

1. Mutations of the TRβ gene

a. RTH. RTH is a syndrome characterized by reduced sensitivity of tissues to the actions of thyroid hormone (100, 101). The hallmark of RTH is elevated thyroid hormone associated with nonsuppressible TSH. Other clinical signs are goiter, short stature, decreased weight, tachycardia, hearing loss, attention deficit/hyperactivity disorder, decreased IQ, and dyslexia (100, 101). The clinical manifestations vary between families with different mutations, between families with the same mutation, and also between members of the same family with identical mutations. Most patients are heterozygous, with only one mutated TRβ gene, and their clinical symptoms are mild (100, 101). Only one patient homozygous for a mutant TRβ has been reported (102). This patient, who died young, displayed an extraordinary and complex phenotype of extreme RTH with very high levels of thyroid hormones and TSH (102).

Two knockin mutant mice harboring mutations of the TRβ gene have been created to understand the molecular basis of RTH, one harboring a carboxyl-terminal 14-amino acid frame-shift mutation (TRβPV mouse) (103) and the other a Δ337T mutation (TRβΔ337T mouse) (104). These two knockin mice exhibit RTH phenotypes including dysregulation in the pituitary-thyroid axis, abnormal regulation of serum cholesterol, and neurological dysfunction (36, 104–106). Consistent with phenotypes

of RTH patients, TRβPV mice also exhibit growth retardation (103), hearing defects (107), and thyrotoxic skeletal phenotype (108).

Using TRβPV mice, Zhang *et al.* (109) elucidated the molecular mechanisms of the dominant activity of TRβ mutants *in vivo*. In the liver nuclear extracts of $TR\beta^{PV/+}$ mice, PV forms not only TRE-bound homodimers, but also TRE-bound heterodimers with TRβ1, TRα1, or RXR. In $TR\beta^{PV/PV}$ mice, in addition to PV/PV homodimers, the lack of wild-type TRβ1 facilitates the formation of TRE-bound PV/TRα1 and PV/RXR heterodimers. Therefore, *in vivo*, PV competes with TRβ or wild-type TRα1 for binding to TRE and for heterodimerization with RXRs (109). Such competition leads to repression of the T_3-positively regulated target genes—S14, malic enzyme, and type 1 deiodinase—in the liver of TRβPV mice. These studies demonstrate that one of the molecular mechanisms by which TRβ mutants exert their dominant-negative activity *in vivo* is through competition of inactive PV dimers with TRs for binding to TRE and of the mutant PV with RXR for binding to TRE of T_3-target genes.

Although most heterozygous RTH patients are clinically euthyroid, some are hypothyroid, and some may appear thyrotoxic. Intriguingly, the same individual may present evidence of hypothyroidism in one tissue, whereas showing signs of thyrotoxicosis in other tissues (100, 101). Using TRβPV mice, Zhang *et al.* (109) showed that differential expression of TR isoforms in tissues contributes to variable clinical manifestations in RTH. Using mice from the cross of TRβPV mice and SRC-1-deficient mice, Kamiya *et al.* (105) showed that lack of SRC-1 modulates the degree of resistance to thyroid hormone in a target tissue-dependent manner and alters abnormal expression patterns of several T_3 target genes in tissues. Thus, differential expression of CoAs such as SRC-1 also contributes to the variable clinical manifestations in RTH.

To test whether TRα1 plays a compensatory role in maintaining the normal physiological functions of T_3 in RTH patients who are clinically euthyroid, Suzuki and Cheng crossed TRβPV mice with mice deficient in TRα1 (110) and compared the phenotypes of TRβPV mice with or without TRα1 (110). Lack of TRα1 worsens the RTH symptoms and suggests that TRα1 plays an important and previously unrecognized compensatory role in maintaining the physiological functions of T_3 in heterozygous patients with RTH. It is clear from these studies that complex regulation of actions of TRβ mutants and cellular context lead to varied manifestations of the RTH phenotype.

b. Thyroid cancer. Despite reports to indicate a close association of TRβ mutants in human cancers (111, 112), direct evidence to support their oncogenic actions is lacking. The remarkable discovery that $TR\beta^{PV/PV}$ mice spontaneously develop follicular thyroid carcinoma has provided an unprecedented opportunity to elucidate the oncogenic actions of TRβ mutants *in vivo* (113). The pathological progression from hyperplasia to capsular invasion, vascular invasion, anaplasia, and metastasis to the lung is similar to human thyroid cancer (113). The mutation of TRβPV is highly penetrated, as evidenced by the fact that by 1 yr of age, all $TR\beta^{PV/PV}$ mice have developed thyroid cancer.

cDNA microarray analysis identified altered expression of 280 genes during thyroid carcinogenesis (114), indicating that complex alterations of multiple signaling pathways induced by TRβPV could contribute to thyroid carcinogenesis. Among the altered signaling pathways, the repression of the PPARγ (19) is particularly relevant in view of findings that altered PPARγ expression and function could be an important risk factor in the development of human thyroid carcinomas (115–118). Further biochemical and molecular analysis indicates that TRβPV acts not only to repress the expression of PPARγ, but also to inhibit its transcriptional activity. Such repression of PPARγ activity leads to the activation of the nuclear factor κB downstream signaling, thereby promoting tumor cell proliferation and inhibiting apoptosis. Via such actions, TRβPV functions as an oncogene to mediate thyroid carcinogenesis (119).

The oncogenic actions of TRβPV are not limited via transcription regulation initiated at the nucleus site. Molecular analyses showed that TRβPV could also act via nongenomic actions to activate other oncogenes and key effectors of critical cellular signaling pathways to promote carcinogenesis. Several interacting oncogenes and key regulators have been identified. They are the regulatory subunit p85α of PI3K (67, 120), PTTG (91), and β-catenin (95). The physical interaction of TRβPV with the regulatory subunit p85α of PI3K leads to the activation of PI3K-AKT-mammalian target of rapamycin-$p70^{S6K}$ pathway to promote tumor cell proliferation and organ growth (67, 120). This interaction also leads to activation of the PI3K-integrin-linked kinase-matrix metalloproteinase-2 pathway to increase cell invasion and metastasis (120). The physical association of TRβPV with PTTG blocks the degradation of PTTG via proteasome machinery, resulting in an aberrant accumulation of cellular PTTG to disrupt mitotic progression and chromosomal abnormalities (91). The physical interaction of TRβPV with β-catenin prevents the degradation of β-catenin, leading to sustained activation of β-catenin-mediated downstream target gene expression to contribute to thyroid carcinogenesis (93). Thus, TRβPV via nucleus-initiated transcription as well as nongenomic signaling pathways functions as an oncogene in thyroid carcinogenesis.

c. Pituitary tumors. In addition to RTH and thyroid carcinoma, TR$\beta^{PV/PV}$ mice also spontaneously develop TSH-secreting pituitary adenomas (TSH-omas) (121). TSH-omas are tumors that constitutively secrete TSH. The molecular genetics underlying this abnormality are not well understood. Clues about genetic alterations leading to TSH-omas began to emerge when mutated TRβ was identified in several patients with TSH-omas (122, 123). The TR$\beta^{PV/PV}$ mouse has provided an opportunity to address the role of TRβ mutants in the pathogenesis of TSH-omas (121). Extensive biochemical and cell-based studies indicated that TRβPV increases expression of cyclin D1 at both the mRNA and protein levels, leading to the activation of the CDK/retinoblastoma (Rb)/E2F pathway that mediates, at least in part, the aberrant proliferation of thyrotrophs in TR$\beta^{PV/PV}$ mice. Thus, these findings provide mechanistic insights into the pathogenesis of TSH-omas in patients and raise the possibility that the mutated TRβ could serve as a molecular marker for diagnosis.

2. Mutations of the TRα gene

Dwarfism and metabolic disorders. The intriguing fact that no mutations of the TRα gene have ever been identified in RTH patients has perplexed investigators for years and raised the fundamental issue of whether mutations of the TRα gene are lethal or can cause other human diseases. This perplexing question led to the creation of knockin mice harboring the same PV mutation in the TRα gene locus (TRα1PV mice) (124) to unequivocally resolve this fundamental issue. The mutation of both alleles of the TRα gene is not lethal to the embryos, although neonates die shortly after birth. The mutation of one TRα allele results in dwarfism and other abnormalities that are clearly distinct from those caused by mutations of the TRβ gene. Notably, TR$\alpha^{PV/+}$ mice exhibit no apparent abnormalities in thyroid function tests. These differences in the pituitary-thyroid axis are consistent with the fact that no TRα mutations have been identified in RTH patients. These results indicate that mutated TRα1 and TRβ have distinct biological functions *in vivo* and thus lead to different diseases.

That mutations of the TRα gene lead to phenotypes differing from the mutations of the TRβ gene was also confirmed in two other knockin mutant mice harboring different mutations, TRα1R384C (125) and TRα1P398H (126). Interestingly, among the three knockin mutant mice, there are differences in phenotypes. TR$\alpha^{R384C/+}$ mice exhibit juvenile growth retardation that is overcome in adult mice. The milder impairment in growth was attributed to a mutant (TRα1R384C) with a weaker dominant-negative activity (125). In contrast to the TRα1PV mutation, with no T_3-binding and transactivation capacity, TRα1R384C only partially lost T_3-binding activity, and its transactivation activity could be restored by increased T_3 concentration. *In vivo*, the growth impairment in TR$\alpha^{R384C/+}$ mice could be rescued by elevated thyroid hormone via an independent mutation in the TRβ gene. In contrast to TRα1PV and TRα1R384C knockin mice that exhibit dwarfism and reduction in fat mass, TRα1P398H mutant mice have increased body fat accumulation and elevated serum levels of leptin, glucose, and insulin. In addition, there is a marked impairment in sympathetic-mediated lipolysis in white adipose tissue (126).

Recent studies indicate that the lean phenotype exhibited by TRα1PV and TRα1R384C knockin mice is partly due to the reduction in fat mass (127, 128). However, the underlying mechanisms that lead to abnormal lipid metabolism differ in these two mutant mice. In TR$\alpha^{PV/+}$ mice, the reduced white adipose mass is due to the repression by TRα1PV of the expression of PPARγ, the key regulator of adipogenesis at both the mRNA and protein levels and the inhibition of the transcription activity of PPARγ. By contrast, TR$\alpha^{R384C/+}$ mice are hypermetabolic, showing increased lipid mobilization and β-oxidation in adipose tissues. The blockade of sympathetic signaling to brown adipose tissue normalized the metabolic phenotype despite a continued perturbed hormone signaling in this cell type (128). In contrast, the brown adipose tissue of TR$\alpha^{PV/+}$ mice is not affected by PV mutation (127).

The manifestation of lean phenotype in TRα1PV (124, 127) and TRα1R384C type (128), but of increased fat accumulation in TRα1P398H mutant mice (126) suggests that the phenotypic expression of a knockin mutant is sensitive to the location and type of mutation in TRα1. These three mutations—TRα1PV, TRα1R384C, and TRα1P398H—are all located in helix 12, which in wild-type TR undergoes dramatic structural reorganization in response to T_3 binding (16), suggesting that the movement and reorganization of helix 12 in relation to the remainder of the TRα1 molecule could be sensitive to mutational alteration. Previously, it has been shown that *in vitro* different RTH TRβ mutants interact differently with CoRs (66, 129, 130). Although it is currently unknown how these TRα1 mutants interact with CoRs *in vivo*, it is conceivable that a different mode of interaction of TRα1 mutants with various CoRs could lead to differential transcriptional repression of different target genes, resulting in the manifestation of different phenotypes. Verification of this possibility in future studies would certainly further advance our understanding of the actions of TRα1 mutants *in vivo* and the molecular basis of diseases due to mutation of the TRα gene.

II. Nongenomic Actions of Thyroid Hormone

A. Initiation sites for nongenomic actions of thyroid hormone: plasma membrane and cytoplasm (Fig. 4)

1. Sites on the plasma membrane at which thyroid hormone actions may be initiated

High-affinity binding sites for thyroid hormone analogs have for many years been recognized on the plasma membrane of erythrocytes (131, 132) and other cells (133, 134) and sometimes have been linked to local membrane functions, such as the calcium pump (132, 135); see *Section II.B.1.a*). There was a reluctance to describe these sites as receptors because: 1) complex cellular functions ascribed to thyroid hormone did not follow hormone-binding to the sites; 2) specific identities of membrane binding sites for iodothyronines were not established; and 3) nuclear receptors for thyroid hormone appeared to account for most of the acknowledged actions of thyroid hormone. Rapid-onset membrane effects, such as stimulation of cellular glucose uptake (136) and changes in sodium current (137), were attributed to thyroid hormone in intact cells but implicated an initiation site at or near the plasma membrane.

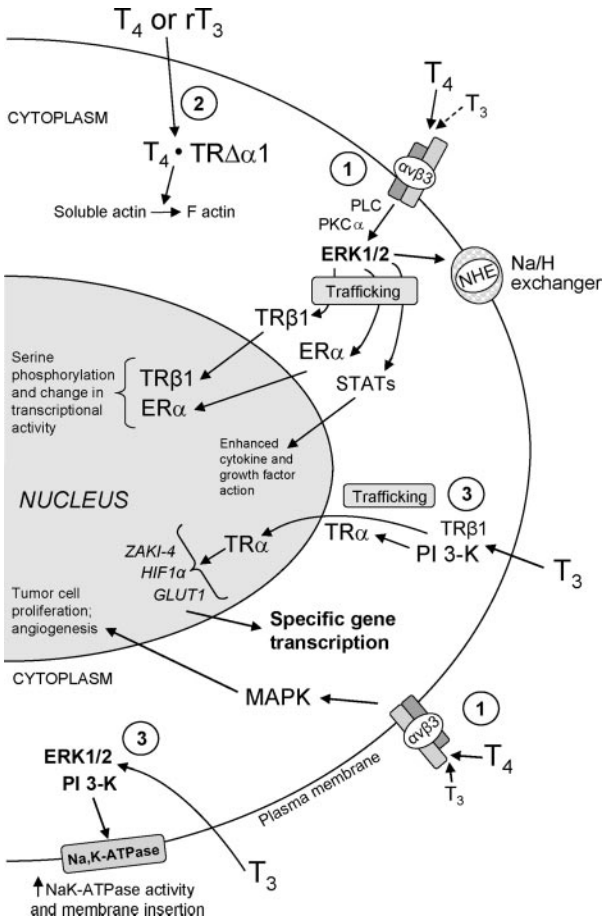

FIG. 4. Nongenomic actions of thyroid hormone that are initiated at the plasma membrane receptor on integrin $\alpha v\beta 3$ or in cytoplasm. Via the integrin receptor, thyroid hormone from the cell surface stimulates MAPK (ERK1/2) through phospholipase C (PLC) and protein kinase C (PKC) (161). Hormone-activated ERK1/2 promotes trafficking of specific proteins resident in cytoplasm to the nuclear compartment and serine phosphorylation of nucleoproteins by activated ERK1/2 also translocated to the nucleus. Target proteins phosphorylated by hormone-directed ERK include estrogen receptor (ER)-α, TR-β1, signal transducing and activator of transcription (STAT)-1α, and CoA protein Trip230. Complex cellular events induced from the cell surface receptor include angiogenesis (endothelial and vascular smooth muscle cells) and tumor cell proliferation (160). In cytoplasm, T_3 can nongenomically activate PI3K and initiate downstream transcription of specific genes. Activation of PI3K can involve TRβ1 or TRα resident in cytoplasm. A truncated form of TRα1 (TR$\Delta\alpha$1) in cytoplasm mediates the action of T_4 and rT_3 on the actin cytoskeleton. T_3 and T_4 may also activate PI3K from the integrin $\alpha v\beta 3$ hormone receptor site (148). GLUT1, Glucose transporter-1.

Recently, a structural protein of the plasma membrane, integrin $\alpha v\beta 3$, has been shown to contain a binding domain for iodothyronines that is an initiation site for hormone-directed complex cellular events, such as cell division (138) and angiogenesis (139). This qualifies the binding site for characterization as a receptor. The receptor has been localized to the Arg-Gly-Asp (RGD) recognition site on the integrin that is important to the binding of a number of extracellular matrix proteins (140) and growth factors (141–143). From this site, the thyroid hormone signal is transduced by MAPK (ERK1/2) into angiogenesis in endothelial cells (139) and cell proliferation of tumor cell lines (138, 144, 145) and tumor xenografts (146, 147). T_4 in concentrations that approximate physiological (10^{-10} M free T_4) and T_3 in supraphysiological concentrations cause ERK-dependent cell proliferation. It is now clear that the hormone receptor domain on the integrin is more complex than initially thought. That is, there is a T_3-specific site in the domain, as well as a site at which both T_4 and T_3 may act (148). The T_3-specific site activates PI3K and is linked not to cell proliferation, but to trafficking of certain intracellular proteins such as shuttling of TRα from cytoplasm to nucleus, and to the transcription of specific genes, such as hypoxia-inducible factor-1α (HIF-1α) (148). T_4 is unable to activate PI3K. A deaminated derivative of T_4, tetraiodothyroacetic acid (tetrac), is an antagonist at the integrin receptor domain and blocks actions of agonist hormone analogs at both the T_4/T_3 site and the T_3-specific site. Interestingly, RGD peptides block the T_3 site, but at the T_4/T_3 site do not inhibit cell proliferation; they selectively inhibit thyroid hormone-enhanced shuttling of TRβ1 to the nucleus from cytoplasm. The effects of these hormone analogs at the integrin receptor domain are reproduced by their reformulation into nanoparticles that prohibit access of the agents to the cell interior.

Other laboratories have in the past 5 yr clearly shown that T_3 can activate the PI3K/protein kinase B/Akt signal transduction pathway (149–151), but have reported that the activation process begins in cytoplasm. The consequences of this action of T_3 include transcription of the *HIF1-α* gene and

activation of plasma membrane Na,K-ATPase and its insertion in the plasma membrane (see *Section II.A.2*).

Binding sites for thyroid hormone have also been described in synaptosomes of chick embryo brain (152). These sites in cerebral cortex have a higher affinity for T_3 than for T_4. Interestingly, the binding capacity of one species of binding sites declines importantly after hatching. A synaptosomal binding site for iodothyronines appears to be associated with one or more G proteins (153). The application of the term "receptor" to these moieties awaits further definition of their function(s).

2. Sites in cytoplasm at which thyroid hormone actions may be initiated

Proteins in cytoplasm that bind iodothyronines are either nuclear receptors that reside in cytoplasm, apparently transiently, or native cytoplasmic proteins. Nuclear thyroid hormone receptors at one time were seen to be functional only within the nuclear compartment, despite their identification in cytoplasm (154–156). However, nuclear TRβ1 detected in cytoplasm and complexed with T_3 has been shown by Cao *et al.* (157) to interact with the p85 regulatory subunit of PI3K, leading downstream to specific gene transcription (157, 158). Among the genes transcribed by this mechanism are ZAKI-4, a calcineurin-like protein, and HIF-1α (149, 158). HIF-1α protein is involved in expression of a series of genes important to carbohydrate metabolism, including glucose transporter-4. TRβ1 was also shown to direct via PI3K the slowing of deactivation of KCNH2 channels in the plasma membrane of pituicytes (151). Lei *et al.* (159) have implicated cytoplasmic TRβ1 and PI3K in modulation of activity of Na, K-ATPase, in insertion of the sodium pump protein in the plasma membrane, and in transcription of the Na, K-ATPase gene. TRα1 may also reside in endothelial cell cytoplasm. Hiroi *et al.* (160) have reported that this receptor, when extranuclear, may activate endothelial cell nitric oxide synthase and is thought to contribute to vasodilatation induced by thyroid hormone. Finally, TR$\Delta\alpha$1 is a truncated form of a nuclear receptor shown to contribute to regulation by thyroid hormone of the state of the actin cytoskeleton (161) (see *Section IV*).

The above effects that include nuclear thyroid hormone receptors that reside in cytoplasm—and, in certain cases, PI3K—are felt to be initiated in cytoplasm and not at the plasma membrane. It should be noted, however, that MAPK (ERK1/2) activation has been shown by Lei *et al.* (162) to precede the stimulation of PI3K by T_3 that leads to changes in plasma membrane Na, K-ATPase activity. This raises the possibility that the cell surface integrin receptor for iodothyronines that is capable of activating both ERK1/2 and PI3K (148), as described in *Section II.A.1*, may be implicated in certain actions of T_3 on Na, K-ATPase. It will be useful to investigate whether tetrac or RGD peptides or antibody to $\alpha v\beta 3$ affect the cytoplasmic nuclear receptor/PI3K-based mechanisms that are involved in gene transcription and modulation of plasma membrane Na, K-ATPase activity.

Several proteins that reside largely, if not exclusively, in cytoplasm are known to bind iodothyronines and are not nuclear thyroid hormone receptors or hormone receptor-derived (163–167). Hashizume and co-workers (163, 168) described a dimeric 76 kDa rat liver cytosol binding protein for T_3 whose binding capacity for the hormone was enhanced by NADPH. Affinity of the protein for D-T_3 was higher than for L-T_3. A 38-kDa human kidney cytosol protein similar to that described by Hashizume *et al.* (163) was identified by Vie *et al.* (166) that bound T_3 with an affinity comparable to that of nuclear receptors for the hormone. T_3 binding was regulated by NADPH/NADP+. An insight into function of these cytosol thyronine-binding proteins (CTBPs) was provided when NADP was shown to transform the NADPH-activated liver cytosol protein into a form capable of transferring T_3 to the nuclear compartment (164). Cytosolic pyruvate kinase monomers M_1 (PKM_1) and PKM_2 are both capable of binding T_3 (167, 169). The chemistry of the interaction of hormone and kinase is interesting. T_3 inhibits kinase activity of both p58 PKM_1 and p58 PKM_2. At least in the case of PKM_1, enzyme activity is restored by the addition of fructose 1, 6-diphosphate (167).

B. Examples of nongenomic actions of thyroid hormone

1. Actions of thyroid hormone that are expressed at the plasma membrane

a. Ca^{2+}-ATPase activity. The existence of nongenomic actions of thyroid hormone at the plasma membrane was first demonstrated in membranes harvested from mature, *i.e.*, enucleate, human and rat erythrocytes. In studies of such membranes conducted more than 20 yr ago by Galo *et al.* (135), calcium pump (Ca^{2+}-ATPase) activity was shown to be modulated by thyroid hormone. The hormone concentrations used in these studies were near-physiological. Galo *et al.* (135) also showed that whether iodothyronines increased or decreased, the level of activity of the pump depended upon the lipid content of the rodent diet. That is, increased saturated fat intake permitted the stimulation of the calcium pump by T_4 and T_3. This inferred that the lipid microenvironment of the Ca^{2+}-ATPase in rodent red blood cell membranes was a modulator of pump activity.

Other investigators subsequently confirmed this effect of thyroid hormone *in vitro* on calcium pump activity (170, 171), established that the calmodulin-Ca^{2+} complex was essential to thyroid hormone action (170), and

showed that transduction of the hormone signal into Ca^{2+}-ATPase activity required specific kinases (172). Functional significance of the action of the hormone on Ca^{2+}-ATPase activity was revealed when Ca^{2+} efflux from inside-out vesicles of human erythrocytes was shown *in vitro* to increase in response to physiological concentrations of thyroid hormone (173). The mechanism by which thyroid hormone rapidly activates the enzyme *in vitro* appears to involve activation of phospholipase C, release of inositol 3,4,5-trisphosphate, and consequent activation of protein kinase C (172).

The paradigm in these studies of isolated membranes was acute exposure of the preparations *in vitro* to physiological concentrations of T_4 or supraphysiological levels of T_3. Clinical studies revealed that red blood cells obtained from hyper- and hypothyroid patients (174) had membrane Ca^{2+}-ATPase activities that were, respectively, increased and decreased. Another clinical report described hormone effects on Ca^{2+}-ATPase activity and on intracellular free Ca^{2+} concentration in polymorphonuclear leukocytes (175). *In vitro* studies included in the latter report presented evidence for a direct action of the hormone on membrane calcium pump activity.

Several laboratories have explored action of thyroid hormone on Ca^{2+}-ATPase activities in excitable cells of animals. Zinman *et al.* (176) recently showed that T_3 and T_4 acutely reversed calcium overload induced in neonatal rat cardiomyoctes. That this reflected increased pumping of Ca^{2+} from sarcoplasm into sarcoplasmic reticulum (SR) was shown when pharmacological inhibition of SR Ca^{2+}-ATPase blocked reduction of sarcoplasmic [Ca^{2+}] by thyroid hormone. Intracellular calcium overload was induced in these cells by increasing extracellular [Ca^{2+}] or decreasing extracellular [Na^+], where the latter caused reverse-mode Na^+/Ca^{2+} exchange.

This set of observations on Ca^{2+}-ATPase function provides in part an explanation for the inotropic action of thyroid hormone. However, T_3 has also been shown to increase transcription of SR Ca^{2+}-ATPase gene (SERCA2) (177). This genomic action of the hormone obviously contributes to inotropism and to normal diastolic relaxation (178). TRα mediates this genomic effect of the hormone (179).

Ca^{2+}-ATPase activity of myocardiocyte sarcolemma is also subject to modulation by thyroid hormone (180). Although this calmodulin-requiring function of thyroid hormone serves to reduce cardiac sarcoplasmic [Ca^{2+}], its quantitative contribution to regulation of sarcoplasmic [Ca^{2+}] is small. More likely, this action is relevant to local, subsarcolemmal regulation of the Na^+/Ca^{2+} exchanger (181).

The Ca^{2+}-ATPase activity of adult rat cerebrocortical synaptosomes can rapidly increase upon *in vitro* exposure to T_3 (182). Only seconds are required to obtain this effect with concentrations of T_3 that are within the physiological range (Table 1), but the maximum effect is achieved at supraphysiological levels of the hormone. A correlate of the change in enzyme activity is a rise in intrasynaptosomal free ionized [Ca^{2+}] with T_3 treatment (183) and subsequent activation of synaptosomal nitric acid synthase (184). It was felt that T_3 interacted directly with the endofacial aspect of the ATPase. Our knowledge of the thyroid hormone-Ca^{2+}-ATPase relationship in other tissues implicates an intermediate transduction mechanism for the hormone signal, as noted above, but the activation of synaptosomal Ca^{2+}-ATPase occurs sufficiently rapidly that a second and rapid mechanism for stimulation of the ATPase by the hormone may exist (184). Increases in Ca^{2+}-ATPase activity were also obtained *in vivo* when T_3 was administered to intact animals and synaptosomes prepared from brain.

Calmodulin is a cytoplasmic intracellular Ca^{2+}-binding protein that is important to the modulation of plasma membrane Ca^{2+}-ATPase activity and is essential to the ability of thyroid hormone to increase activity of this ATPase (170). Calmodulin is also found in synaptosomes (185). In the case of SR Ca^{2+}-ATPase, pump activity is regulated primarily by phospholamban, a single-pass

TABLE 1. Threshold concentrations of iodothyronines at which selected nongenomic actions of thyroid hormone are detected

Action of thyroid hormone	Thyroid hormone analog	Hormone concentration	Ref.
Membrane Ca^{2+}-ATPase activity	T_4	10^{-10} M	132
2-Deoxyglucose transport	T_3	10^{-9} M	191
Na, K-ATPase activity	T_3	10^{-9} M	159
Na^+ current: myocardiocytes	T_4, T_3	5×10^{-9} M	137
Na^+ current: sensory neurons	T_4	3×10^{-8} M	205
Na^+/H^+ exchanger	T_3	10^{-10} M	197
Cancer cell proliferation	T_4	10^{-10} M free	138
Angiogenesis	T_4	10^{-10} M free	139
TR[a] shuttling to nucleus	T_4	10^{-10} M free	214
Initiation of transcription of *HIF1*α gene	T_3	10^{-9} M	148

[a] Nuclear thyroid hormone receptor resident in cytoplasm.

membrane protein that, unphosphorylated, inhibits calcium uptake by SR. Inhibition is reversed by dual-site phosphorylation at a specific serine and threonine (186). But, as noted above, pharmacological inhibition of calmodulin can block acute stimulation by thyroid hormone of SR Ca^{2+}-ATPase activity in intact cardiac myocytes (176). The basis for this is the observation that calmodulin participates in the regulation of phospholamban activity via phosphorylation of phospholamban that is calmodulin kinase II-dependent at one residue (Thr-17) or is protein kinase A-mediated at Ser-16 (187–189). Because thyroid hormone is capable of nongenomically activating protein kinase A activity (142), as well as calmodulin-dependent action, both phosphorylation steps may be influenced by the hormone. Specific phosphorylation of another serine of phospholamban has also been implicated in the enhancement of SR Ca^{2+}-ATPase activity by phospholamban (187). Actions of thyroid hormone on SR Ca^{2+}-ATPase have the ability to increase the velocity of cardiac muscle relaxation and, by increasing SR [Ca^{2+}] during diastole, enhancing contractility.

b. 2-Deoxyglucose uptake. In a series of papers beginning in 1979, Segal and Ingbar (190–192) showed that thyroid hormone, specifically T_3, rapidly increased the rate of 2-deoxyglucose uptake by thymocytes. The hormone concentration required was near-physiological (Table 1). The molecular basis of this action of the hormone was not fully defined, but an increase in [Ca^{2+}]i preceded glucose uptake induced by T_3 (191). There was also a component to the regulation of this hormonal effect that was contributed by cAMP (192). This raised the possibility that nongenomic transduction of the thyroid hormone signal relevant to other cell processes might involve cyclic nucleotides. This has not been shown widely to be the case.

The functional significance of the action of thyroid hormone on cellular uptake of 2-deoxyglucose is speculative because the action has not been compared quantitatively with that of insulin. However, in addition to its action on thymocytes, T_3 was shown to enhance glucose uptake in heart cells, diaphragm, and fat cells (193). At what cellular site the thyroid hormone effect on glucose uptake is initiated is not known.

c. Na, K-ATPase activity. Lei, Ingbar, Mariash, and co-workers (159, 194) have shown that T_3 at 10^{-9} M can by a nongenomic mechanism increase activity of the plasma membrane Na, K-ATPase (sodium pump) in lung alveolar cells. Transduction of the thyroid hormone signal into sodium pump activity is via both MAPK (162) and PI3K (150, 162). T_3 is also a part of the control process for insertion of Na, K-ATPase units into the cell membrane (159). In the euthyroid intact organism, Na, K-ATPase is tonically exposed to iodothyronines, and thus the basal activity of the pump is in part regulated by ambient levels of T_3.

In contrast to these observations in intact cells, the activity of Na, K-ATPase present in synaptosomes has been shown to decrease in response to T_3 and, to a lesser extent, other thyroid hormone analogs (195). It is possible that this observation and those made in pulmonary alveolar epithelial cells reflect specialized tissue functions that differentially recognize thyroid hormone and that there are different proportions of certain ion transport mechanisms in the synaptosome and in intact cells. Regulation of [Na^+]i or intrasynaptosomal [Na^+] is complex and subject to contributions from the Na^+/Ca^{2+} exchanger and Na^+/H^+ antiporter, as well as sodium current and Na, K-ATPase (see *Section II.B.3.a*). It thus can be difficult to distinguish primary from secondary effects of the hormone on a given ion transport mechanism.

Gick, Ismail-Beigi, and Edelman (196) showed that transcriptional regulation of the Na, K-ATPase gene in rat liver and kidney was a function of T_3 by a genomic mechanism. However, because of the substantial differences that were observed in gene transcription rates and mRNA abundance, these authors proposed that both genomic and nongenomic mechanisms were invoked by T_3. That is, the nongenomic contribution might be via stabilization of mRNA (see *Section II.B.3.a*). By nongenomic and genomic mechanisms, then, thyroid hormone participates in the setting/maintenance of intracellular concentrations of Na^+ and K^+.

d. Na^+/H^+ antiporter. Incerpi *et al.* (197) established that the sodium-proton exchanger (Na^+/H^+ antiporter) was subject to regulation by T_3. The model tissue was rat skeletal muscle (L6 myoblasts). The rapid onset of this action supported a nongenomic mechanism for the effect of the iodothyronine, and subsequent studies by this group showed that the hormonal effect was mediated by MAPK (ERK1/2) (198). Approximately physiological concentrations of T_3 were effective in this system (Table 1) (197). An implication of this set of observations is that ambient thyroid hormone, by contributing to the setpoint of the antiporter, in part defines the capacity of cells to recover from acid loads. In the L6 myoblast, for example, the presence of T_3 accelerated return to normal intracellular pH after an NH_4Cl load (198). This observation is likely to be of relevance to hyopoxic/ischemic stress in cardiac and striated muscle. The exchange of Na^+ for protons by the antiporter serves to acidify the immediate environment of the cell and to increase [Na^+]i. The latter result may

be an indirect mechanism by which plasma membrane Na, K-ATPase activity is enhanced in cells exposed to thyroid hormone.

The activity of multidrug resistance (P-glycoprotein) pumps (199) in the plasma membranes of cancer cells reduces the intracellular residence times of certain cancer chemotherapeutic agents. Tetrac is a deaminated analog of T_4 that inhibits binding of agonist thyroid hormone analogs, such as T_4 and T_3, to the cell surface receptor for thyroid hormone on integrin $\alpha v\beta 3$ (161, 200, 201). Tetrac, itself, is not an agonist at the integrin receptor. It has been shown that tetrac increases the residence time of doxorubicin in doxorubicin-resistant human breast cancer (MCF-7) cells (146) *in vitro* and thus is capable of reversing chemoresistance in such cells. It may be postulated that, at least in part, this action of tetrac is due to its inhibition of the contribution of thyroid hormone to basal activity of the Na^+/H^+ exchanger and fostering of a decrease in intracellular pH (202).

The increase in $[Na^+]i$ that is the result of stimulation of Na^+ current by thyroid hormone or of Na^+/H^+ exchange by iodothyronines may secondarily increase $[Ca^{2+}]i$ via stimulation of reverse-mode Na^+/Ca^{2+} exchange (176). It was noted above that reverse-mode sodium/calcium exchange may cause intracellular calcium overload when extracellular $[Na^+]$ is reduced. Without acting directly on Na^+/Ca^{2+} exchange, then, the hormone may influence this cellular mechanism.

e. Na^+ current. Craelius, Green, and Harris (137, 203) have described a rapid onset effect of thyroid hormone analogs on slowing of the inactivation of the Na^+ current. The model cell was neonatal rat myocardiocytes. A hormonal effect was apparent within 1 min of exposure of the cells to T_3. Concentrations of T_4 and T_3 were effective in this model (Table 1) (137). The increased inward flux of Na^+ that results from this hormonal action amplifies cell depolarization and may also contribute to activation of membrane Na, K-ATPase or Na^+/Ca^{2+} exchange. This action of the hormone on the sodium current is a mechanism by which thyroid hormone in excess may increase cell excitability. In the case of the heart, this action of iodothyronines is antagonized by lidocaine (203, 204) and may be postulated to contribute to abnormal cardiac rhythms.

Huang *et al.* (204) also carried out a structure-activity analysis of iodothyronines in this model of hormone action. T_3 and T_4 were equally active at 10 nM. Deaminated hormone analogs such as tetrac and triiodothyroacetic acid did not affect Na^+ current, but pretreatment of cells with rT_3 inhibited the effects of T_4 and T_3.

Recent voltage clamp study by Yonkers and Ribera (205, 206) documented chronic and acute effects of T_4 (10^{-8} M) (Table 1) on sodium current (I_{Na}) in developing sensory neurons in the zebrafish. T_3 was not active in this model system. Hormonal action on I_{Na} in the neuron was inhibited by tetrac and by function-blocking antibody to integrin $\alpha v\beta 3$, indicating that the effect of T_4 was initiated at the cell surface integrin receptor for the hormone. As is the case in regulation of tumor or endothelial cell proliferation and of the state of the actin cytoskeleton discussed in *Section IV*, T_4 may act in this neuronal sodium current paradigm as a hormone, rather than a prohormone precursor to T_3.

f. Endocytosis. Endocytosis is an inherent property of the lipid-enriched plasma membrane and is requisite to the metabolism/degradation of cell membrane components and to trafficking of specific membrane component proteins within the cell. For example, thyroid hormone promotes the endocytosis of type 2 (207) and type 3 iodothyronine 5′-monodeiodinases (208), enzyme family members that are responsible for the conversion of T_4 to T_3. The molecular mechanisms involved in this action of thyroid hormone are incompletely understood. However, the state of actin is a component of endocytosis (209), and regulation of the actin cytoskeleton is a role of iodothyronines (see *Section IV*).

g. Epidermal growth factor (EGF) receptor (EGFR) activity. The EGFR in the plasma membrane transduces the EGF signal at the target cells of the growth factor. This receptor has been of special interest to oncology research because of the EGF dependence of proliferation that has been defined in a variety of tumor cells (210, 211). Pharmacological and antibody inhibitors of EGFR tyrosine kinase activity have recently been developed as cancer chemotherapeutic agents (212, 213).

It has been shown that thyroid hormone is capable of refining the function of the EGFR. For example, the presence of thyroid hormone in *in vitro* experiments permits the EGFR in tumor cells to distinguish between EGF and TGF-α, two natural ligands of the receptor (142). In these HeLa cell studies, the downstream consequences of TGF-α-binding and EGF-binding at the receptor are identical in the absence of thyroid hormone, but different when physiological levels of thyroid hormone are present, *e.g.*, on c-*fos* expression. This action of thyroid hormone on EGFR may be relevant to the proliferative effect of the hormone on tumor cells.

2. Complex cellular actions initiated at or modulated by the integrin receptor for thyroid hormone

a. Protein trafficking. It was mentioned above that iodothyronines can nongenomically influence the internalization of plasma membrane proteins, such as the 5′-iodothyro-

nine monodeiodinase (207). However, these observations provide only a limited insight into the effects of the hormone on movement of specific proteins throughout the cell. Thus, Baumann *et al.* (154) and Zhu *et al.* (155) detected nuclear TRβ1 in cytoplasm and showed a decade ago that T_3 caused movement of a readily detectable TR-green fluorescent protein (GFP) chimera into the nucleus. Physiological concentrations of thyroid hormone may support cytoplasm-to-nucleus shuttling of TR (214) (Table 1). The finding of the nuclear receptor in cytoplasm was somewhat surprising, given the canonical view that the receptor was exclusively contained within the nucleus. However, nascent receptor must move through the cytoplasm from endoplasmic reticulum, where it is synthesized, to the nucleus, thus providing one explanation for the presence of TR in cytoplasm. Subsequently, Moeller *et al.* (149) showed that TRβ1 in cytoplasm was functional and capable of binding in T_3-treated cells to p85α, the regulatory subunit of the signal transducing protein, PI3K, a step premonitory to downstream transcription of genes important to glucose metabolism in cells (Fig. 4).

TRα1 in cytoplasm may also interact with the regulatory subunit of PI3K in endothelial cells (160). Translocation has also been described of cytoplasmic TRα1 to the nucleus in thyroid hormone-treated cells (148), and a truncated isoform of the TRα1 is also found in cytoplasm and mediates the action of T_4 and rT_3 on the actin cytoskeleton (see *Section IV*).

From the standpoint of a mechanism by which thyroid hormone may affect trafficking of TR, Cao *et al.* (214) have disclosed that this process is modulated from the plasma membrane by the thyroid hormone receptor on integrin $\alpha v\beta 3$. T_4 and T_3 both promote translocation of cytoplasmic TRβ1-GFP to the cell nucleus by a mechanism that is inhibited by tetrac, by RGD peptide, and by antibody to integrin $\alpha v\beta 3$. The thyroid hormone receptor is located at the RGD recognition site on the integrin that is critical to integrin-extracellular matrix protein interactions (140). Interestingly, exposure to thyroid hormone of normal cells engineered to contain TRβ1-GFP and fluorescently-labeled MAPK (ERK1/2) shows that complexes of cytoplasmic MAPK-TR and nuclear MAPK-TR develop rapidly (214). It has been proposed that the interaction of TR and MAPK in cytoplasm is a prerequisite to transfer of TR to the nucleus (214). An ERK1/2 docking site on TRβ1 has been identified (215), and thyroid hormone-activated ERK1/2 is known to phosphorylate TRβ1 at Ser-142 (156). Studied in the cell nucleus, this phosphorylation has functional consequences, including the recruitment of CoA proteins (Fig. 4). The observation that TRβ1 and ERK1/2 form a complex in cytoplasm raises the possibility that phosphorylation may occur outside the nucleus.

Several other proteins move between cellular compartments in response to thyroid hormone. Trip230 is an activator protein whose transport from the Golgi apparatus to the cell nucleus is facilitated by T_3 (216). The translocation of signal transducer and activator of transcription-1α (STAT-1α) from cytoplasm to nucleus is also enhanced by treatment of cells with thyroid hormone (217). The STAT family is important to the transduction of a number of polypeptide or protein factor signals at the cell surface into cell responses. These factors include EGF (218) and interferon-γ (IFN-γ). In the case of IFN-γ, exposure of HeLa cells to thyroid hormone leads to activation of MAPK (ERK1/2), formation of STAT1α-MAPK nuclear complexes, specific serine phosphorylation (residue 727) of the already tyrosine-phosphorylated STAT1α, and material potentiation of the transduction of the IFN signal. That is, the tyrosine phosphorylation of STAT1α is required for signal transduction, but concomitant serine phosphorylation amplifies the transduction of signals by the protein. By this mechanism, thyroid hormone is capable of enhancing the antiviral activity of IFN-γ by up to 100-fold (217).

ERα may also translocate to the nucleus in thyroid hormone-treated breast cancer cells (144), but no other members of the nuclear hormone receptor superfamily have as yet been reported to move into the nucleus in response to cell exposure to thyroid hormone. However, MAPK-docking sites exit on several other superfamily members (145). These receptors thus are subject to serine (or threonine) phosphorylation by MAPK (ERK1/2) in thyroid hormone-treated cells.

Another nongenomic protein trafficking observation in thyroid hormone-treated cells is the transfer of the oncogene suppressor protein, p53, from cytoplasm to the nucleus (219). p53 is another protein that is subject to serine phosphorylation by iodothyronine-activated MAPK (ERK1/2). Once in the nucleus, p53 can be recovered in protein complexes that include MAPK and TR (219). Activated p53 is transcriptionally active, and it is reasonable to assume that this action of thyroid hormone on p53 is protective, that is, it contributes to the cellular defense against oncogenesis. It has not been shown, however, that this action of the hormone is a cellular defense mechanism. In fact, this p53-related observation needs to be reconciled with the fact that thyroid hormone is antiapoptotic (144) and is a proliferation factor for certain tumor cells. The latter include human breast cancer (145), glioma/glioblastoma (138, 220), thyroid cancer (144), and head-and-neck cancer (H. Y. Lin, F. B. Davis, and P. J. Davis, unpublished observations). A distinction must be drawn here between

oncogenesis and proliferation. There is little evidence that the hormone is oncogenic; rather, the proliferation of already-established cancer cells is promoted by T_4 and T_3.

MAPK (ERK1/2) is activated (tyrosine-phosphorylated) by exposure of cells to thyroid hormone, as noted above. Activated MAPK rapidly translocates to the nucleus, regardless of the activating factor. The ability to promote this translocation thus is not a specific attribute of thyroid hormone.

b. Cell migration. Migration of neuronal and glial cells has been shown by Farwell *et al.* to be regulated by thyroid hormone (221). T_4 and rT_3 are important here, whereas T_3 does not influence motility of these cells. Migration is dependent upon the presence of sufficient cytoskeletal F-actin in cells to support cell motility. Leonard and co-workers (222) have also described a role of thyroid hormone in the conversion of soluble actin into F-actin (see *Section IV*).

c. Platelet aggregation. Platelets bear integrin $\alpha v\beta 3$ and have been studied by Mousa *et al.* (223) to determine whether thyroid hormone, acting via this integrin, is capable of modifying platelet function. Human platelets agglutinate in response to physiological concentrations of free T_4 and, as a biological indicator of agglutination, secrete ATP in response to the hormone. To support the concept that this hormonal action occurs via a cell surface receptor, the investigators reproduced with agarose-T_4 the effect of unmodified T_4. RGD peptide blocked this action of T_4 and agarose-T_4, indicating that the action of the hormone was initiated at the RGD recognition site on the heterodimeric integrin, where the thyroid hormone receptor is located. T_3, however, did not cause platelet agglutination or ATP secretion, nor did diiodothyropropionic acid, another iodothyronine analog with thyroid hormone agonist properties. Where angiogenesis is desired in the setting of tissue, *e.g.*, mammalian limb, ischemia (224), a proangiogenic thyroid hormone analog would be desirable that lacks the ability to aggregate platelets (see *Section II.B.2.d*).

d. Cell proliferation: angiogenesis. Several laboratories have shown that thyroid hormone fosters new blood vessel formation. Tomanek *et al.* (225) studied angiogenesis in the setting of experimental myocardial infarction and found that administration of thyroid hormone in above-physiological concentrations in the rat resulted in an increase in abundance of new blood vessels, compared with untreated controls. The mechanism involved accumulation of basic fibroblast growth factor (bFGF). The desirability of new blood vessel formation in the setting of tissue damage is apparent in terms of limiting future, recurrent ischemia in the areas of tissue vulnerability. Gerdes and co-workers have shown that iodothyronines increase new blood vessel growth in brain (226), as well as myocardium (227).

Davis and co-workers have defined the proangiogenic activity of thyroid hormone in two standard experimental models, the chick chorioallantoic membrane (CAM) system (139, 228) and the human dermal microvascular endothelial cell (HDMEC) microtubule assay (229). In these models, T_4 at 10^{-10} M (Table 1) and T_3 at higher concentrations are both active. These studies of the action of T_4 have included propylthiouracil to prevent conversion of T_4 to T_3 by cellular 5′-iodothyronine monodeiodinase. The importance of this manipulation is that: 1) it supports intrinsic proangiogenic activity of T_4; and 2) it opposes the impression that T_4 is solely a prohormone for T_3. Both the CAM and HDMEC microtubule models reveal that RGD peptide (but not the control RGE moiety), tetrac, and monoclonal antibody to integrin $\alpha v\beta 3$ serve to block thyroid hormone-induced angiogenesis. The results indicate that the proangiogenic action of thyroid hormone is initiated at its plasma membrane receptor. The angiogenic response is dependent upon activation of MAPK (ERK1/2) by the hormone-integrin complex. In addition, exposure of the CAM to thyroid hormone results in the release about the treated cells of bFGF (FGF2) (228) that is seen, in an autocrine manner, to cause endothelial cell proliferation. Addition of bFGF antibody to the model blocks the angiogenic response to the hormone. At least in part, then, the angiogenic response to thyroid hormone is mediated by release of one or more vascular growth factors.

Tetrac inhibits iodothyronine-induced angiogenesis by blocking binding of agonist thyroid hormones to the integrin receptor. However, tetrac will block the angiogenic properties of several polypeptide or protein vascular growth factors in the absence of thyroid hormone. These factors include, as noted above, vascular endothelial growth factor and bFGF. It has also been shown that angiopoietin-2 (*Ang-2*) gene expression (229) is increased by thyroid hormone, but not the expression of *Ang-1*. The significance of the Ang-1 *vs.* Ang-2 observations is that Ang-2 protein destabilizes the vascular microtubules, a step premonitory to vascular endothelial growth factor action, whereas Ang-1 stabilizes microtubles.

It is assumed that the antiangiogenic activity of tetrac is expressed via the RGD recognition site on integrin $\alpha v\beta 3$ at which the thyroid hormone receptor is located. The vascular growth factors whose actions are inhibited by tetrac contain an RGD sequence; the sequence must be appreciated by the integrin before the factors become capable of acting at their specific receptors that are anatomically close to the integrin. That is, tetrac may interfere

with crosstalk between the integrin and specific vascular growth factor receptors geographically clustered on the endothelial cell surface.

e. Cell proliferation: cancer cells. The possibility that thyroid hormone in physiological concentrations may support tumor cell proliferation is evident from several experimental studies (138, 144, 145) and from limited clinical evidence (220, 230). In patients with advanced glioblastoma multiforme, reduction by pharmacological means of circulating thyroid hormone levels had a significant therapeutic benefit (220), and a retrospective analysis of experience at the M.D. Anderson Cancer Center with spontaneous hypothyroidism and breast cancer suggested that hypothyroidism delayed tumor appearance and decreased aggressiveness (230). On the other hand, several authors and the American Thyroid Association concluded more than two decades ago that thyroid hormone replacement therapy did not affect breast cancer clinical behavior (231).

The experimental studies that have supported the existence of a proliferative effect of thyroid hormone *in vitro* include those on glioma cells (138), on human breast cancer cells (145), and on human thyroid cancer cell lines (144). In all such studies thyroid hormone was tested in physiological concentrations. The fact that tetrac opposed the trophic effect of agonist thyroid hormone analogs indicated that the cell surface receptor for the hormone might mediate the proliferative effect. This conclusion was supported by additional experiments in which agarose-T_4, which does not gain access to the cell interior, mimicked unmodified T_4. The proliferative effect of iodothyronines has been blocked by RGD peptide and antibody to integrin $\alpha v\beta 3$. Inhibition of the MAPK signal transduction pathway with PD 98059–which inhibits the cascade at MAPK kinase—also blocks the action of thyroid hormone on tumor cell division (144, 145).

Exactly what the events downstream of MAPK (ERK1/2) are in tumor cell proliferation caused by integrin-initiated thyroid hormone action is not clear in the various types of tumor cells mentioned above. Activated MAPK translocates to the cell nucleus, as noted earlier, and it is likely that nuclear MAPK serine phosphorylates transcriptionally-active nucleoproteins, as has been shown for STAT1α and TRβ1 (156, 217). In the case of human breast cancer (MCF-7) cells, it has been shown that thyroid hormone-activated MAPK phosphorylates nuclear ERα at Ser-118 (145), and this is an antecedent of hormone-induced cell division. This mimics precisely the action of estradiol on MCF-7 cells.

Another mechanism by which thyroid hormone may stimulate cancer cell proliferation is by potentiation of the action of EGF on tumor cells. EGF is widely understood to be a proliferative factor (212). As noted above, the hormone can modify the actions of EGF at its plasma membrane receptor (219).

Thyroid hormone has been shown to be antiapoptotic (144). This action of the hormone supports cancer cell proliferation in the setting of proapoptotic agents, such as the stilbene, resveratrol. Such observations suggest that circulating levels of thyroid hormone in patients enrolled in clinical trials could blunt the activity of proapoptotic drugs. This conjecture has not yet been investigated in animal models of cancer. The mechanism of the antiapoptotic effect at least in part involves inhibition of p53 activation in tumor cell nuclei (H. Y. Lin, F. B. Davis, and P. J. Davis, unpublished observations). On the other hand, tetrac is proapoptotic and blocks the antiapoptotic effects of T_4 (144, 146, 232).

3. Nongenomic cellular actions of thyroid hormone whose site(s) of initiation are not yet known

a. Stabilization of mRNA by thyroid hormone. It has been known for more than a decade that the genomic activity of thyroid hormone was complimented by a nongenomic effect of the hormone on the half-lives of mRNAs transcribed from thyroid hormone-responsive genes. Among the transcripts so affected by the hormone are acetylcholinesterase mRNA (233), apolipoprotein AI (234), and uncoupling protein (UCP) mRNA (235), as well as Na, K-ATPase mRNA (196), as mentioned above. The mechanism is not yet clear for stabilization of mRNAs by thyroid hormone treatment of cells, but a serine-threonine kinase pathway has been implicated in the process (233). This prolongation of mRNA half-life is in contrast to the effects of the hormone on gene products, where iodothyronines generally increase protein turnover.

b. Actions of thyroid hormone in cytoplasm. Viewed in the context of genomic actions of thyroid hormone, the existence of the hormone in cytoplasm may be conceived as a transient phenomenon, reflecting transport from the cell surface and cytoplasm into the nucleus. However, several cytoplasmic proteins are known to bear binding sites for iodothyronines, and these were identified above in *Section II.A.2*, which describes putative receptor sites in cytoplasm for thyroid hormone. Pyruvate kinase monomers M_1 and M_2 have their enzyme activities modulated when they bind T_3 (167, 236, 237), and a dimeric CTBP may facilitate nuclear uptake of T_3 (163, 164). The regulation by the intracellular ratio of NADP/NADPH of the activity of CTBP has been described (163, 166). Somewhat surprising in studies of CTBP, however, was that the protein had a higher affinity for D-T_3 than for L-T_3 (166, 168).

III. Thyroid Hormone and Mitochondria

The ability of thyroid hormone to regulate energy utilization was first recognized more than 100 yr ago and is still a contemporary topic in thyroid research (238, 239). To date, we still do not understand the molecular events by which thyroid hormone controls this essential function. Energy, in the form of ATP, is the currency required for life, and the mitochondrion is its principal source. This fact has made this organelle an obvious target for thyroid hormone action. In addition to providing about 90% of the energy currency of the cell, these acquired organelles also play essential roles in cell signaling and cell survival, two other cell regulatory cascades that are possible targets for thyroid hormone action. In this section we will focus on the effects of thyroid hormone on mitochondrial energetics and mitochondriogenesis.

Several provisos should be kept in mind when evaluating the influence of thyroid hormone on metabolism. First, most studies have focused exclusively on T_3—at pharmacological levels—and have ignored the other iodothyronine metabolites found within cells, specifically, T_4, rT_3, and 3,3′-diiodothyronine. Secondly, the conventional view that thyroid hormone action is mediated by altered gene expression has often been used to dismiss actions that very likely are mediated by direct/nongenomic actions that occur within cellular compartments other than the nucleus. Finally, the impression that the mitochondrion is a ubiquitous organelle of bacterial origin often confounds the real differences that exist between these organelles in different tissues. For example, functional analysis of liver mitochondria is very likely to differ from that of mitochondria isolated from skeletal muscle or fat because mitochondria share only about 60% of their proteomes; up to 40% is derived in a tissue-specific fashion from the cells in which they reside. Such tissue-specific specialization generates mitochondria that are unique to their tissue of origin (240, 241). Too often, reductionism obscures the biology of hormone action by extending tissue-specific observations to the whole organism, especially for mitochondrial functions.

A. Mitochondrial energetics and thyroid hormone

1. ATP generation

The mitochondrion consumes metabolic fuels and generates ATP by oxidative phosphorylation. Oxidation of energy substrates at the inner mitochondrial membrane generates a proton gradient that is used by ATP synthase to phosphorylate ADP, and the newly generated ATP is then exported across the inner membrane by the ADP/ATP translocator. The coupling of fuel consumption to ATP generation is tightly controlled but is not absolute, and uncoupling of substrate oxidation to ATP generation produces heat. In large part, it is the heat generated by uncoupled oxidative phosphorylation in mitochondria that constitutes the basal metabolic rate, the physiological process influenced by thyroid hormone. Because ATP generation depends upon the proton gradient across the inner mitochondrial membrane, disturbing this gradient by "proton leak" is one biochemical mechanism that uncouples fuel oxidation from ATP generation leading to the production of heat. Proton leaks can be generated by altering proton trafficking across the inner membrane or by covalent modification of the ADP/ATP translocator (242); both factors uncouple fuel oxidation from ATP generation and lead to heat generation.

2. Heat generation

There are two components to mitochondrial heat generation: 1) basal proton leak, which accounts for up to 30% of oxygen consumption and is thought to affect whole-body energy utilization; and 2) inducible proton leaks—proton leaks mediated by dynamically regulated UCPs that belong to the superfamily of mitochondrial anion carriers (reviewed in Refs. 243 and 244). Although it is the latter family of UCPs that have received attention because of their role in brown adipose tissue, it appears that the "basal proton leak" component of mitochondrial heat generation is the component that is influenced by thyroid hormone (245). In rodents, the catecholamine and thyroid hormone-dependent expression of UCP1 is a major player for facultative thermogenesis by brown fat mitochondria, but UCP1 is unique to this tissue and its sisters; principally UCP2 and UCP3 found in skeletal muscle do not appear to serve the same heat-generating function (246–248). Thus, the effects of thyroid hormone on basal proton conductance in mitochondria, outside of brown fat, remain an unknown in need of further study.

B. Thyroid hormone and mitochondriogenesis

The ability of thyroid hormone to increase the number of mitochondria in a cell is well known and, like its effects on energetics, remains poorly understood. Clearly, an increase in the number of mitochondria in a cell provides the machinery required to enhance ATP generation, consume more oxygen, and generate more heat. The direct effects of thyroid hormone on mitochondriogenesis are complicated by the fact that much of the mitochondrial proteome is imported from genes located in the cell nucleus. At least two general mitochondrial transcription factors encoded by the cell genome, the cold-induced CoA of nuclear receptors, PPAR gamma coactivator-1 (PGC-1) (249), and the mitochondrial transcription factor A (250, 251), appear to be thyroid hormone dependent (252). Direct actions of thyroid hormone on the mitochondrial genome

have recently received a boost by the identification of validated thyroid hormone "receptors" in the mitochondrial matrix. The recent demonstration that truncated forms of the TRα1 (253, 254) and TRβ1 (255) are specifically imported into the mitochondrion, show high-affinity T_3 binding, and stimulate generalized transcription of the mitochondrial genome provides an attractive mechanism by which thyroid hormone can directly affect mitochondrial replication. The fact that both nuclear and mitochondrial genomes are targets for thyroid hormone activation and can ultimately lead to the propagation of mitochondria is not unsurprising and is just another example of the communication network that exists between the mitochondrion and nuclear genomes that cooperate to reproduce new, fully functional mitochondria.

C. Thyroid hormone-dependent induction of mitochondrial DNA

Work from Wrutniak and co-workers (253) and Lechleiter and colleagues (256, 257) has demonstrated definitively that TRα1 lacking the A/B domain is imported into the mitochondrion and: 1) impacts global gene expression by the mitochondrial genome (253, 256); 2) participates in the changes in proton gradient; and 3) enhances inositol-1,4,5-trisphosphate-mediated calcium signaling (257). This work is consistent with that of others that indicate that the A/B domain is required for nuclear targeting of the TR gene products (255). Once inside the mitochondria, TRα1 interacts with a common transcriptional machinery and initiates global increases in mitochondrial gene expression by interacting with two authentic TREs located in the D-loop, an element of the mitochondrial genome that also contains the promoters for the general mitochondrial transcription factor A. In addition to the TREs found in the D-loop, authentic TREs are also located in the mitochondrial genes encoding the 12S and 16S rRNA in mitochondria (253). Thus, the N-terminal truncated TRα1 can serve as a T_3-dependent transcription factor that initiates global mitochondrial transcription.

D. Thyroid hormone-dependent nongenomic actions in mitochondria

An intriguing recent finding is that the N terminus of Xenopus xTR$_\beta$A1 is nearly identical to that of the mitochondrial targeted, A/B domain-deficient TRα1 encoded by transcripts originating from an internal AUG start site in exon 3 of the mouse TRα gene. Expression of xTR$_\beta$A1 in frog oocytes or in mammalian cells leads to its appearance in the mitochondria matrix where it: 1) increases the proton gradient; and 2) inhibits cytochrome c release in thyroid hormone-dependent fashion. Not surprisingly, ligand-dependent suppression of cytochrome c release mediated by xTR$_\beta$A1 leads to a fall in apoptotic activity *in vitro* and in CV1 cells (256). The ability of the T_3-liganded xTR$_\beta$A1 to increase mitochondrial membrane potential is counterintuitive, especially when an elevated proton leak is thought to mediate the thyroid hormone-dependent heat generation. However, Lechleiter and colleagues (257) argue that these T_3-dependent effects observed in isolated mitochondria eliminate mitigating factors present in other cellular compartments and restrict those actions of T_3 to mitochondria alone, possibly through interactions with electron chain components. These findings, together with the tissue-specific diversity of mitochondrial proteome, suggest that there is much more to be learned before the role of thyroid hormone on mitochondrial function is understood.

IV. Actions of Thyroid Hormone on the Cytoskeleton, Cell Migration

The ability of T_4 to influence actin polymerization and, thereby, the physical state of a key component of the cytoarchitecture of cells is another novel, nonenomic action of thyroid hormone (222, 258, 259). The microfilament network is one of the major structural components of the cell and is composed of a dynamic, continuously remodeled fiber network composed of filamentous actin bundles, F-actin. In hypothyroid rodents, both neurons and astrocytes have poorly developed actin cytoskeletons that cannot be restored to normal by T_3 replacement. By contrast, both T_4 and rT_3 initiate the reappearance of filamentous actin bundles in both cell types within minutes and without any change in total actin mRNA or protein content (222, 258).

A. Astrocytes

The loss of the actin cytoskeleton has significant consequences on cerebellar maturation, rendering the astrocyte incapable of depositing neuronal guidance protein(s), such as laminin, onto their cell surface. This, in turn, impairs the recognition of guidance cues by the actin-anchored, transmembrane recognition apparatus in the neuronal growth cone (259). Laminin is an astrocyte-derived extracellular matrix protein that is secreted by the cell and deposited onto its surface in specific patterns during periods of neuronal migration (260–266). These astrocyte-based laminin arrays are assembled by the binding of secreted laminin to receptor-bearing integrins that span the astrocyte plasma membrane and then cluster into macromolecular complexes known as focal contacts (267, 268). These focal contacts comprise a functional signaling unit that transduces mechanical force through their C-terminal intracellular tail to the actin cytoskeleton and serves as the

proximal sensor in a complex signaling cascade that provides contextual information about the immediate environment of the cell. Loss of the actin cytoskeleton in the thyroid hormone-deficient or T_3-treated astrocyte prevents focal contact formation and prevents the cells from attaching to laminin-coated surfaces; T_4 replacement fully restores focal contact assembly, cell attachment, and growth (269, 270). The inability to cluster integrins also prevents the hypothyroid or T_3-treated astrocyte from holding on to its newly secreted laminin (221, 271, 272). As is the case for cell attachment, both T_4 and rT_3 replacement restore the ability of astrocytes to deposit laminin arrays on their cell surface.

These *in vitro* findings have an *in vivo* counterpart in the developing rodent cerebellum where hypothyroidism leads to the loss of laminin-derived migration pathways in the molecular layer during the critical period of granule cell migration from d 8–14 of life (221, 271). Interestingly, all of the thyroid hormone influence on laminin deposition observed in cultured astrocytes occurs without any changes in laminin mRNA abundance, laminin protein synthesis, or the rate of laminin secretion (221).

B. Neurons

The loss of the actin cytoskeleton in the neuronal growth cone also directly impacts neuronal migration. Normal brain development requires the developing neuron to migrate over long distances and to project axons along specific pathways toward target cells (221, 273, 274). Interactions between the actin cytoskeleton and integrins located in the growth cone are essential for interpretation by integrins of extracellular guidance cues (273–278). Chemical disruption of the actin cytoskeleton markedly impairs neuronal growth cone pathfinding and motility (278–281). Work done in cerebellar explants has revealed that thyroid hormone-dependent changes in the actin cytoskeleton modulate neuronal process formation (282) and that the loss of the actin cytoskeleton, due to a lack of thyroid hormone, severely impaired neurite outgrowth and markedly suppressed granule cell migration (282). These developmental defects are not corrected by T_3 replacement but can be completely reversed by the addition of either T_4 or rT_3 to the explants (282). *In vivo*, a similar consequence of thyroid hormone deficits is observed in the neonatal cerebellum where hypothyroidism results in the loss of laminin-derived migration pathways in the molecular layer just during the critical period of granule cell migration (272). Thus, the available data argue that direct T_4-dependent regulation of the actin cytoskeleton in both astrocytes and neurons is likely to modulate the formation and recognition of critical extracellular guidance cues— cues necessary for normal neuronal migration and neuronal process formation—and provides a potential sequence of events that could explain the developmental defects observed in the cretinous brain.

C. The role of TRΔα1 gene in T_4-dependent actin polymerization

Analysis of the ability of a family of thyroid hormone analogs to promote actin polymerization revealed that the ligand-binding site of the putative effector molecule differed from that of all other iodothyronine-binding proteins (283). Based on these earlier findings, a unique affinity-labeling molecule was constructed, and a specifically labeled small polypeptide was identified on pull-down assay as the 16-kDa TRΔα1. TRΔα1 and its partner, TRΔα2, are 16- and 26-kDa polypeptides, respectively; are encoded by transcripts originating from an internal transcription start site located in intron 7 of the TRα gene; and are composed of the C-terminal portion of the LBD (284). Neither binds T_3, both lack a nuclear localization signal (A/B domain), and both are found in the gut, brain, and lung of the rat (284). Although early work showed that overexpression of TRΔα1 interferes with transcription *in vitro* (12, 284), the absence of a nuclear localization signal and the failure to identify TRΔα1 in the nucleus suggests that this effect may be due to faulty intracellular sorting, a likely artifact of overexpression studies.

Preliminary data suggest that TRΔα1 has all of the properties required of the mediator of T_4- and rT_3-regulated actin polymerization in the developing cerebellum. Native TRΔα1 is found in the extranuclear compartment of both astrocytes and neurons and shows the ligand affinity and specificity required for the physiological regulation of actin polymerization, *i.e.*, it binds T_4 and rT_3 with high affinity while failing to bind T_3. Structural analysis of the TRα1 LBD shows that the hydrophobic pocket cradling the ligand remains intact, and that Phe^{405} in helix 12 "locks" the thyroid hormone into the binding site by hydrophobic bonds with the 5′ iodine of T_4 and rT_3.

Preliminary studies done in astrocytes from two different TRα1 knockout mice ($TR\alpha1^{-/-}$ and TR00) yielded interesting data linking TRΔα1 to TH-dependent regulation of actin polymerization. TR00 animals lack all TRα1-derived gene products (24, 285), whereas $TR\alpha1^{-/-}$ express both TRα2 and TRΔα2 (285). Even at supraphysiological levels of T_4 and T_3, astrocytes from both TR mutants have a poorly developed actin cytoskeleton that is identical in appearance to that in thyroid hormone-deficient cells. Gene replacement with TRΔα1 restored the ability of the actin cytoskeleton to respond to T_4 and rT_3 in astrocytes from both TR mutants, whereas restoring T_3-dependent gene expression with the full-length TRα1 transfactor had no effect on the actin cytoskeleton. Although granule cell migration in the de-

veloping cerebellum of TR mutants was reported to be normal (286), closer examination reveals that apoptosis of the migrating granule cells, especially in the cerebellum, is markedly protracted and increased compared with the wild-type mice, illustrating that defects in cell trafficking are present. These initial data will surely spur further work to define the role of the novel TR$\Delta\alpha$1 in the nongenomic actions of thyroid hormone and its participation in the developmental program of the brain.

Congenital hypothyroidism is the major preventable cause of mental retardation in the world today, and the molecular events by which thyroid hormone regulates neuronal migration and neuronal process projection in the brain are complex and poorly understood. With the identification of the actin cytoskeleton as a site of thyroid hormone action, we have a new handle on the cellular machinery that participates in this key developmental plan.

V. Concentrations of Thyroid Hormone at Which Molecular Actions of the Hormone Are Measured

A wide range of hormone concentrations have been used *in vitro* to define genomic and nongenomic actions of iodothyronines. Important genomic actions have been described at 10–100 nM levels of T_3 (287, 288) that are four or five orders of magnitude above total and free hormone concentrations defined in intact organisms, usually human subjects. In some cases, hormone receptor K_d values are defined in the picomolar range, but studies in the same report that are mediated by the receptor are conducted at the submicromolar level (289). Some genomic actions have been characterized at subnanomolar hormone concentrations (290–292).

The nongenomic actions of T_4 reviewed here have largely been described at total or free concentrations (10^{-10} M; Table 1) that approximate physiological free levels of the hormone (10^{-11} M). Nongenomic effects initially demonstrated at 10^{-6} M T_3 (154) have now been reproduced at lower concentrations, *i.e.*, 10^{-7} M total and 10^{-10} M free T_4 (214). Using hormone-stripped bovine serum for supplementation of culture medium in studies of nongenomic actions of the hormone in cultured cells, we have shown that addition of physiological 10^{-7} M T_4 yields a directly measured total free T_4 of 10^{-10} M (156) that approximates physiological.

Use of hormone-stripped fetal bovine serum for culture medium supplementation is desirable in cell culture studies in which thyroid hormone is to be added and its effects studied. However, we have recently pointed out that stripping of serum with charcoal or anion-exchange resin will not only remove thyroid hormone and the principal steroids, but also will materially reduce serum content of folate, vitamin B_{12}, magnesium, phosphate, and potassium (293). Thus, this useful product introduces several variables that are not routinely taken into consideration in the design of cell culture experiments.

In interpreting studies carried out in the artificial construct of the culture medium and cultured cells, we have emphasized those that rely on hormone concentrations in the nanomolar range. When the *in vitro* studies have been extended to the intact organism, we have included the results, although dosing in the intact animal may be subject to criticisms similar to those applied to *in vitro* experiments.

A. Deiodinases

Extrathyroidal conversion of T_4 to T_3 by deiodinases in various tissues precedes many actions of thyroid hormone. Discussion of deiodinase activities is beyond the scope of the current review and is recently and extensively presented elsewhere (294–296).

B. Thyroid hormone transporters

This review is focused on molecular actions of thyroid hormone. There is substantial information now available about plasma membrane transporters that import the hormone. The description and function of the transporters in anticipation of genomic, mitochondrial and certain cytoplasmic actions of iodothyronines is beyond the scope of the present review. Visser and colleagues (297, 298) have recently reviewed the topic of transporters.

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