1-THYROID HORMONE VS. 3,5,3'-TRIODO-L-THYRONINE AND CELL PROLIFERATION: ACTIVATION OF MITOGEN-ACTIVATED PROTEIN KINASE AND PHOSPHATIDYLINOSITOL 3-KINASE

Hung-Yun Lin,1 Mingzeng Sun,1 Heng-Yuan Tang,1 Cassie Lin,1 Mary K. Luidens,2 Shaker A. Mousa,3 Sandra Incerpi,4 George L. Drusano,1 Faith B. Davis,1 and Paul J. Davis1,2

1The Signal Transduction Laboratory, Ordway Research Institute, 2Albany Medical College, and 3Pharmaceutical Research Institute, Albany College of Pharmacy, Albany, New York; and 4Department of Biology, University of Rome “Roma Tre,” Rome, Italy

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Lin H, Sun M, Tang H, Lin C, Luidens MK, Mousa SA, Incerpi S, Drusano GL, Davis FB, Davis PJ. 1-THYROID HORMONE VS. 3,5,3'-TRIODO-L-THYRONINE AND CELL PROLIFERATION: ACTIVATION OF MITOGEN-ACTIVATED PROTEIN KINASE AND PHOSPHATIDYLINOSITOL 3-KINASE. Am J Physiol Cell Physiol 296: C980–C991, 2009. First published January 21, 2009; doi:10.1152/ajpcell.00305.2008.–3,5,3'-Triiodo-L-thyronine (T3), but not l-thyroxine (T4), activated Src kinase and, downstream, phosphatidylinositol 3-kinase (PI3-kinase) by means of an α,β3 integrin receptor on human glioblastoma U-87 MG cells. Although both T3 and T4 stimulated extracellular signal-regulated kinase (ERK) 1/2, activated ERK1/2 did not contribute to T3-induced Src kinase or PI3-kinase activation, and an inhibitor of PI3-kinase, LY-294002, did not block activation of ERK1/2 by physiological concentrations of T3 and T4. Thus the PI3-kinase, Src kinase, and ERK1/2 signaling cascades are parallel pathways in T3-treated U-87 MG cells. T3 and T4 both caused proliferation of U-87 MG cells; these effects were blocked by the ERK1/2 inhibitor PD-98059 but not by LY-294002. Small-interfering RNA knockdown of PI3-kinase confirmed that PI3-kinase was not involved in the proliferative action of T3 on U-87 MG cells. PI3-kinase-dependent actions of T3 in these cells included shuttling of nuclear thyroid hormone receptor-α (TRα) from cytoplasm to nucleus and accumulation of hypoxia-inducible factor (HIF)-1α mRNA; LY-294002 inhibited these actions. Results of studies involving α,β3 receptor antagonists tetraiodothyroacetic acid (tetrac) and Arg-Gly-Asp (RGD) peptide, together with mathematical modeling of the kinetics of displacement of radiolabeled T3 from the integrin by unlabeled T3 and by unlabeled T4, are consistent with the presence of two iodothyronine receptor domains on the integrin. A model proposes that one site binds T3 exclusively, activates PI3-kinase via Src kinase, and stimulates TRα trafficking and HIF-1α gene expression. Tetrac and RGD peptide both inhibit T3 action at this site. The second site binds T3 and T4, and, via this receptor, the iodothyronines stimulate ERK1/2-dependent tumor cell proliferation. T3 action here is inhibited by tetrac alone, but the effect of T4 is blocked by both tetrac and the RGD peptide.

The phosphatidylinositol 3-kinase (PI3-kinase)/protein kinase B (Akt) pathway is an important regulator of cellular growth, metabolism, and survival (13, 19). Studies of Storey et al. (38) indicate that T3 activates PI3-kinase to slow the deactivation of potassium KCNH2 channels. Treatment of endothelial cells with T3 increases the association of TRα1 with the p85α subunit of PI3-kinase, leading to the phosphorylation and activation of Akt and endothelial nitric oxide synthase (15). Furuya et al. (14) have also pointed out that the TRβ mutant TRβPV, via activation of the PI3-kinase-regulatory subunit p85α, may affect PI3-kinase downstream signaling in both nuclear and extranuclear compartments, thus contributing to thyroid carcinogenesis. PI3-kinase appears to play a role in rapid nongenomic (15) and genomic actions of thyroid hormone (32), even though the significance and molecular mechanisms of many of these nongenomic actions are incompletely understood. Src kinase, another integrin α,β3-regulated kinase, has also been reported to be involved in T3-induced Na-K-ATPase activation and expression in alveolar epithelial cells (22).

In the current study, we have addressed the contributions of both ERK1/2 and PI3-kinase signal transduction pathways in the actions of T3 and T3 to specifically assess whether the cell proliferation effects of iodothyronines utilize both pathways. We find that both T4 and T3 activate ERK1/2 and cause cell proliferation, indicated by accumulation of proliferating cell nuclear antigen (PCNA) and radiolabeled thymidine incorporation, in a human glioma (U-87 MG) cell line that has been studied extensively by others (4, 21, 34, 42). However, only T3 causes tyrosine phosphorylation (activation) of PI3-kinase. This latter cell surface action of T3, however, does not contribute to cell proliferation; instead, its consequences include translocation of the nuclear hormone receptor, TRα1, from cytoplasm to the nucleus of hormone-treated cells and increased expression of the hypoxia-inducible factor (HIF)-1α gene.

METHODS

Cell line. Human glioma U-87 MG cells (4, 21, 30, 35) were purchased from ATCC (Rockville, MD) and maintained in EMEM supplemented with 10% FBS and incubated in a 95% air-5% CO2 incubator at 37°C. Before treatment, cells were exposed for 2 days to 0.25% hormone-stripped FBS-containing medium.

Address for reprint requests and other correspondence: F. B. Davis, Ordway Research Institute, 150 New Scotland Ave., Albany, NY 12208 (e-mail: fdavis@ordwayresearch.org).
Reagents and antibodies. Ta, Tβ, tetraiodothyroacetic acid (tetrac), and Arg-Gly-Asp (RGD) and arginine-glycine-glutamate (RGF) peptides were obtained from Sigma Chemical (St. Louis, MO). Polyclonal rabbit antibodies to phospho-(p)-ERK1/2, phospho-Src kinase (Y416), p85-Pi3-kinase, and tyrosine-phosphorylated p85-Pi3-kinase were purchased from Cell Signaling Technology (Beverly, MA), and monoclonal mouse anti-PCNA was purchased from Santa Cruz (Santa Cruz, CA). Goat anti-rabbit IgG and rabbit anti-mouse IgG were purchased from Dako (Carpenteria, CA). The chemiluminescence reagent was from ECL (Amer sham, Piscataway, NJ), and the specific ERK1/2 activation inhibitor PD-98059, PI3-kinase inhibitor LY-294002, and Src inhibitor PP2 were obtained from Calbiochem (San Diego, CA). These inhibitors were added to cells 30 min before hormone addition.

Cell fractionation. Fractionation in a microfuge and preparation of nucleoproteins was by our previously reported methods (8, 27, 28). Nuclear extracts were prepared by resuspension of the crude nuclei in high-salt buffer (hypotonic buffer containing 420 mM NaCl and 20% glycerol) at 4°C with rocking for 1 h. The supernatants were collected after centrifugation at 10,000 g for 15 min. The pellet obtained was resuspended in 500 μl of a buffer containing 250 mM sucrose, 10 mM EDTA, 5 mM HEPES, pH 7.5, and 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The samples were centrifuged two times at 100,000 g for 15 min. The precipitate was washed two times with cold ethanol; 2% SDS (1 ml) was added to each well, and the TCA-precipitable radioactivity was quantitated in a liquid scintillation counter.

Radioiodinated binding assay. Membranes from U-87 MG cells were prepared by method as described previously (27). The harvested cells were washed with PBS, harvested, and resuspended in ice-cold 250 mM sucrose, 10 mM EDTA, pH 7.5, 1 mM EGTA, containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The samples were centrifuged two times at 500 g for 15 min at 4°C. Na2CO3 (100 mM) was added to the resulting supernatant and shaken for 45 min at 4°C, and then centrifuged at 100,000 g for 15 min. The pellet obtained was resuspended in 500 μl of a buffer containing 250 mM sucrose, 10 mM HEPES, pH 7.5, and 1 mM MgCl2 and separated into aliquots for future use. Protein content was quantitated using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA), with BSA as a standard. Thyroid hormone binding displacement studies were performed as follows: stock solutions of T3 and T4 were diluted to their final concentrations in 0.04 N KOH with 0.4% polyethylene glycol to ensure that the effect was independent of the solvent used. Purified cell membrane protein preparation (50 μg) was mixed with 10 μl of 125I-labeled hormone at 2 μCi for 10 min, and the indicated concentrations of test compounds were then added; the mixture was allowed to incubate an additional 30 min at room temperature. The mixtures were precipitated with 1 ml 5% TCA at 4°C for 30 min. The precipitate was then washed two times with cold ethanol, and the resulting TCA-precipitable radioactivity was quantitated in a liquid scintillation counter.

Quantitation of results and statistical analysis. Immunoblot densitometry was measured with a Storm 860 phosphorimager followed by analysis with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Unpaired Student’s t-test, with P < 0.05 as the threshold for significance, was used to evaluate the significance of the hormone and inhibitor effects.

Mathematical modeling of the binding site-ligand interactions was by nonlinear least-square regression, using the ADAPT II package of programs of D’Argenio and Schumitzky (6). Weight was as the inverse of the point estimate of the observation variance to best approximate the homoscedastic assumptions. The Akaike Information Criterion was calculated for both one- and two-site models, and discrimination was based on an F-test.

RESULTS

Thyroid hormones activate diverse signal transduction pathways in human glioma U-87 MG cells. In cells treated with 10−10 to 10−6 M T3, ERK1/2 activation and cellular PCNA accumulation were seen in nuclear fractions (Fig. 1A). Cytoplastic PI3-kinase accumulation and tyrosine phosphorylation of GTC-ATCCCTAGGCCCTAG-3′ (forward) and 5′-GGGT-GTCGTGGTGTAGCTAGC-3′ (reverse)]. The PCR cycle was an initial step of 95°C for 3 min, followed by 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, then 25 cycles and a final cycle of 72°C for 8 min. PCR products were separated by electrophoresis through 2% agarose gels containing 0.2 μg of ethidium bromide/ml. Gels were visualized under ultraviolet light and photographed with Polaroid film (Polaroid, Cambridge, MA). Photographs were scanned under direct light for quantitation and illustration. Results from PCR products were normalized to the GAPDH signal.

Thymidine incorporation. U-87 MG cells were seeded in 24-well trays and placed in medium containing 0.25% hormone-stripped FBS for 2 days. Cells were then placed in 10% hormone-stripped serum-containing medium before experiments were started. Aliquots of cells were treated with reagents as indicated and incubated with 1 μCi [3H]thymidine (final concentration, 13 nM) for 24 h. Cells were then washed with cold PBS, 5% trichloroacetic acid (TCA, 1 ml) was added, and the plate was held at 4°C for 30 min. The precipitate washed two times with cold ethanol; 2% SDS (1 ml) was added to each well, and the TCA-precipitable radioactivity was quantitated in a liquid scintillation counter.
p85-PI3-kinase were induced by T3. In addition, total cellular Src kinase phosphorylation was increased although total Src kinase did not change (Fig. 1A). These effects of T3 were hormone concentration-dependent.

After treatment of U-87 MG cells with T4, 10^{-10} to 10^{-6} M for 30 min, there was concentration-dependent activation of ERK1/2 (pERK1/2) from 10^{-9} to 10^{-6} M (Fig. 1B). PCNA accumulation in 24 h paralleled activation of ERK1/2 with T4 in concentrations of 10^{-9} to 10^{-7} M. There was no detectable PI3-kinase or Src activation in the cytosol of T4-treated U-87 MG cells, compared with results of T3 treatment shown in Fig. 1A.

Fig. 1. L-Thyroxine (T4) and 3,5,3'-triiodo-L-thyronine (T3) cause activation of extracellular signal-regulated kinase (ERK) 1/2 and cell proliferation, but only T3 activates p85-phosphatidylinositol 3-kinase (PI3-kinase) in human glioblastoma U-87 MG cells. A, cells cultured in MEM starvation medium for 2 days were treated with 10^{-10} to 10^{-6} M T3 for 30 min. Cells were then harvested, and nuclear and cytosolic proteins were prepared and processed for gel electrophoresis and immunoblotting. To show Src kinase activation, whole cell lysate proteins were separated by electrophoresis. T3, 10^{-9} to 10^{-7} M, caused ERK1/2 activation (phosphorylated (p)ERK1/2) in 30 min (P < 0.02) and stimulated cell proliferation after a 24-h incubation, as detected by anti-proliferating cell nuclear antigen (PCNA) (P < 0.02). T3 also induced PI3-kinase phosphorylation (pTyr-p85-PI3-kinase), total PI3-kinase accumulation (P < 0.05), and Src kinase activation, indicated by pSrc accumulation (P < 0.05). The derivation of the relative integrated optical densities (IOD) shown in this and Figs. 2–11 is described in METHODS.

In this and bar graphs in Figs. 1–11, the mean ± SE of values for pERK1/2 are always on left, and the remaining proteins are listed in left-to-right order corresponding to the key on each graph. The protein on top in the key is at the left of each group of bars. B, cells grown in MEM starvation media for 2 days were treated with 10^{-10} to 10^{-6} M T4 for 30 min. Nuclear and cytosolic proteins of cells treated with T4 for 30 min were also separated by electrophoresis and examined as in A. T3, 10^{-7} and 10^{-6} M, caused dose-dependent nuclear accumulation of pERK1/2 and cell proliferation after incubation for 24 h (P < 0.05). Phosphorylation of PI3-kinase and Src by T3 is seen in representative blots on far right, whereas T4 did not cause phosphorylation of PI3-kinase or Src.
In additional studies, the responses of U-87 MG cells to T4 and T3 separately or together were examined. Although the addition of T4 to T3 enhanced the effect on pERK1/2 accumulation, there was no enhancement or inhibition of the T3 effect on cell proliferation or pTyr-p85-PI3-kinase levels in cells also treated with T4 (Fig. 2).

Role of integrin αβ3 in thyroid hormone-induced signal transduction activation. We have previously shown that T4, via binding to cell membrane integrin αβ3, activates ERK1/2 and that this activation can be blocked by an RGD peptide (3, 8). To examine the role of integrin αβ3 in T3-induced activation of ERK1/2 and PI3-kinase, U-87 MG cells were incubated with RGD or RGE peptide for 30 min before treatment with 10^{-9} M T3 for 30 min. ERK1/2 activation was induced by T3, but this effect was not inhibited by preincubation with either RGD or RGE peptides (Fig. 3A). Levels of PCNA accumulation in T3-treated cells were also unaffected by the peptides. In contrast, activation of PI3-kinase and Src kinase by T3 was blocked by preincubation with the RGD peptide although levels of ERK1/2 activation and PCNA accumulation remained the same (Fig. 3A). These observations are consistent with the existence of two discrete binding sites for T3 on the integrin, one of which activates PI3-kinase and the second of which is linked to ERK1/2 activation.

Further studies were carried out with the thyroid hormone analog tetrac, which, like the RGD peptide, also inhibits thyroid hormone binding to integrin αβ3 (3, 8). Tetrac alone did not induce ERK1/2 activation, cell proliferation, or tyrosine phosphorylation of PI3-kinase and Src kinase (Fig. 3B). Addition of tetrac (10^{-9} M) to cells pretreated with T3 (10^{-9} M) resulted in inhibition of T3-induced ERK1/2 activation and PCNA expression, as well as inhibition of PI3-kinase and Src activation (Fig. 3B). As anticipated from our previous studies (3, 7, 8), T4-stimulated cell proliferation and ERK1/2 activation were completely blocked by tetrac (Fig. 3B).

To examine further the possibility that T3 shares a cell surface binding site with T4, 50 µg of plasma membrane protein/sample were incubated with [125I]T3 for 10 min, and varying concentrations of unlabeled T3 or T4 were then added. Bound/free separation was by protein precipitation. Membrane-bound radiolabeled T3 was displaced by unlabeled T4 in a concentration-dependent manner (Fig. 4A), and unlabeled T3 was also effective in displacing [125I]T3 binding to U-87 MG plasma membranes (Fig. 4B). Statistical modeling by F-test of the binding data shown in Fig. 4 favored a one-site model for displacement of [125I]T3 by unlabeled T4. However, for displacement of [125I]T3, binding by unlabeled T3, a two-site model was significantly favored by F-test. This binding kinetic analysis supports our impression from tetrac and RGD inhibition studies that T3 may bind to integrin αβ3 at two different sites.

Activation of ERK1/2 but not PI3-kinase is necessary for thyroid hormone-induced proliferation of U-87 MG cells. Cells were treated with the ERK1/2 activation inhibitor PD-98059 for 30 min before further addition of 10^{-9} M T3 or 10^{-7} M T4 for 30 min to detect ERK1/2, PI3-kinase, and Src activation or for 24 h for detection of PCNA. Both T3 and T4 stimulated ERK1/2 activation and cell proliferation; these effects were inhibited by PD-98059 (PD, Fig. 5A). In parallel studies of cytosol fractions, PI3-kinase phosphorylation by 10^{-9} M T3, but not by T4, occurred in U-87 MG cells, and PD-98059 did not block that T3-induced effect (Fig. 5A), indicating that ERK1/2 activation does not contribute to PI3-kinase activation. Activation of Src by T3 was similarly unaffected by inhibition of ERK1/2 activation.

Transfection with ERK1/2 siRNA was used to further confirm that ERK1/2 activation does not play a role in T3-induced PI3-kinase activation. Results presented in Fig. 5B indicate that nuclear ERK1/2 decreased by 60% in ERK1/2 siRNA-transfected cells exposed to T3 compared with levels in cells transfected with scRNA, as measured in three experiments. There was no difference in PI3-kinase activation by 10^{-9} or 10^{-7} M T3 when comparing scRNA- and ERK1/2 siRNA-transfected cells, indicating again that activation of ERK1/2 is not essential for T3-induced PI3-kinase activation.

In further experiments, we examined the possible role of PI3-kinase in ERK1/2 activation and PCNA expression. The PI3-kinase inhibitor LY-294002 was added to U-87 MG cell media 30 min before addition of 10^{-9} M T3 or 10^{-7} M T4. Treatment with LY-294002 did not inhibit nuclear ERK1/2 activation.
activation in the presence of T₃ or T₄ (Fig. 5C). In contrast, PI3-kinase phosphorylation by T₃ was inhibited by LY-294002, although Src phosphorylation was not affected by the LY-294002 compound. However, T₃-induced PCNA expression was increased by the PI3-kinase inhibitor (Fig. 5C). When cells were pretreated with the Src activation inhibitor PP2, PI3-kinase activation by T₃ was blocked, as shown in Fig. 5D.

Thymidine incorporation studies comparing the results of T₄ and T₃ in the presence of the inhibitors PD-98059 or LY-294002 support the PCNA results presented above: cell proliferation in T₃-treated cells did not change with the addition of LY (Fig. 6, lanes 2 and 3 compared with lanes 6 and 7), whereas T₄-induced thymidine uptake increased in the presence of LY-294002 (Fig. 6, lane 8). In contrast, PD-98059 partially suppressed thymidine incorporation by both T₃ and T₄ because of the dependence of the proliferative action of both hormones on ERK1/2 activation.

The minimal role of PI3-kinase in cell proliferation was further confirmed in cells transfected with siRNA of the p85-PI3-kinase regulatory domain. Results of these studies demon-
3-kinase-dependent nature of this response. Results of these microscopic studies were confirmed by immunoblotting of nucleoproteins with TRα antibody (Fig. 8B), again showing that TRα translocation to nuclei relates directly to T3 activation of PI3-kinase.

Neither PD-98059 (Fig. 9A) nor siRNA-ERK1/2 (Fig. 9B) affected T3-induced nuclear accumulation of TRα. In contrast, 10⁻⁷ M T4 had no effect on TRα translocation but did induce ERK1/2-dependent TRβ1 nuclear accumulation (Fig. 9A). PD-98059 inhibited T4-stimulated nuclear accumulation of TRβ1, as we have demonstrated previously (28). At concentrations of 10⁻⁹ and 10⁻⁷ M, T3 did not promote translocation of TRβ1 to the nucleus (Fig. 9A). At higher concentrations of 10⁻⁶ or 10⁻⁵ M, however, T3 does promote nuclear accumulation of TRβ1 (results not shown), as reported by others (2).

\[ \text{PI3-kinase activation is required for T3-induced expression of the HIF-1α gene.} \]

The role of PI3-kinase in the expression of selected thyroid hormone-inducible genes has also been examined. U-87 MG cells were treated with 10⁻⁹ or 10⁻⁷ M T3 in the presence or absence of the PI3-kinase inhibitor LY-294002. Results from a representative experiment, presented in Fig. 10A, indicate that HIF-1α expression was induced by T3 and that this action was PI3-kinase-dependent. HIF-1α gene expression has been shown by Moeller and coworkers (32, 33) to be nongenomically modulated by T3. We further examined the role of ERK1/2 activation on thyroid hormone-induced HIF-1α expression, and results indicated that PD-98059 did not affect the expression of HIF-1α induced by T3 (Fig. 10B). Of interest was that PD did inhibit the stimulatory effect of T4 on HIF-1α expression; we have previously demonstrated that T4-induced gene expression, mediated via TRβ1, is inhibited by PD-98059 (28).

**DISCUSSION**

The genomic actions of thyroid hormone involve primary interaction of the hormone T3 in the cell nucleus with a heterodimer of TRβ1 and another member of the superfamily of hormone receptors such as retinoid X receptor to initiate transcription of thyroid hormone-responsive genes (41). The process involves the shedding of corepressor proteins and the recruitment of coactivators (1, 25). Nongenomic actions of thyroid hormone include changes in activity of ion pumps in the plasma membrane (9, 18), changes in cytosolic signaling initiated at the plasma membrane (3, 8–12), actions that involve mitochondria (31), and stimulation of intracellular protein trafficking from cytosol or organelles into the nucleus (10, 28, 39).

Complex interfaces of nongenomic and genomic mechanisms of thyroid hormone action have also been described (11). These include activation at the cell surface by thyroid hormone of the ERK1/2 signal transduction pathway and subsequent ERK1/2-dependent phosphorylation of specific serines of nucleoproteins. Among the proteins involved are TRα1 (28), the nuclear estrogen receptor-α (39), and p53 (37).

Another example of interplay between nongenomic and genomic mechanisms is activation of PI3-kinase in cytoplasm by direct interaction of thyroid hormone-ligated, extranuclear TRα1 with the p85 regulatory subunit of the kinase, resulting in transcription of a number of specific genes (16). Lei et al.
have implicated Src (22), PI3-kinase (22, 23) and ERK1/2 (24) in the nongenomic action of T3 on insertion of Na-K-ATPase units into the plasma membrane and enhancement of the activity of the sodium pump. An additional example of nongenomic genomic cross talk is thyroid hormone-stimulated cell proliferation. The latter is ERK1/2-dependent, requires new blood vessel formation (7), and has been described in human breast cancer cells (39) and glioma cells (8).

We have recently described a plasma membrane receptor for T4 and T3 on integrin αβ3 (3). Thyroid hormone binding at this receptor has been shown to activate ERK1/2 and promote angiogenesis (7) and cell proliferation (8). That a receptor for thyroid hormone might exist on an integrin was suggested more than a decade ago by Farwell et al. (12) in studies of glial cells. Tetrac is a deaminated T4 analog that has no agonist function at the integrin receptor but blocks hormone binding at this site and suppresses the proliferative activity of thyroid hormone on cancer cells (8). Tetrac is therefore a probe of the involvement of the integrin receptor in cellular actions of thyroid hormone. An RGD peptide provides an additional probe, since the hormone receptor is at or near the RGD recognition site on the integrin (3, 8) that is critical to the binding of extracellular matrix proteins by the integrin (36).

Reports from other laboratories have implicated several kinases, including ERK1/2, Src kinase, and PI3-kinase, in the nongenomic mechanisms of thyroid hormone action (22, 24, 33). Integrin αβ3 is known to activate Src (17). Against this background, we conducted the present experiments to compare the ability of T4 and T3 to activate via αβ3, the ERK1/2, Src kinase, and PI3-kinase signal transduction cascades. We also determined whether ERK1/2 and PI3-kinase both contribute to the proliferative activity of thyroid hormone. These studies carried out in a human glioblastoma cell line confirm that both T4 and T3 activate the ERK1/2 pathway and cause cell proliferation, whereas only T3 is also capable of enhancing Src kinase and PI3-kinase activities. Because both hormone analogs interact with the integrin receptor (3), this observation suggests that the receptor domain can discriminate between T4 and T3. Further support for this conclusion was the observation that tetrac inhibited pERK1/2 activation (nuclear accumulation) and cancer cell proliferation by both T4 and T3, whereas the RGD peptide inhibited the action of T4, but not that of T3, on ERK1/2 activation and cell proliferation. The RGD peptide did, however, block activation by T3 of Src kinase and PI3-kinase, suggesting that there may be two integrin binding sites for T3, as shown schematically in Fig. 11.

We also determined the kinetics of displacement of radiolabeled T3 from the membrane receptor domain by unlabeled T4 and by unlabeled T3. Mathematical modeling of the results of these displacement studies by one of the authors (Drusano) significantly favored the existence of two sites capable of binding T3, only one of which also bound T4. Thus several lines of evidence (modeling of the kinetics of binding of T3 and T4, differential activation of PI3-kinase and of ERK1/2 by T3 and T4, and binding inhibitor studies with RGD peptide and tetrac) infer the existence of two T3-binding sites on the integrin, one of which is shared with T4.

When we looked downstream of the receptor to cell proliferation; using either PCNA accumulation or labeled thymidine incorporation, we observed that both T4 and T3 were proliferative factors for glioblastoma cells via an ERK1/2-dependent pathway. T3, but not T4, was found to be capable of activating...
PI3-kinase, and this led to nonproliferative downstream functions, i.e., trafficking of TRα and expression of HIF-1α. Thus there was segregation of downstream consequences of hormone action initiated at the plasma membrane. Tetrac and RGD peptide are two inhibitors of binding of iodothyronines to the integrin receptor (3, 8, 11) whose application in the current studies clarified the distinction between the two T3 binding sites.

In Fig. 11, we propose a two-site model of the actions of thyroid hormone at the receptor domain of integrin αvβ3. At one site (S1), T3 alone is bound and activates Src kinase and PI3-kinase; this leads to nuclear uptake of TRα and transcription of HIF-1α. Tetrac and RGD peptide are inhibitors at this site. At site S2, Tα and T3 act via ERK1/2 activation to cause tumor cell proliferation. The effects of tetrac and RGD peptide are discrete at S2; both block cell proliferation caused by T4, whereas only tetrac inhibits the proliferative, ERK1/2-dependent effect of T3. In this model, we assume that the binding of T3 and T4 has different allosteric consequences at the receptor site.

Recent reports of the participation of extranuclear TRα (11, 16) in nongenomic actions of iodothyronines were the rationale for our focus on the trafficking of TRα in thyroid hormonetreated U-87 MG cells. Our confocal microscopy studies revealed that the TRα receptor moved from cytoplasm to the nucleus of T3-treated cells and that LY-294002 inhibited that trafficking, indicating that the process was PI3-kinase-dependent. That the trafficking promoted by T3, compared with T4, may affect different proteins was shown above by the translo-
from cytoplasm into the nucleus, as Baumann et al. (2) have shown.

The induction of HIF-1α expression by T₃ was included in the present studies as a nongenomically initiated endpoint of thyroid hormone action because of a recent description of this mechanism (32, 33). This effect of the hormone is an example of the genomic consequences of a nongenomic action of the hormone. In the original description, this T₃ action was seen to be initiated in cytoplasm (32), but the present studies offer the possibility that the plasma membrane integrin receptor for thyroid hormone may be involved in the hormonal effect.

These observations provide novel insights into both the complex transduction of thyroid hormone signals by the ERK1/2 and PI3-kinase pathways and the nongenomic activities of T₃ and T₄. From a cell surface integrin receptor, T₃ may

Fig. 9. Activation of ERK1/2 is not required for T₃-induced TRα nuclear accumulation but is required for T₄-induced nuclear accumulation of TRβ1 in U-87 MG cells. A: U-87 MG cells were treated with 10⁻⁹ or 10⁻⁷ M T₃ or 10⁻⁷ M T₄ in the presence or absence of PD-98059 (30 μM) for 24 h. T₃ caused nuclear accumulation of TRα, which was not affected by PD-98059. At the concentrations used, T₄ did not stimulate nuclear accumulation of TRβ1. Treatment with 10⁻⁷ M T₄ caused nuclear accumulation of TRβ1, but no effect on TRα location in cells. The addition of PD-98059 did inhibit the T₄ effect, as we have reported previously (37). B: U-87 MG cells transfected with ERK1/2 siRNA or scRNA were treated with 10⁻⁹ or 10⁻⁷ M T₃ for 24 h. Lane 4 provides a normalized control value of 1 for lanes 5 and 6, and lane 7 for lanes 8 and 9. Comparing results in lanes 4–6 (scRNA) with those in lanes 7–9 (siRNA), it is again apparent that ERK1/2 activation does not play a principal role in the intracellular transfer of TRα to the nucleus in the presence of T₃.

Fig. 10. PI3-kinase activation contributes to T₃-induced expression of hypoxia-inducible factor-α (HIF-α) in U-87 MG cells. A: U-87 MG cells were treated with 10⁻⁹ or 10⁻⁷ M T₃ in the presence or absence of LY-294002 for 24 h. T₃-stimulated expression of HIF-1α was inhibited by LY-294002, indicating dependence on PI3-kinase activation by the hormone. B: cells were treated with either 10⁻⁹ or 10⁻⁷ M T₃ or 10⁻⁷ M T₄ in the presence or absence of 30 μM PD-98059 for 24 h. Although both hormones stimulated expression of the HIF-1α gene, the effect of T₄ was inhibited by PD-98059, indicating an ERK1/2-mediated process. The effect of T₃ was not impaired by inhibition of ERK1/2 activation.
regulate very specific gene transcription via PI3-kinase; others have also implicated PI3-kinase in nongenomic induction of gene transcription (22–24, 32, 33), although not from an initiation point on the cell surface. In the current studies, T3 also stimulated tumor cell proliferation, as did T4, via an initiation point on the cell surface. In the current studies, T3 is unaffected by this pathway, T3 (T4) and T3 both bind to S2 to cause ERK1/2-dependent U-87 MG cell proliferation, and this effect is blocked by the ERK1/2 inhibitor PD-98059. The T4 effect on cell proliferation is inhibited by both tetrac and RGD peptide, but the action of T3 on cell proliferation via S2 is inhibited only by tetrac. This may reflect allosteric changes in the integrin site that are distinctive for the liganding of T3 and T4 and S2. At S2, the disproportionate sizes of “T3” and “T4,” reflect the greater effectiveness of T4 at this site in terms of TRβ1 trafficking and cell proliferation.

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Fig. 11. Proposed pathways by which thyroid hormones promote U-87 MG cell proliferation and intracellular trafficking of the thyroid hormone receptors TRα and TRβ1. Two hormone-binding sites [site 1 (S1) and site 2 (S2)] are proposed within the iodothyronine receptor domain on integrin αvβ3. T3 (T4) interacts with S1 to activate the PI3-kinase signal transduction pathway via Src kinase activation. Downstream consequences are shutting of cytoplasmic TRα to the nucleus and transcription of the HIF-1α gene. T3-initiated action at S1 is inhibited by tetrac and the RGD peptide. Inhibition of PI3-kinase activation by LY-294002 downstream of Src blocks TRα shuttling and HIF-1α transcription, and inhibition of Src kinase activation by PP2 prevents hormone activation of PI3-kinase and consequent cellular actions. Proliferation of U-87 MG cells in response to T3 is unaffected by this pathway. T3 (T4) and T3 both bind to S2 to cause ERK1/2-dependent U-87 MG cell proliferation, and this effect is blocked by the ERK1/2 inhibitor PD-98059. The T4 effect on cell proliferation is inhibited by both tetrac and RGD peptide, but the action of T3 on cell proliferation via S2 is inhibited only by tetrac. This may reflect allosteric changes in the integrin site that are distinctive for the liganding of T3 and T4. At S2, the disproportionate sizes of “T3” and “T4,” reflect the greater effectiveness of T4 at this site in terms of TRβ1 trafficking and cell proliferation.


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