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# Thyroid Hormone Stimulates Protein Synthesis in the Cardiomyocyte by Activating the Akt-mTOR and p70$^{S6K}$ Pathways*

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**Thyroid hormones affect cardiac growth and phenotype; however, the mechanisms by which the hormones induce cardiomyocyte hypertrophy remain uncharacterized. Tri-iodo-L-thyronine (T3) treatment of cultured cardiomyocytes for 24 h resulted in a 41 ± 5% ($p < 0.001$) increase in [$^3$H]leucine incorporation into total cellular protein. This response was abrogated by the phosphatidylinositol 3-kinase (PI3K) inhibitor, wortmannin. Co-immunoprecipitation studies showed a direct interaction of cytosol-localized thyroid hormone receptor TR$\alpha$1 and the p85$\alpha$ subunit of PI3K. T3 treatment rapidly increased PI3K activity by 52 ± 3% ($p < 0.005$), which resulted in increased phosphorylation of downstream kinases Akt and mammalian target of rapamycin (mTOR). This effect was abrogated by pretreatment with wortmannin or LY294002. Phosphorylation of p70$^{S6K}$, a known target of mTOR, occurred rapidly following T3 treatment and was inhibited by rapamycin and wortmannin. In contrast, phosphorylation of the p85 variant of S6K in response to T3 was not blocked by LY294002, wortmannin, or rapamycin, thus supporting a T3-activated pathway independent of PI3K and mTOR. 40 S ribosomal protein S6, a target of p70$^{S6K}$, and 4E-BP1, a target of mTOR, were both phosphorylated within 15–25 min of T3 treatment and could be inhibited by wortmannin and rapamycin. Thus, rapid T3-mediated activation of PI3K by cytosolic TR$\alpha$1 and subsequent activation of the Akt-mTOR-S6K signaling pathway may underlie one of the mechanisms by which thyroid hormone regulates physiological cardiac growth.**

The observation that thyroid hormone treatment of patients and experimental animal models of heart failure can improve cardiac function has been attributed in part to its regulation of cardiac genes (1–5). It has also been well documented that thyroid hormones stimulate physiologic cardiac hypertrophy (6); however, the intracellular mechanisms underlying this response remain poorly defined. Patients with chronic hyperthyroidism experience a marked reduction in systemic vascular resistance with increased cardiac contractility and cardiac output, often associated with ventricular hypertrophy (7, 8). However, the role of thyroid hormones on gene expression fails to satisfactorily explain the effects observed on cardiomyocyte growth. Recent published studies supporting the cytosolic localization and non-transcriptional activities of thyroid hormone receptors may underlie thyroid hormone-induced physiological growth (9–11). Evidence of protein-protein interactions between cytosolic thyroid hormone receptors and the p85$\alpha$ regulatory subunit of phosphatidylinositol 3-kinase (PI3K),[2] and activation of PI3K activity by T3 treatment have been reported (12, 13). Activation of the PI3K$\alpha$-Akt-mTOR pathway by IGF-1 and transgenic animal models expressing molecular components of this pathway have implicated the PI3K-Akt signaling pathway in determining heart size and physiologic cardiac growth (14–18). Recently, Kuzman *et al.* (19) reported that the Akt-mTOR signaling pathway was activated in the hypertrophied hearts of hyperthyroid animals. Furthermore, activation of PI3K and Akt appears to be a common feature of the cardioprotective mechanisms of numerous peptide hormones and growth factors including IGF-1, insulin, adrenomedullin, and estrogen (20–23). It remains to be ascertained whether activation of this pathway by thyroid hormones explains some of its cardioprotective effects and unresolved non-nuclear mechanisms of action (24–27).

The objective of the current study was to determine whether T3-induced physiologic cardiac growth is mediated via the PI3K-Akt-mTOR signaling pathway by the activation of PI3K through a direct interaction with TR$\alpha$1. Thus, cytosolic localization of TR$\alpha$1 in cardiomyocytes may serve a role in regulating protein synthesis, maintaining growth, and cell survival.

## MATERIALS AND METHODS

*Isolation, Culture, and Viral Transduction of Neonatal Rat Ventricular Myocytes*—Animals were treated in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Department of Health and Human Services Publication 85–23), and study protocols were approved by the Institutional Animal Care and Use Committee. Ventricular myocytes were isolated from hearts of 2-day-old rats by collagenase digestion as we have previously described (9). Myocytes were plated at ~1.5 × $10^4$/cm$^2$ on collagen-

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[2] The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; m.o.i., multiplicity of infection; MyHC, myosin heavy chain; IP, immunoprecipitate; T3, tri-iodo-L-thyronine.

coated 6-well plates or 60-mm dishes and cultured for the first 20 h in Dulbecco's modified Eagle's medium/F12 medium containing 10% fetal bovine serum, L-glutamine, cytosine β-D-arabino-furanoside (10 μM), and antibiotics. After the first 20 h, the neonatal rat ventricular myocyte cultures were washed with Hanks' balanced salt solution and transduced with adenovirus-expressing TRα1 (2 m.o.i.) as we have previously published (9). Select cultures were co-transduced with adenovirus expressing a mutant p85α subunit of PI3K that lacks the p110-binding site (Ad-Δp85PI3K, kindly provided by Dr. M. Kasuga, Kobe University, Japan). In most experiments, cultures were transduced with Ad-TRα1 unless indicated otherwise. Cells were washed and maintained in serum-free medium (containing transferrin (5 mg/liter), selenium (5 μg/liter) for 48 h prior to experimentation as indicated under "Results." Reagents used in cell culture were the highest quality available from Calbiochem or ICI Biomedicals Inc. (Aurora, OH). Final concentrations were: T3 ($10^{-8}$ M), LY294002, ($5 \times 10^{-5}$ M), wortmannin ($2 \times 10^{-6}$ M), rapamycin ($2.5 \times 10^{-8}$ M), IGF-1 ($10^{-8}$ M).

*Measurement of Protein Synthesis*—After 24 h in serum-free medium, the cardiomyocytes were treated with T3 ($10^{-8}$ M) for 24 h or left untreated. In some cultures, wortmannin was added either with T3 for 24 h or for the final 6 h of the experiment. 6 h before harvest, L-[3,4,5-$^3$H]leucine (5 uCi/ml; 117 Ci/mmol) was added to the culture medium to measure incorporation into newly synthesized protein. Total cellular proteins were precipitated in ice-cold 10% trichloroacetic acid and collected by centrifugation (14,000 × *g*, 10 min, 4 °C). The protein pellets were washed twice by resuspension in cold 10% trichloroacetic acid and collected by centrifugation. The final pellets were dissolved in 0.2 N NaOH by incubation at 60 °C for 30 min. Protein concentrations were determined by the bicinchoninic acid protein assay (Micro BCA assay; Pierce Biotechnology) and the radioactivity measured by liquid scintillation counting.

*Measurements of Protein and DNA*—Cardiomyocytes were quantitatively scraped from culture dishes into 0.2 N perchloric acid and collected by centrifugation (10,000 × *g* for 10 min.). The resulting precipitate was dissolved in 0.3 N KOH by incubation at 60 °C for 20 min. Aliquots were used for analysis of total protein by the BCA method and for DNA determination by fluorescence spectrophotometry using 33258 Hoechst dye and calf thymus DNA as standard (DNA assay kit; Sigma).

*RNA Analysis*—Total cellular RNA was prepared from ~$10^6$ cardiomyocytes using a commercially available kit (RNeasy mini kit; Qiagen, Valencia, CA). Northern blotting methods were used for mRNA analysis of cardiac α- and β-myosin heavy chains (MyHC) and sarcoplasmic reticulum calcium-activated ATPase as previously published (10).

*Cell Fractionation and Immunoblot Analysis*—Cells were homogenized in buffer containing 20 mM Hepes, pH 7.9, 10 mM KCl, 1 mM EDTA, 10% glycerol, 0.2% Nonidet P-40 plus protease and phosphatase inhibitors and fractionated by centrifugation at 12,000 × *g* for 1 min at 4 °C. The resulting supernatant was used as the cytosolic fraction. Protein concentrations were determined by Micro BCA assay, and equal amounts of protein were analyzed by Western blotting as previously published (9, 10). Antibodies used were: anti-TRα1 (PA1-211A; Affinity BioReagent, Golden, CO), anti-PI3Kp85 (Upstate Cell Signaling Solutions, Lake Placid, NY), and anti-Akt, p-Akt(Ser-473), p-Akt(Thr-308), mTOR, p-mTOR(Ser-2448), p70$^{S6K}$, p-p70$^{S6K}$(Thr-389), 4E-BP1, p-4E-BP1(Ser-65), S6 ribosomal protein, p-S6(Ser-235/236) from Cell Signaling Technology (Beverly, MA). Secondary antibodies used were either goat anti-rabbit or goat anti-mouse IgG conjugated with horseradish peroxidase. Protein bands were detected using chemiluminescence reagent and visualized by exposure to x-ray film. Protein band intensity was quantified by laser scanning densitometry (GS-800 calibrated densitometer; Bio-Rad).

*Measurement of PI3 Kinase Activity*—Cardiomyocytes were treated with T3 ($10^{-8}$ M) or IGF-1 ($10^{-8}$ M) for 10 min or left untreated. Cells were lysed in ice-cold buffer containing 137 mM NaCl, 20 mM Tris·HCl, pH 7.4, 1 μM $CaCl_2$, 1 μM $MgCl_2$, 1% Nonidet P-40, protease and phosphatase inhibitors, and the soluble fraction obtained by centrifugation at 10,000 × *g* was used for immunoprecipitation. Cytosolic protein (500 μg) in 1 ml of final volume was incubated overnight with 8 μg of anti-PI3Kp85 antibody at 4 °C. The immune complex was precipitated using TrueBlot anti-rabbit Ig IP beads (eBioscience, San Diego, CA) and retrieved by centrifugation at 1000 × *g* for 1 min. The immunoprecipitate was washed and resuspended in PI3K assay buffer (20 mM Tris·HCl, pH 7.4, 100 mM NaCl, 0.5 mM EGTA) and preincubated for 5 min with 10 μg of L-α-phosphatidylinositol and L-α-phosphatidyl-serine. The reaction was initiated by the addition of Mg-ATP mixture containing 50 mM $MgCl_2$, 125 μM ATP, 5 uCi of [γ-$^{32}$P]ATP/reaction. The reaction was terminated by the addition of 15 μl of 4 N HCl and 130 μl of chloroform/methanol (1:1). Phospholipids were extracted and separated by thin layer chromatography in chloroform: methanol:4 N ammonia (45:35:10). Radiolabeled product L-α-phosphatidylinositol-4-phosphate was identified by exposure to x-ray film and quantified by laser scanning densitometry (GS-800; Bio-Rad densitometer).

*Co-immunoprecipitation Analysis*—Total cell lysate (500 μg) was first precleared using 50 μl of TrueBlot anti-rabbit Ig IP beads (eBioscience) and then incubated with either anti-PI3Kp85 or anti-TRα1 antibody for 1 h, 4 °C. The antibody complex was precipitated with anti-rabbit Ig IP beads and collected by centrifugation (2500 × *g*, 5 min). In some experiments, agarose-conjugated P13K-p85α antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used. Precipitates were extensively washed prior to resolution by electrophoresis. Nonspecific immune precipitation was determined using anti-rabbit IgG. Immunoblot analysis with TrueBlot horseradish peroxidase anti-rabbit IgG (used according to the manufacturer's instructions; eBioscience) was required to eliminate interference with the detection of TRα1 protein.

*Statistical Analysis*—All data are presented as mean ± S.E. derived from a minimum of two separate cell preparations. One-way analysis of variance was used for statistical analysis of mean values between experimental groups, and Student-Newman-Keuls was used for pairwise multiple comparisons. Differences between means were considered significant at

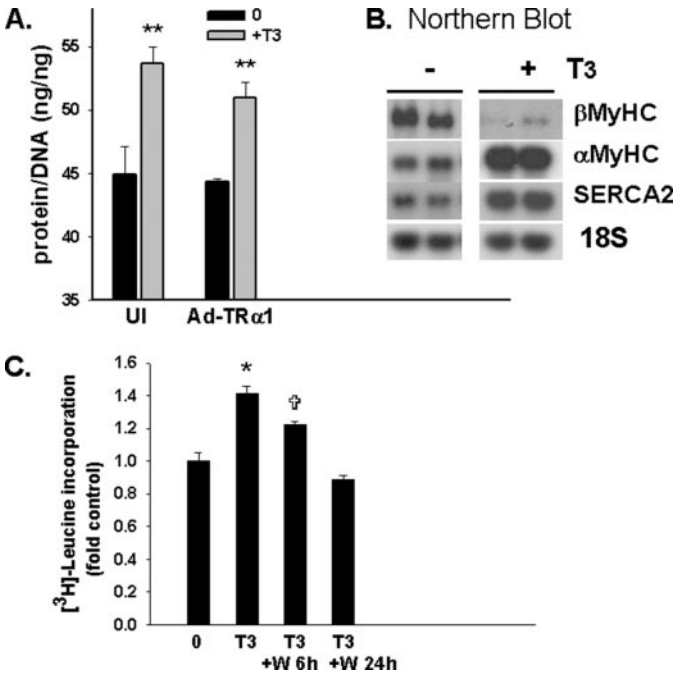


FIGURE 1. **T3-stimulated protein synthesis is inhibited by wortmannin.** *A*, neonatal rat cardiomyocytes were either transduced with replication-deficient adenovirus-expressing TR$\alpha$1 (*Ad-TR$\alpha$1*, 2 m.o.i.) or left untreated (*UI*) and then cultured in serum-free medium for 24 h prior to treatment with T3 (10 nM) or vehicle (0) for another 24 h. Cultures were harvested for total cell protein and DNA analysis and results expressed as protein/DNA ratios. Data are means ± S.E., $n = 6$ per treatment. **, $p < 0.02$ *versus* 0 T3. *B*, Northern blot showing mRNA expression for $\beta$- and $\alpha$-myosin heavy chain (*$\beta$MyHC*, *$\alpha$MyHC*), sarcoplasmic reticulum calcium-activated ATPase (*SERCA2*), and ribosomal 18 S RNA after 24 h of T3 treatment (10 nM). Cardiomyocytes were transduced with Ad-TR$\alpha$1 24 h prior to T3 treatment (+) or no treatment (−). *C*, cardiomyocytes were transduced with Ad-TR$\alpha$1 24 h prior to T3 treatment for 24 h. Control cultures (*0*) were left untreated. Wortmannin (2 $\mu$M) was added either simultaneously with T3 (*+W 24h*) or for the final 6 h (*+W 6h*) before harvest. Protein synthesis was measured by the incorporation of [$^3$H]leucine into total cell protein by adding it to the medium for the last 6 h of the experiment. $n = 12$ cultures/group from two different cell isolations. *Bar graph* is mean ± S.E. Data were analyzed by analysis of variance, Student-Newman-Keuls test. *, $p < 0.001$ *versus* 0, T3+W 6h, and T3+W24h; *cross*, $p < 0.01$ *versus* 0 and T3+W24h.

$p < 0.05$. Data were analyzed using SigmaStat 3.1 (Systat Software, Inc., Richmond, CA).

## RESULTS

*T3-stimulated Protein Synthesis Is Mediated by PI3K Activation*—We determined that T3 treatment of cultured cardiomyocytes for 24 h resulted in significant cell growth as measured by the 15–20% increase ($p < 0.02$) in protein to DNA ratios (Fig. 1*A*). Similar effects were seen in cardiomyocytes that were transduced with Ad-TR$\alpha$1 and cultures that were not transduced. We evaluated whether this growth effect of T3 treatment resulted from an increase in rate of protein synthesis by measuring the incorporation of [$^3$H]leucine into total cell protein over a 6-h time period. Using Ad-TR$\alpha$1-transduced cultures, we showed that T3 significantly increased protein synthesis by 41 ± 5% and that this increase could be prevented by inhibition of PI3K activity by simultaneous treatment of the cells with wortmannin (*W*) for either 6 or 24 h (Fig. 1*C*). These data support a role for the PI3K pathway in T3 stimulation of protein synthesis and cardiomyocyte growth. Furthermore, Northern blot analysis ascertained that T3 treatment of the Ad-TR$\alpha$1-transduced cardiomyocytes resulted in a physiological hypertrophic phenotype with stimulation of $\alpha$-myosin heavy chain ($\alpha$MyHC) and sarcoplasmic reticulum calcium-activated ATPase mRNA expression and repression of $\beta$MyHC (Fig. 1*B*). These T3-induced phenotypic changes were similar to those observed in non-transduced cardiomyocytes (data not shown) and in cardiomyocytes transduced with control Ad-ne$\beta$gal (nuclear localized $\beta$-galactosidase) as we have previously published (10).

*TR$\alpha$1 Complexes with p85$\alpha$ Subunit of PI3K, and T3 Stimulates PI3K Activity*—Because our previous studies had shown that the TR$\alpha$1 isoform localized to both nuclear and cytoplasmic compartments of the cardiomyocyte, we investigated whether the receptor interacted directly with the p85$\alpha$ subunit of PI3K as had been previously reported in human skin fibroblasts (12). To address this possibility, cardiomyocytes were transduced with adenovirus-expressing TR$\alpha$1 and cultured for 48 h prior to harvest. Cytosolic fractions were subjected to immunoprecipitation with either anti-TR$\alpha$1 or anti-P13Kp85$\alpha$ antibodies and probed for the presence of both proteins in the immune complex by Western blot analysis. As shown in Fig. 2*A*, immune complexes containing the two proteins were observed both in the presence and absence of T3, suggesting that complex formation was ligand independent. Furthermore, we were able to show immune complex formation of endogenous TR$\alpha$1 and p85$\alpha$ proteins when immunoprecipitated with the P13Kp85 antibody (*UI*) (Fig. 2*A*). However, the low affinity of commercially available anti-TR$\alpha$1 antibodies for effective use in immunoprecipitation of the low amounts of endogenous TR$\alpha$1 precluded successful immunoprecipitation of the TR$\alpha$1-p85 complex using anti-TR$\alpha$1 antibodies.

To determine whether T3 stimulated PI3K enzymatic activity, cardiomyocytes transduced with Ad-TR$\alpha$1 were treated with T3 ($10^{-8}$ M) or vehicle for 10 min prior to harvest. Cytosolic fractions were incubated overnight with anti-PI3K-p85 antibodies, and then the precipitated immune complexes were used for enzymatic analysis. Results in Fig. 2*B* show that T3 rapidly increased PI3K activity by 52 ± 3% ($p < 0.005$) compared with untreated myocytes. Shown for comparison is the stimulatory effect of IGF-1 on PI3K activity showing ~2-fold increase. As stated above, we were unsuccessful in using the anti-TR$\alpha$1 antibodies to immunoprecipitate sufficient TR$\alpha$1-PI3K complex for enzymatic analysis.

*T3 Rapidly Induces Phosphorylation of Akt and p70$^{S6K}$ That Is Sustained for 24 Hours*—We examined whether the observed T3-stimulated PI3K activity could result in activation of the Akt-S6K signaling pathway as has been shown in IGF-1-mediated physiological cardiac hypertrophy and cardioprotection (15, 16). As shown in Fig. 3, Ser-473 phosphorylation of Akt was detected at 15 min after addition of T3 and phosphorylation was maintained up to 24 h, whereas total Akt protein levels were unaltered. Similarly, rapid and sustained phosphorylation of p70$^{S6K}$ occurred after T3 treatment, thus supporting a potential role of these signaling proteins in mediating a non-genomic cytosolic T3 response. Downstream targets of activated Akt and p70$^{S6K}$ pathways include several proteins directly involved in protein translation, such as 4E-BP1 and 40 S ribosomal protein S6. Rapid and persistent phosphorylation

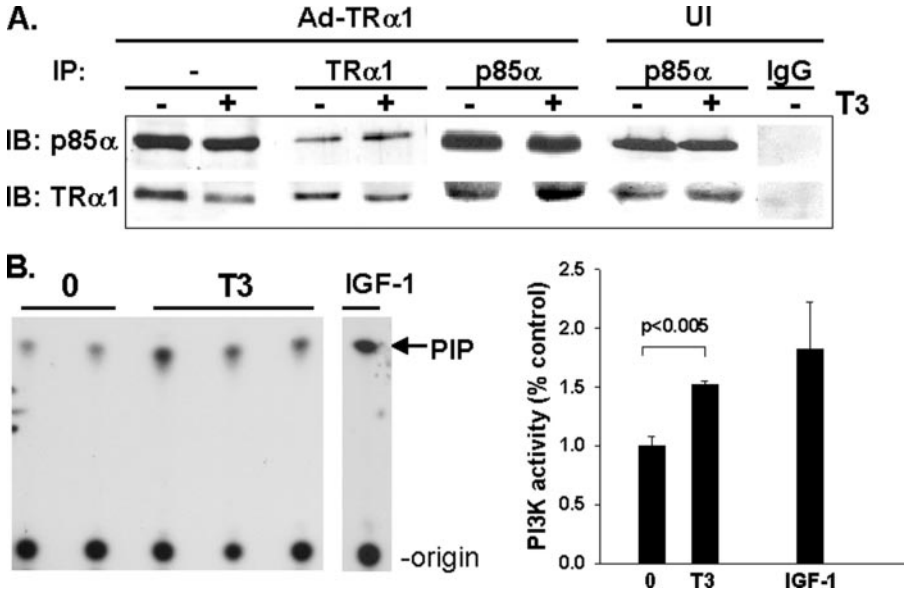


FIGURE 2. **TRα1 forms a complex with p85 subunit of PI3K, and T3 activates PI3 kinase activity.** *A*, cultured cardiomyocytes were either not transduced (*UI*) or transduced with Ad-TRα1 (2 m.o.i.) and cultured in serum-free medium for 48 h. Some cultures were treated with T3 (10 nM) (+) for 30 min prior to harvest. Cell lysates from treated and untreated cultures were immunoprecipitated (*IP*) with either anti-TRα1, anti-p85αPI3K antibodies, or control IgG and then subjected to immunoblot (*IB*) analysis with antibodies as indicated. Cell lysates without IP (−) were used to show cellular content of p85α and TRα1. Blots are representative of eight samples/treatment group from three separate cell isolations. *B*, *left*, cardiomyocytes transduced with Ad-TRα1 were cultured as above and treated with T3 (10 nM) or IGF-1 (10 nM) for 10 min prior to cell lysis as described under "Methods." Immunoprecipitated PI3K from treated and untreated cultures were used to measure PI3 kinase activity by formation of radiolabeled L-α-phosphatidylinositol-4-phosphate (*PIP*) as detected by exposure of the TLC plate to x-ray film. *Right*, PIP product was quantified by laser densitometry and is shown graphically. $p < 0.005$ T3 *versus* untreated group (*0*). *Bar graph* represents mean ± S.E., $n = 6–8$ from two cell isolations.

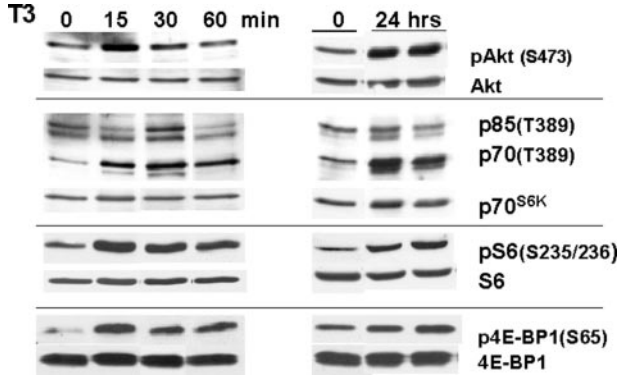


FIGURE 3. **T3 rapidly activates Akt and S6 kinase.** Cardiomyocytes were cultured under serum-free conditions for 24 or 48 h prior to treatment with T3 over a 60-min period or for 24 h. Proteins from whole cell lysates were resolved by electrophoresis, and cell signaling protein activation was determined by Western blot analysis of phosphorylated Akt, p70$^{S6K}$, ribosomal S6 protein, and 4E-BP1 and then reanalyzed for the corresponding total protein (non-phosphorylated and phosphorylated). Blots are representative of four separate cell isolations, $n = 8–12$/time point.

of these two proteins was observed following treatment with T3 (Fig. 3), providing evidence that rapid non-genomic responses to T3, potentially acting through cytosol-localized TRα1, may stimulate protein synthesis resulting in cardiomyocyte growth.

*T3 Activates mTOR through Phospho-Akt*—We further studied the rapid responses to T3 using cardiomyocytes that were transduced with Ad-TRα1. As with cardiomyocytes not subject to Ad-TRα1 transduction (Fig. 3) or transduced with Ad-β-galactosidase (data not shown), phosphorylation of Akt at Ser-473 and Thr-308 occurred rapidly within 7 min of T3 treatment (Fig. 4*A*). Cell fractionation studies showed that only low amounts of phosphorylated Akt were present in the nucleus in unstimulated conditions. However, as phosphorylation of cytosolic Akt occurred in response to T3, increasing amounts of pAkt appeared in the nucleus whereas pAkt content in the cytoplasm decreased, suggesting that T3 induced nuclear translocation of pAkt. Inhibition of PI3K activity by LY294002 (*LY*) attenuated the T3-mediated phosphorylation and nuclear translocation of Akt (Fig. 4*A*). Wortmannin similarly blocked the T3 response. Also shown is the well known robust effect of IGF-1 on Akt phosphorylation (Fig. 4*A*).

Akt activation has been implicated in numerous cellular functions, including activation of protein translation through its role in phosphorylation and activation of mTOR (reviewed in Ref. 28). Fig. 4*B* shows that mTOR phosphorylation (*Ser-2448*) in response to T3 treatment occurred more slowly than Akt phosphorylation as would be expected if mTOR were downstream of Akt in the signaling pathway. Furthermore, mTOR phosphorylation was sustained and significantly higher ($p < 0.01$) after 60 min of T3 treatment. Quantitation of the Western blot analyses illustrates the sequential phosphorylation and activation of these proteins within the signaling pathway (Fig. 4*D*). As with Akt, mTOR phosphorylation in response to T3 was abrogated by wortmannin, thus supporting a role of PI3K in this pathway (Fig. 4*B*).

*T3-activated mTOR Phosphorylates 4E-BP1 and p70$^{S6K}$*—In response to growth factor stimulation, mTOR has been shown to increase protein translation by phosphorylating 4E-BP1, and thus prevent its association with the translation initiation factor eIF4E, and by activating p70$^{S6K}$ protein kinase (reviewed in Ref. 29). As shown in Fig. 4*C*, 4E-BP1 phosphorylation was evident within 25 min of T3 treatment, clearly after the phosphorylation and activation of mTOR, supporting its location downstream of mTOR. Furthermore, this effect of T3 was completely inhibited by pretreatment with wortmannin.

T3-mediated phosphorylation of the p70 and p85 variants of S6 kinase (S6K) are shown in Fig. 5, *A* and *B*. Phosphorylation of both S6K isoforms occurred rapidly and peaked at ~25–40 min of T3 treatment. Pretreatment of the cardiomyocytes with inhibitors of PI3K (LY294002, *LY*; wortmannin, *W*) or inhibitors of mTOR (rapamycin, *R*) prevented T3-induced phosphorylation of the p70, but not the p85 S6K variant, supporting distinct T3-mediated pathways activating these two S6K proteins (Fig. 5, *A* and *B*). Thus, the T3-mediated phosphorylation of p85$^{S6K}$ appears independent of the PI3K-mTOR signaling

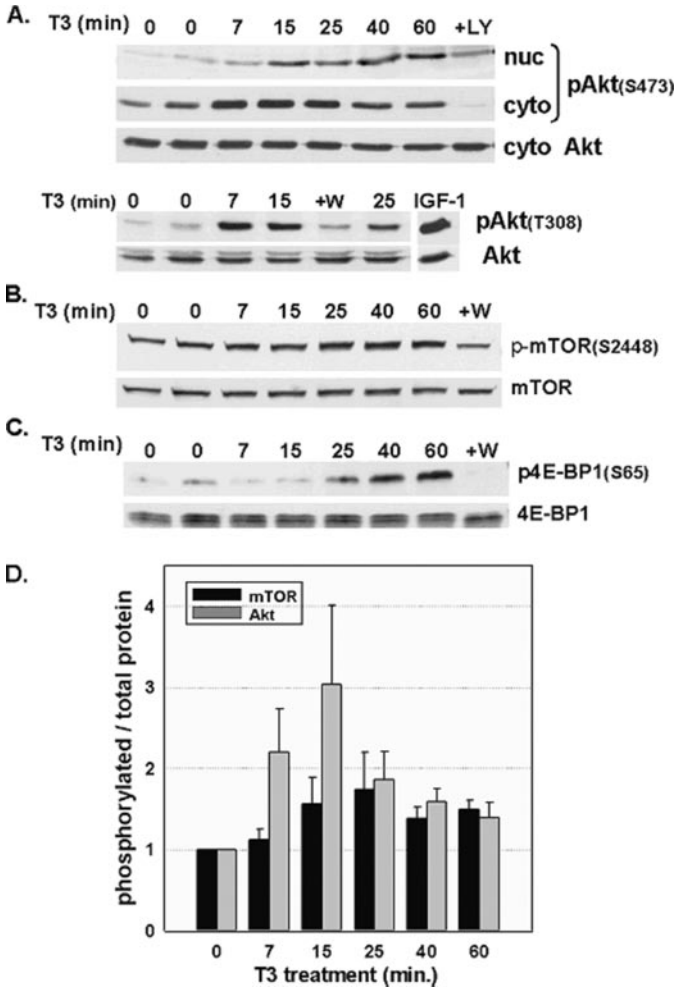


FIGURE 4. **Time course of T3-induced phosphorylation of Akt, mTOR, and 4E-BP1.** Cardiomyocytes transduced with Ad-TR$\alpha$1 were cultured in serum-free medium for 48 h prior to treatment with T3 (10 nM) and harvested at the time points indicated. IGF-1 ($10^{-8}$ M) treatment was for 15 min. *A*, nuclear (*nuc*) and cytoplasmic (*cyto*) fractions were isolated for protein analysis by Western blotting methods for detection of nuclear translocation of phosphorylated Akt in response to T3 treatment. Total cytosolic Akt shows equal sample loading at each time point. Cytosolic proteins were analyzed for phosphorylated and total mTOR (*B*) and 4E-BP1 (*C*) as described under "Methods." Some cultures were pretreated with wortmannin (*W*) or LY294002 (*LY*) for 30 min prior to treatment with T3 for 15 min. *D*, densitometric quantitations of Western blots of cytosolic Akt and mTOR from four separate cell isolations were combined and are shown graphically; *bar graph* represents mean ± S.E. relative to untreated group (0 min T3), $n = 8$/time point. Phospho-mTOR/mTOR at 15, 25, 40, and 60 min of T3 treatments are significantly different ($p < 0.01$ *versus* 0 min); pAkt/Akt at 7, 15, 25 min ($p < 0.05$ *versus* 0 min T3).

pathway. In contrast, phosphorylation of both p70 and p85 S6K variants by IGF-1 was completely prevented by pretreatment with wortmannin (Fig. 5*A*).

*T3-activated p70$^{S6K}$ Results in Phosphorylation of 40 S Ribosomal Protein S6*—p70$^{S6K}$ is recognized as the kinase that phosphorylates 40 S ribosomal subunit protein S6 that enables polyribosomal association with mRNAs containing 5′-terminal oligopyrimidine tracks (reviewed in Ref. 28). As shown in Fig. 5*C*, phosphorylation of S6 protein was first observed at 15 min and sustained to 1 h of T3 treatment. This effect was blocked by pretreatment with wortmannin or rapamycin, suggesting that T3-mediated activation of p70$^{S6K}$ by the PI3K-mTOR signaling pathway was potentially responsible for ribosomal S6 protein phosphorylation and stimulation of protein translation.

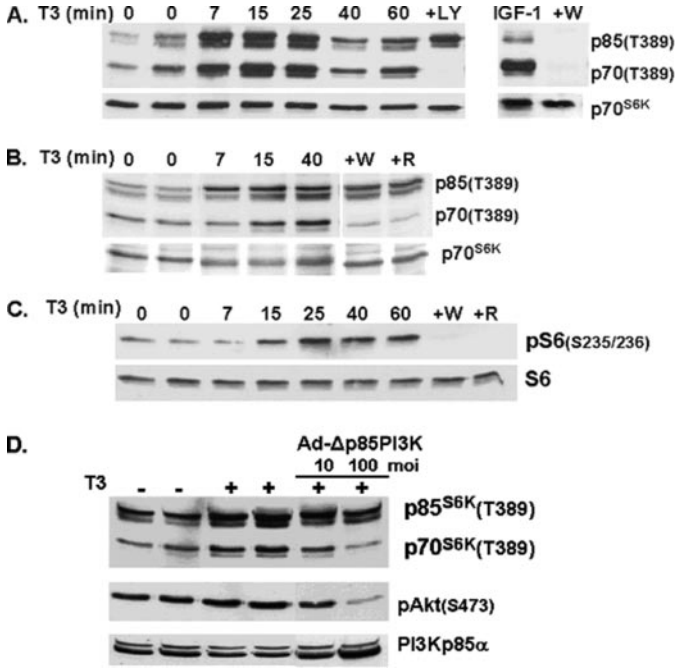


FIGURE 5. **T3-stimulated phosphorylation of S6K and 40 S ribosomal protein S6 is inhibited by wortmannin and rapamycin and by overexpression of a dominant negative mutant PI3K.** Ad-TR$\alpha$1-transduced cardiomyocytes were cultured and treated with T3, IGF-1, and inhibitors as described in Fig. 4. Pretreatment with rapamycin (*R*) was for 60 min prior to T3 treatment for 40 min. *A* and *B*, immunoblot analyses of cytoplasmic fractions were probed with antibodies detecting phosphorylated and total S6K proteins. Phospho-S6K(T389) antibody recognizes both p70 and p85 variants of S6K, whereas anti-S6K antibody preferentially detects p70$^{S6K}$. *C*, the same samples as above were used for immunoblot analysis of phosphorylated 40 S ribosomal protein S6 in response to T3, showing complete inhibition by PI3K inhibitor wortmannin and mTOR inhibitor rapamycin. Western blots are representative of samples from four separate cell isolations. *D*, cultured cardiomyocytes were transduced with Ad-TR$\alpha$1 and adenoviruses expressing mutant p85PI3K (*Ad-Δp85PI3K*, at 10 and 100 m.o.i.) and then cultured for 48 h in serum-free medium prior to T3 (10 nM) treatment for 15 min. Cytoplasmic fractions were analyzed for phospho-S6K(T389) and phospho-Akt(S473) content by immunoblot analysis. Increased expression of the p85$\alpha$ subunit of PI3K in Ad-Δp85PI3K-transduced cells was verified by anti-PI3Kp85 antibody.

*T3 Effects Are Blocked by Overexpression of a Mutant p85α PI3K*—To further corroborate the results showing T3-mediated activation of the PI3K-Akt pathway, we overexpressed a mutant form of the p85$\alpha$ subunit of PI3K (Ad-Δp85PI3K). As shown in Fig. 5*D*, overexpression of the mutant p85$\alpha$ by adenoviral-mediated transfer into cardiomyocytes at two different multiplicities of infection (10 and 100 m.o.i.) prevented T3-induced phosphorylation of Akt(S473) and p70$^{S6K}$. The effects of T3 on the phosphorylation of the p85$^{S6K}$ variant appeared unaffected by the mutant p85PI3K, further supporting distinct mechanisms of activation of these two proteins.

## DISCUSSSION

The present studies have provided insight into the molecular mechanisms by which thyroid hormones induce physiologic cardiac hypertrophy and potentially function to maintain normal cell growth. Although previous studies have documented increased rates of protein synthesis and decreased protein degradation rates in cardiac tissue in response to thyroid hormone, the mechanisms underlying these responses have not been elucidated (6). Heretofore, biological effects of thyroid hormone

have been largely attributed to nuclear transcriptional mechanisms of action that promote a normal cardiac phenotype but fail to fully explain its growth-promoting effects (30, 31). However, recent reports have documented cytosolic and membrane-initiated effects of thyroid hormones that do not involve transcriptional activity (reviewed in Refs. 32 and 33). Kinugawa *et al.* (11) published that the stress-activated p38$^{MAPK}$ pathway was involved in overexpressed TR$\alpha$1-induced pathologic, but not physiologic, cardiomyocyte hypertrophy. Kuzman *et al.* (19) showed increased phosphorylation of Akt, p70$^{S6K}$, and mTOR in hypertrophied hearts of thyroxine-treated animals. These latter observations are consistent with recent reports supporting a role for the PI3K-Akt-mTOR pathway in regulating mammalian cell size and in promoting physiologic cardiac hypertrophy in response to insulin and IGF-1 (34). Thyroid hormones have been reported to stimulate PI3K activity in skin fibroblasts and endothelial cells by non-nuclear mechanisms (12, 13). These observations together with our recently published data showing cytoplasmic localization of TR$\alpha$1 (9, 10) provided the rationale to propose that thyroid hormone-induced physiologic cardiac growth is mediated by activating the PI3K-Akt signaling pathway through cytosol-localized TR$\alpha$1.

In support of this hypothesis the present study shows co-immunoprecipitation of TR$\alpha$1 and the p85$\alpha$ subunit of PI3K. Using anti-p85 antibodies, sufficient endogenous TR$\alpha$1 protein could be co-immunoprecipitated. However, no commercially available anti-TR$\alpha$1 antibodies proved satisfactory in the immunoprecipitation of endogenous TR$\alpha$1, so that we transduced the cardiomyocytes with replication-deficient adenovirus-expressing TR$\alpha$1 at very low multiplicity of infection (2 m.o.i.). As the results show, this approach provided substantive data to support a direct protein-protein interaction between TR$\alpha$1 and p85$\alpha$. This protein interaction appeared to be independent of ligand binding; however, treatment with T3 rapidly activated PI3K activity to ~140% of the activity in untreated cardiomyocytes. Concurrent with this effect was the ability of inhibitors of PI3K (wortmannin) to inhibit the T3-induced increase in protein synthesis, lending support for a role of PI3K in this response. A downstream target of PI3K and dependent on its enzymatic product, L-$\alpha$-phosphatidylinositol-3,4,5-triphosphate, is the phosphoinositide-dependent kinase, PDK1, and its target, Akt (29, 35). We were able to show that T3 rapidly phosphorylated Akt and that pAkt was translocated to the nucleus. Both phosphorylation and nuclear translocation of pAkt in response to T3 could be blocked by inhibition of PI3K. Recent studies have shown that nuclear-targeted Akt has potent anti-apoptotic effects with significant protective effects on reducing myocardial infarct size (18). We showed that phosphorylation and activation of Akt was sustained even after 24 h of T3 treatment, further supporting its role in T3-mediated maintenance of cell growth and, potentially, cell survival.

mTOR has been shown to be important in maintaining cell size by regulating ribosomal biogenesis and protein translation (reviewed in Refs. 29, 36). Regulation of mTOR signaling is linked to PI3K/Akt by the tuberous sclerosis complex. As might be expected, activation of Akt by T3 resulted in the phosphorylation of mTOR in a time frame consistent with its location downstream of Akt in the signaling pathway. This effect was inhibited by wortmannin, further supporting the observed stimulatory effect of T3 on PI3K activity. Two downstream targets of mTOR, 4E-BP1 and S6K1, are important regulators of protein translation, and their regulation by phosphorylation enables protein synthesis to proceed commensurate with growth factor and nutrient availability (28). Thus, phosphorylation of 4E-BP1 can be used as a measure of signaling through the mTOR pathway. In response to T3, phosphorylation of 4E-BP1 was observed at a later time point than mTOR phosphorylation (25 *versus* 15 min). 4E-BP1 plays a key regulatory role in the initiation of protein translation by binding to and inhibiting eIF4E initiation complex formation when it is hypophosphorylated, as would occur in cells deprived of growth factors (reviewed in Refs. 28, 29, 36). Phosphorylation of 4E-BP1 relieves its inhibitory effect, thus enabling translation initiation to occur as would potentially be the case in T3-treated cardiomyocytes.

The S6 kinases are also important regulators of protein translation by virtue of their ability to phosphorylate a 40 S ribosomal subunit protein, S6, which enables the translation of mRNAs containing a 5′-terminal oligopyrimidine track (37). In the present studies, T3 stimulated phosphorylation of p70$^{S6K}$, followed by sustained phosphorylation of S6 protein. These effects were blocked by both wortmannin and rapamycin, suggesting that T3-mediated phosphorylation and activation of PI3K and mTOR were necessary for this response. These data support a direct role of T3 in stimulating translation initiation and perhaps a more important function of thyroid hormone in maintaining a basal level of protein synthesis under physiological euthyroid conditions.

The p85 variant of S6K1 was also phosphorylated by T3 treatment, but unlike p70$^{S6K}$ this response was not inhibited by PI3K inhibitors (LY294002 or wortmannin) or by inhibition of mTOR by rapamycin. Furthermore, overexpression of a PI3K mutant (Δp85PI3K) blocked T3-induced phosphorylation of p70$^{S6K}$ but was without effect on the p85$^{S6K}$ variant. Therefore, these data suggest that p85$^{S6K}$ may not be involved in the phosphorylation of ribosomal S6 protein and that the T3-induced phosphorylation of this S6K variant occurs via a mechanism that is distinct from the PI3K-mTOR signaling pathway. The function of this protein is largely unknown, except that it differs from p70$^{S6K}$ by the presence of an amino-terminal nuclear localization signal (38). Studies are currently underway to determine the function of this protein in response to T3.

Recent studies addressing the non-nuclear, non-transcriptional actions of thyroid hormones, whether through cytosolic receptors or through membrane-initiated processes (39, 40), may provide answers to many previously unexplained actions of these hormones (24–27). Similar to other steroid hormones like estrogens (23), the nuclear and cytosolic activities of thyroid hormones are likely to be a part of a complementary regulatory network involved in the maintenance of normal cellular function. Results from the present study support cytosolic actions of thyroid hormone that can be rapid in onset but potentially have long-term effects in the maintenance of normal cell growth.

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