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# Integrin $\alpha_V\beta_3$ Contains a Cell Surface Receptor Site for Thyroid Hormone that Is Linked to Activation of Mitogen-Activated Protein Kinase and Induction of Angiogenesis

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**Integrin $\alpha_V\beta_3$ is a heterodimeric plasma membrane protein whose several extracellular matrix protein ligands contain an RGD recognition sequence. This study identifies integrin $\alpha_V\beta_3$ as a cell surface receptor for thyroid hormone [L-$T_4$ ($T_4$)] and as the initiation site for $T_4$-induced activation of intracellular signaling cascades. Integrin $\alpha_V\beta_3$ dissociably binds radiolabeled $T_4$ with high affinity, and this binding is displaced by tetraiodothyroacetic acid, $\alpha_V\beta_3$ antibodies, and an integrin RGD recognition site peptide. CV-1 cells lack nuclear thyroid hormone receptor, but express plasma membrane $\alpha_V\beta_3$; treatment of these cells with physiological concentrations of $T_4$ activates the MAPK pathway, an effect inhibited by tetraiodothyroacetic acid, RGD peptide, and $\alpha_V\beta_3$ antibodies. Inhibitors of $T_4$ binding to the integrin also block the MAPK-mediated proangiogenic action of $T_4$. $T_4$-induced phosphorylation of MAPK is inhibited by small interfering RNA knockdown of $\alpha_V$ and $\beta_3$. These findings suggest that $T_4$ binds to $\alpha_V\beta_3$ near the RGD recognition site and show that hormone-binding to $\alpha_V\beta_3$ has physiological consequences. (*Endocrinology* 146: 2864–2871, 2005)**

THE MOLECULAR MECHANISMS of the numerous cellular actions of thyroid hormone have been widely studied (1, 2). These mechanisms largely involve hormone-stimulated changes in gene transcription and protein expression that are mediated by one or more isoforms of a specific nuclear transcription factor, the thyroid hormone receptor (TR). The latter preferentially binds L-$T_3$ ($T_3$), a thyroid hormone derived by tissue deiodination of circulating L-$T_4$ ($T_4$) (2). Recent studies have identified monocarboxylate transporter 8 as a specific thyroid hormone transporter, favoring the uptake of $T_3$ over $T_4$ (3, 4). However, activation of intracellular signaling cascades by agarose-linked $T_4$, which cannot gain access to the cell interior, implies the existence of a plasma membrane receptor for thyroid hormone that is independent of hormone transport into the cell. The ability of $T_4$ and $T_3$ to activate intracellular signal transduction cascades, independently of TR, has recently been described by several laboratories (5–8). Acting independently of TR, thyroid hormone also modulates the activity of the plasma membrane $Na^+/H^+$ exchanger (9, 10), $Ca^{2+}$-stimulable adenosine triphosphatase (11–14), several other ion pumps or channels (15–17), and the guanosine triphosphatase activity of synaptosomes (18). A cell surface receptor for thyroid hormone that accounts for these TR-independent actions of the hormone has not previously been described. In this report we disclose that activation by thyroid hormone of the MAPK signal transduction pathway and consequent MAPK-dependent proangiogenic actions of the hormone are linked to a novel hormone receptor site on a specific plasma membrane integrin.

Our laboratory has shown in the CV-1 monkey fibroblast cell line, which lacks functional TR, and in other cells that $T_4$ activates the MAPK (ERK1/2) signaling cascade and promotes the phosphorylation and nuclear translocation of MAPK as early as 10 min after application of a physiological concentration of $T_4$ (6, 19). In nuclear fractions of thyroid hormone-treated cells, we have described complexes of activated MAPK and transactivator nucleoproteins that are substrates for the serine kinase activity of MAPK. These proteins include signal transducer and activator of transcription-1$\alpha$ (STAT-1$\alpha$) (6), STAT3 (19), p53 (20), estrogen receptor-$\alpha$ (21), and, in cells containing TR, the nuclear thyroid hormone receptor for $T_3$ (TR$\beta$1) (22). Thyroid hormone-directed MAPK-mediated phosphorylation of these proteins enhances their transcriptional capabilities (6, 19–22). The effects of $T_4$-induced MAPK activation are blocked by inhibitors of the MAPK signal transduction pathway and by tetraiodothyroacetic acid (tetrac) (6, 19–22), a thyroid hormone analog that inhibits $T_4$ binding to the cell surface (23). Thyroid hormone-activated MAPK may also act locally at the plasma membrane, *e.g.* on the $Na^+/H^+$ antiporter (10), rather than when translocated to the cell nucleus. A cell surface

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Abbreviations: CAM, Chorioallantoic membrane; FBS, fetal bovine serum; mAb, monoclonal antibody; siRNA, small interfering RNA; STAT, signal transducer and activator of transcription; TBST, Tris-buffered saline containing 1% Tween 20; tetrac, tetraiodothyroacetic acid; TR, thyroid hormone receptor.

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receptor for $T_4$ that is linked to activation of the MAPK cascade has not previously been identified.

Integrins are a family of transmembrane glycoproteins that form noncovalent heterodimers. Extracellular domains of the integrins interact with a variety of ligands (24), including extracellular matrix glycoproteins, and the intracellular domain is linked to the cytoskeleton (25). Thyroid hormone was shown a decade ago to influence the interaction of integrin with the extracellular matrix protein, laminin (26), but the mechanism of the interaction was not known. Integrin $\alpha_V\beta_3$ has a large number of extracellular protein ligands, including growth factors and extracellular matrix proteins, and upon ligand binding can activate the MAPK cascade (27, 28). Several of the integrins contain an RGD recognition site that is important to the binding of matrix and other extracellular proteins that contain an Arg-Gly-Asp sequence (24). Recently, Hoffman *et al.* (29) showed that blocking the integrin RGD site prevented bone resorption stimulated by $T_4$. These observations raised the possibility that the cell surface receptor for $T_4$ might be located on an integrin.

Using the chick chorioallantoic membrane (CAM) assay, we have demonstrated that $T_4$ treatment results in increased angiogenesis, *i.e.* an increased number of blood vessel branch points, that is independent of $T_4$ conversion to $T_3$ (30). The mechanism of $T_4$-induced angiogenesis requires MAPK activity, is inhibited by tetrac, and is reproduced by $T_4$-agarose. These observations coupled with the ability of an $\alpha_V\beta_3$ antagonist to inhibit thyroid hormone-induced bone resorption (29) supported the possibility that $\alpha_V\beta_3$ is a cell surface receptor for $T_4$.

We show in this report that integrin $\alpha_V\beta_3$ specifically binds $T_4$, that the integrin and integrin-thyroid hormone complex are required for activation of MAPK by physiological concentrations of $T_4$, and that occlusion by antagonists of the RGD site on $\alpha_V\beta_3$ inhibits $T_4$-induced, MAPK-mediated angiogenesis in the CAM assay. The combination of specific binding of hormone by this integrin and the functional consequences of *in vivo* interference with formation of the $\alpha_V\beta_3$-$T_4$ complex support a role for the integrin as a cell surface thyroid hormone receptor.

## Materials and Methods

### *Reagents*

$T_4$ (≥98% pure by HPLC), $T_3$, tetrac, propylthiouracil, RGD-containing peptides, and RGE-containing peptides were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Monoclonal antibodies to $\alpha_V\beta_3$ (SC7312) and α-tubulin (E9) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Normal mouse IgG and horseradish peroxidase-conjugated goat antirabbit Ig were purchased from DakoCytomation (Carpinteria, CA). Monoclonal antibodies to $\alpha_V\beta_3$ (LM609) and $\alpha_V\beta_5$ (P1F6) as well as purified $\alpha_V\beta_3$ were purchased from Chemicon International (Temecula, CA). L-[$^{125}$I]$T_4$ (specific activity, 1250 μCi/μg) was obtained from PerkinElmer (Boston, MA). $\alpha_V$, $\beta_3$, and scrambled negative control small interfering RNA (siRNAs) were all purchased from Ambion, Inc. (Austin, TX).

### *Cell culture*

The African green monkey fibroblast cell line, CV-1 (American Type Culture Collection, Manassas, VA), which lacks the nuclear receptor for thyroid hormone, was plated at 5000 cells/cm$^2$, maintained in DMEM, supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. All culture reagents were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). Cultures were maintained in a 37 C humidified chamber with 5% $CO_2$. The medium was changed every 3 d, and the cell lines were passaged at 80% confluence. For experimental treatment, cells were plated in 10-cm cell culture dishes (Corning, Inc., Corning, NY) and allowed to grow for 24 h in 10% FBS-containing medium. The cells were then rinsed twice with PBS and fed with serum-free DMEM supplemented with penicillin, streptomycin, and HEPES. After 48-h incubation in serum-free medium, the cells were treated with a vehicle control [final concentration of 0.04 N KOH with 0.4% polyethylene glycol (vol/vol)] or $T_4$ (diluted to its final concentration from a $10^{-3}$ M stock, using the vehicle as a diluent) for 30 min. Media were then collected, and free $T_4$ levels were determined by enzyme immunoassays. All experimental cultures were treated with 1 mM propylthiouracil to prevent the 5′-monodeiodination of $T_4$ into $T_3$. Cultures incubated with $10^{-7}$ M total $T_4$ have $10^{-9}$ to $10^{-10}$ M free $T_4$, consistent with normal physiological levels. After treatment, the cells were harvested, and nuclear proteins were prepared as previously described (6).

### *Transient transfections with siRNA*

CV-1 cells were plated in 10-cm dishes (150,000 cells/dish) and incubated for 24 h in DMEM supplemented with 10% FBS. The cells were rinsed in Opti-MEM (Ambion, Inc.) and transfected with siRNA (100 nM final concentration) to $\alpha_V$, $\beta_3$, or $\alpha_V$ and $\beta_3$ together using siPORT (Ambion, Inc.) according to the manufacturer's directions. Additional sets of CV-1 cells were transfected with a scrambled siRNA to serve as a negative control. Four hours after transfection, 7 ml 10% FBS-containing medium was added to the dishes, and the cultures were allowed to incubate overnight. The cells were then rinsed with PBS and placed in serum-free DMEM for 48 h before treatment with $T_4$.

### *RNA isolation and RT-PCR*

Total RNA was extracted from cell cultures 72 h after transfection using the RNeasy kit from Qiagen (Valencia, CA) according to the manufacturer's instructions. Two hundred nanograms of total RNA were reverse transcribed using the Access RT-PCR system (Promega Corp., Madison, WI) according to the manufacturer's directions. Primers were based on published species-specific sequences: $\alpha_V$ (accession no. NM_002210): forward, 5′-TGGGATTGTGGAAGGAG; reverse, 5′-AAATCCCTGTCCATCAGCAT (319-bp product); $\beta_3$ (NM_000212): forward, 5′-GTGTGAGTGCTCAGAGGAG; reverse, 5′-CTGACTCAATCTCGTCACGG (515-bp product); and glyceraldehyde-3-phosphate dehydrogenase (AF261085): forward, 5′-GTCAGTGGTGGACCTGACCT; reverse, 5′-TGAGCTTGACAAAGTGGTCG (212-bp product). RT-PCR was performed in the Flexigene thermal cycler (TECHNE, Burlington, NJ). After a 2-min incubation at 95 C, 25 cycles of the following steps were performed: denaturation at 94 C for 1 min, annealing at 57 C for 1 min, and extension for 1 min at 68 C. The PCR products were visualized on a 1.8% (wt/vol) agarose gel stained with ethidium bromide.

### *Western blotting*

Nuclear proteins were harvested as previously described (6, 19, 31). Aliquots of nuclear proteins (10 μg/lane) were mixed with Laemmli sample buffer and separated by SDS-PAGE (10% resolving gel), then transferred to nitrocellulose membranes. After blocking with 5% nonfat milk in Tris-buffered saline containing 1% Tween 20 (TBST) for 30 min, the membranes were incubated with a 1:1000 dilution of a monoclonal antibody (mAb) to phosphorylated p44/42 MAPK (Cell Signaling Technology, Beverley, MA) in TBST with 5% milk overnight at 4 C. After three 10-min washes in TBST, the membranes were incubated with horseradish peroxidase-conjugated goat antirabbit Ig (1:1000 dilution; DakoCytomation, Carpinteria, CA) in TBST with 5% milk for 1 h at room temperature. The membranes were washed three times for 5 min each time in TBST, and immunoreactive proteins were detected by chemiluminescence (ECL, Amersham Biosciences, Arlington Heights, IL). Band intensity was determined using the VersaDoc 5000 Imaging system (Bio-Rad Laboratories, Hercules, CA).

### *Radioligand binding assay*

The assay was performed following the basic-native gel protocol from the Protein Purification Facility (www.ls.huji.ac.il/~purification/Protocols/PAGE_Basic.html). All test compounds were diluted to their final concentration in 0.04 N KOH with 0.4% polyethylene glycol to ensure that the effect was independent of the solvent used. Two micrograms of purified $\alpha_V\beta_3$ (stock concentration, 0.3–0.5 $\mu$g/$\mu$l) were mixed with the indicated concentrations of test compounds and allowed to incubate for 30 min at room temperature. [$^{125}$I]$T_4$ (2 $\mu$Ci) was then added, and the mixture was allowed to incubate an additional 30 min at room temperature. The samples were mixed with sample buffer [50% glycerol, 0.1 M Tris-HCl (pH 6.8), and bromophenol blue] and run out on a 5% basic-native gel for 24 h at 45 mA in the cold. The apparatus was disassembled, and the gels were placed on filter paper, wrapped in plastic wrap, and exposed to film. Band intensity was determined with the VersaDoc 5000 Imaging system.

The dissociation constant ($K_d$) and $EC_{50}$ were determined using the PRISM software bundle (GraphPad, San Diego, CA). $K_d$ was determined by nonlinear regression, using the programmed homologous competitive binding curve with one class of binding sites equation. Nonspecific binding was held constant at 15. The constant Hot nM was set at 0.13, as determined by the equation Hot nM = hot cpm/(specific activity × incubation volume × 1000). $EC_{50}$ was determined using nonlinear regression with the programmed equation for sigmoidal dose response.

### *Chick CAM assay*

Ten-day-old chick embryos were purchased from SPAFAS (Preston, CT) and were incubated at 37 C with 55% relative humidity. Chick CAM assays were performed as previously described (30, 32, 33). Briefly, a hypodermic needle was used to make a small hole in the blunt end of the egg, and a second hole was made on the broad side of the egg, directly over an avascular portion of the embryonic membrane. Mild suction was applied to the first hole to displace the air sac and drop the CAM away from the shell. Using a Dremel model craft drill (Dremel, Racine, WI), an approximately 1.0-cm$^2$ window was cut in the shell over the false air sac, allowing access to the CAM. Sterile disks of no. 1 filter paper (Whatman, Clifton, NJ) were pretreated with 3 mg/ml cortisone acetate and 1 mM propylthiouracil and air dried under sterile conditions. Thyroid hormone, control solvents, and the mAb LM609 were applied to the disks and subsequently dried. The disks were then suspended in PBS and placed on growing CAMs. After incubation for 3 d, the CAM beneath the filter disk was resected and rinsed with PBS. Each membrane was placed in a 35-mm petri dish and examined under an SV6 stereomicroscope at ×50 magnification. Digital images were captured and analyzed with Image-Pro software (Media Cybernetics, Silver Spring, MD). The number of vessel branch points contained in a circular region equal to the filter disk was counted. One image from each of eight to 10 CAM preparations for each treatment condition was counted, and in addition, each experiment was performed three times.

## Results

### *$T_4$ is a ligand of $\alpha_V\beta_3$ integrin*

To determine whether $T_4$ is a ligand of the $\alpha_V\beta_3$ integrin, 2 $\mu$g commercially available purified protein were incubated with [$^{125}$I]$T_4$, and the mixture was run out on a nondenaturing polyacrylamide gel. $\alpha_V\beta_3$ Bound radiolabeled $T_4$, and this interaction was diminished by unlabeled $T_4$, which was added to $\alpha_V\beta_3$ before the [$^{125}$I]$T_4$ incubation, in a concentration-dependent manner (Fig. 1). The addition of unlabeled $T_4$ reduced binding of integrin to the radiolabeled ligand by 13% at a total $T_4$ concentration of $10^{-7}$ M ($3 \times 10^{-10}$ M free $T_4$) and by 58% at a total concentration of $10^{-6}$ M ($1.6 \times 10^{-9}$ M free), and inhibition of binding was maximal (85%) with $10^{-5}$ M unlabeled $T_4$ ($2.1 \times 10^{-8}$ M free). Using nonlinear regression, the interaction of $\alpha_V\beta_3$ with free $T_4$ was determined to have a $K_d$ of 333 pM and an $EC_{50}$ of 371 pM. Unlabeled $T_3$ was less effective in displacing [$^{125}$I]$T_4$ binding

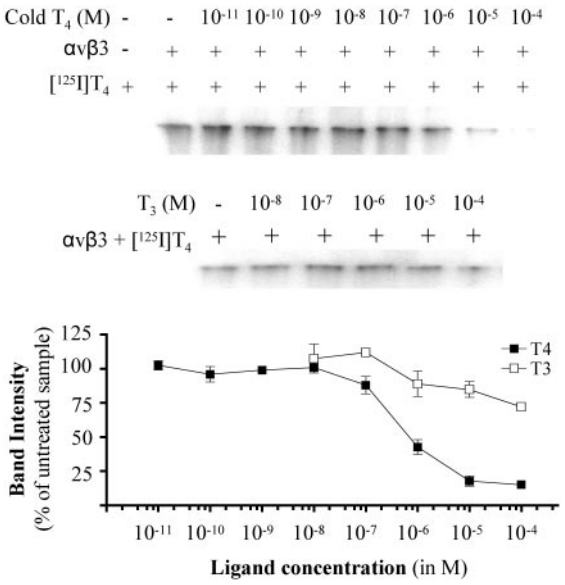



FIG. 1. Unlabeled $T_4$ and $T_3$ displace [$^{125}$I]$T_4$ from purified integrin. Unlabeled $T_4$ ($10^{-11}$–$10^{-4}$ M) or $T_3$ ($10^{-8}$–$10^{-4}$ M) were added to purified $\alpha_V\beta_3$ integrin (2 $\mu$g/sample) before the addition of [$^{125}$I]$T_4$. [$^{125}$I]$T_4$ binding to purified $\alpha_V\beta_3$ was unaffected by unlabeled $T_4$ in the range of $10^{-11}$–$10^{-7}$ M, but was displaced in a concentration-dependent manner by unlabeled $T_4$ at concentrations of $10^{-6}$ M or more. $T_3$ was less effective at displacing $T_4$ binding to $\alpha_V\beta_3$. Graphic presentation of the $T_4$ and $T_3$ data shows the mean ± SD of three independent experiments.

to $\alpha_V\beta_3$, reducing the signal by 28% at $10^{-4}$ M total $T_3$. Similar results were observed when binding assays were performed with addition of the radiolabeled ligand to the integrin for 30 min before addition of unlabeled $T_4$ (data not shown).

### *$T_4$ binding to $\alpha_V\beta_3$ is blocked by tetrac, RGD peptide, and integrin antibody*

We have shown previously that $T_4$-stimulated signaling pathways activated at the cell surface can be inhibited by the iodothyronine analog tetrac, which is known to prevent binding of $T_4$ to the plasma membrane (23). In our radioligand binding assay, although $10^{-8}$ M tetrac had no effect on [$^{125}$I]$T_4$ binding to purified $\alpha_V\beta_3$, the association of $T_4$ and $\alpha_V\beta_3$ was reduced by 38% in the presence of $10^{-7}$ M tetrac and by 90% with $10^{-5}$ M tetrac (Fig. 2). To determine the specificity of the interaction, an RGD peptide, which binds to the extracellular matrix binding site on $\alpha_V\beta_3$, and an RGE peptide, which has a glutamic acid residue instead of an aspartic acid residue and thus does not bind $\alpha_V\beta_3$, were added in an attempt to displace $T_4$ from binding with the integrin. Application of an RGD peptide, but not an RGE peptide, reduced the interaction of [$^{125}$I]$T_4$ with $\alpha_V\beta_3$ in a dose-dependent manner (Fig. 2).

To further characterize the interaction of $T_4$ with $\alpha_V\beta_3$, antibodies to $\alpha_V\beta_3$ or $\alpha_V\beta_5$ were added to purified $\alpha_V\beta_3$ before addition of [$^{125}$I]$T_4$. Addition of 1 $\mu$g/ml $\alpha_V\beta_3$ mAb LM609 reduced complex formation between the integrin and $T_4$ by 52% compared with untreated control samples. Increasing the amount of LM609 to 2, 4, and 8 $\mu$g/ml diminished band intensity by 64%, 63%, and 81%, respectively (Fig. 3). Similar results were observed when a different $\alpha_V\beta_3$ mAb,

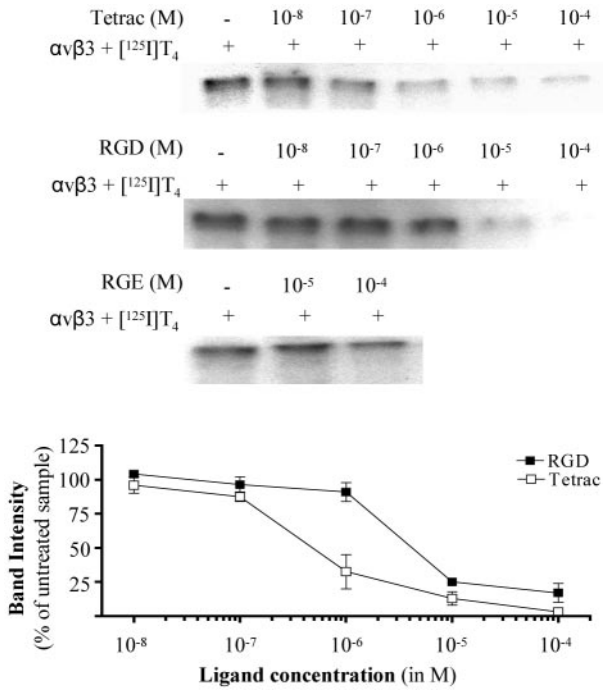



FIG. 2. Tetrac and an RGD-containing peptide, but not an RGE-containing peptide, displace $T_4$ binding to purified $\alpha_V\beta_3$. Preincubation of purified $\alpha_V\beta_3$ with tetrac or an RGD-containing peptide reduced the interaction between the integrin and $[^{125}I]T_4$ in a dose-dependent manner. Application of $10^{-5}$ and $10^{-4}$ M RGE peptide, as controls for the RGD peptide, did not diminish labeled $T_4$ binding to purified $\alpha_V\beta_3$. Graphic presentation of the tetrac and RGD data indicates the mean ± SD of results from three independent experiments.

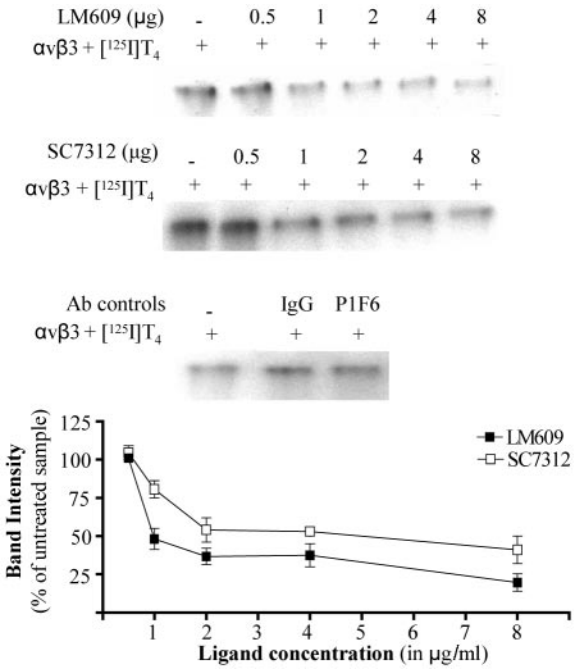



FIG. 3. Integrin antibodies inhibit $T_4$ binding to $\alpha_V\beta_3$. The antibodies LM609 and SC7312 were added to $\alpha_V\beta_3$ at the indicated concentrations (micrograms per milliliter) 30 min before the addition of $[^{125}I]T_4$. Maximal inhibition of $T_4$ binding to the integrin was reached when the concentration of LM609 was 2 $\mu$g/ml and was maintained with antibody concentrations as high as 8 $\mu$g/ml. SC7312 reduced $T_4$ binding to $\alpha_V\beta_3$ in a dose-dependent manner. As a control for antibody specificity, 10 $\mu$g/ml anti-$\alpha_V\beta_5$ mAb (P1F6) and 10 $\mu$g/ml mouse IgG were added to $\alpha_V\beta_3$ before incubation with $T_4$. The graph shows the mean ± SD of data from three independent experiments.

SC7312, was incubated with the integrin. SC7312 reduced the ability of $T_4$ to bind $\alpha_V\beta_3$ by 20% with 1 $\mu$g/ml antibody present, 46% with 2 $\mu$g, 47% with 4 $\mu$g, and 59% with 8 $\mu$g/ml antibody present. Incubation with mAbs to $\alpha_V$ and $\beta_3$ separately did not affect $[^{125}I]T_4$ binding to $\alpha_V\beta_3$ (data not shown), suggesting that the association requires the binding pocket generated from the heterodimeric complex of $\alpha_V\beta_3$ and not necessarily a specific region on either monomer. To verify that the reduction in band intensity was due to specific recognition of $\alpha_V\beta_3$ by antibodies, purified $\alpha_V\beta_3$ was incubated with a mAb to $\alpha_V\beta_5$ (P1F6) or mouse IgG before addition of $[^{125}I]T_4$, neither of which influenced complex formation between the integrin and radioligand (Fig. 3).

### *$T_4$-stimulated MAPK activation is blocked by inhibitors of hormone binding and of integrin $\alpha_V\beta_3$*

Nuclear translocation of phosphorylated MAPK (pERK1/2) was studied in CV-1 cells treated with physiological levels of $T_4$ ($10^{-7}$ M total hormone concentration; $10^{-10}$ M free hormone) for 30 min. Consistent with results we have previously reported (6, 22), $T_4$ induced nuclear accumulation of phosphorylated MAPK in CV-1 cells within 30 min (Fig. 4). Preincubation of CV-1 cells with the indicated concentrations of $\alpha_V\beta_3$ antagonists for 16 h reduced the ability of $T_4$ to induce MAPK activation and translocation. Application of an RGD peptide at $10^{-8}$ and $10^{-7}$ M had a minimal effect on MAPK activation. However, $10^{-6}$ M RGD peptide inhibited MAPK phosphorylation by 62% compared with control cultures, and activation was reduced maximally when $10^{-5}$ M RGD (85% reduction) and $10^{-4}$ M RGD (87% reduction) were present in the culture medium. Addition of the nonspecific RGE peptide to the culture medium had no effect on MAPK phosphorylation and nuclear translocation after $T_4$ treatment in CV-1 cells.

Tetrac, which prevents the binding of $T_4$ to the plasma membrane, is an effective inhibitor of $T_4$-induced MAPK activation (6, 22). When present at a concentration of $10^{-6}$ M with $T_4$, tetrac reduced MAPK phosphorylation and translocation by 86% compared with cultures treated with $T_4$ alone (Fig. 4). The inhibition increased to 97% when $10^{-4}$ M tetrac was added to the culture medium for 16 h before the application of $T_4$. Addition of $\alpha_V\beta_3$ mAb LM609 to the culture medium 16 h before stimulation with $T_4$ also reduced $T_4$-induced MAPK activation. LM609 at 0.01 and 0.001 $\mu$g/ml culture medium did not affect MAPK activation after $T_4$ treatment. Increasing the concentration of antibody in the culture medium to 0.1, 1, and 10 $\mu$g/ml reduced levels of phosphorylated MAPK found in the nuclear fractions of the cells by 29%, 80%, and 88%, respectively, compared with cells treated with $T_4$ alone.

CV-1 cells were transiently transfected with siRNA to $\alpha_V$, $\beta_3$, or both $\alpha_V$ and $\beta_3$ and allowed to recover for 16 h before being placed in serum-free medium. After $T_4$ treatment for 30 min, the cells were harvested, and either nuclear protein or RNA was extracted. Figure 5A demonstrates the speci-

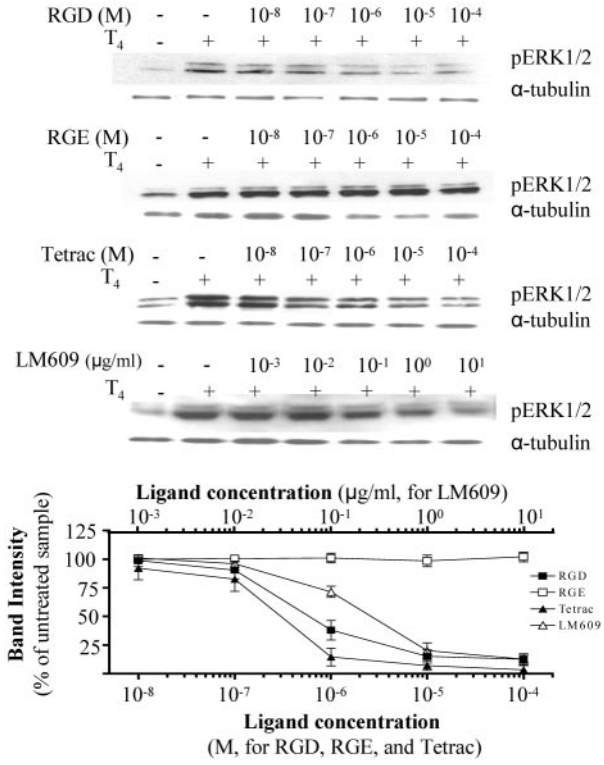



FIG. 4. Effects of RGD and RGE peptides, tetrac, and the mAb LM609 on $T_4$-induced MAPK activation. Nuclear accumulation of pERK1/2 was diminished in samples treated with $10^{-6}$ M or more of RGD peptide, but was not significantly altered in samples treated with up to$10^{-4}$ M RGE. pERK1/2 accumulation in CV-1 cells treated with $10^{-5}$ M tetrac and $T_4$ were similar to levels observed in the untreated control samples. LM609, a mAb to $\alpha_V\beta_3$, decreased accumulation of activated MAPK in the nucleus when it was applied to CV-1 cultures in a concentration of 1 $\mu$g/ml. The graph shows the mean ± SD of data from three separate experiments. Immunoblots with $\alpha$-tubulin antibody are included as gel-loading controls.

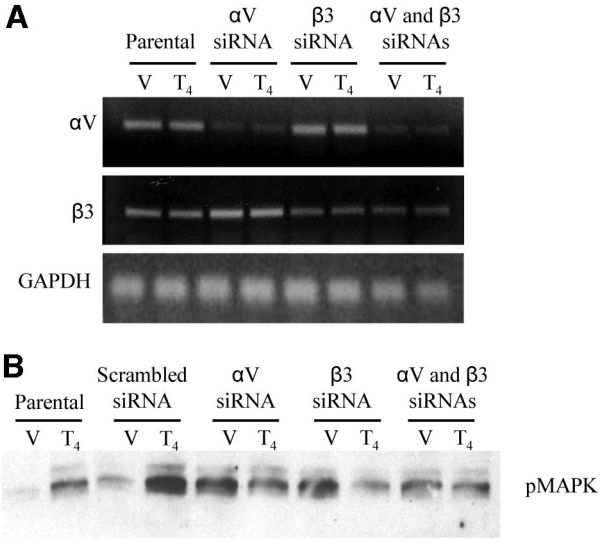



FIG. 5. Effects of siRNA to $\alpha_V$ and $\beta_3$ on $T_4$-induced MAPK activation. CV-1 cells were transfected with siRNA (100 nM final concentration) to $\alpha_V$, $\beta_3$, or $\alpha_V$ and $\beta_3$ together. Two days after transfection, the cells were treated with $10^{-7}$ M $T_4$ or the vehicle control (V) for 30 min. A, RT-PCR was performed with RNA isolated from each transfection group to verify the specificity and functionality of each siRNA. RT-PCR for glyceraldehyde-3-phosphate dehydrogenase was performed on the same samples as a load control. B, Nuclear proteins from each set of transfected cells were isolated, subjected to SDS-PAGE, and probed for pERK1/2 in the presence or absence of treatment with $T_4$. In the parental cells and those treated with scrambled siRNA, nuclear accumulation of pERK1/2 with $T_4$ was evident. Cells treated with siRNA to $\alpha_V$ or $\beta_3$ showed an increase in pERK1/2 in the absence of $T_4$ and a decrease with $T_4$ treatment. Cells containing $\alpha_V$ and $\beta_3$ siRNAs did not respond to $T_4$ treatment.

ficity of each siRNA for the target integrin subunit. CV-1 cells transfected with either the $\alpha_V$ siRNA or both $\alpha_V$ and $\beta_3$ siRNAs showed 87% and 78% decreases, respectively, in $\alpha_V$ subunit RT-PCR products, but there was no difference in $\alpha_V$ mRNA expression when cells were transfected with the siRNA specific for $\beta_3$ or when exposed to the transfection reagent in the absence of exogenous siRNA. Similarly, cells transfected with $\beta_3$ siRNA had reduced levels of $\beta_3$ mRNA by 64% compared with the parental cells, but relatively unchanged levels of $\alpha_V$ siRNA. As expected, the addition of $T_4$ for 30 min did not alter mRNA levels for either $\alpha_V$ or $\beta_3$ regardless of the siRNA transfected into the cells.

Activated MAPK levels were measured by Western blot in CV-1 cells transfected with siRNAs to $\alpha_V$ and $\beta_3$, either individually or in combination (Fig. 5B). CV-1 cells treated with scrambled negative control siRNA had slightly elevated levels of $T_4$-induced activated MAPK compared with the parental cell line. Cells exposed to the transfection reagent alone displayed similar levels and patterns of MAPK phosphorylation as nontransfected CV-1 cells (data not shown). When either $\alpha_V$ siRNA or $\beta_3$ siRNA, alone or in combination, was transfected into CV-1 cells, the level of phosphorylated MAPK in vehicle-treated cultures was elevated, but the ability of $T_4$ to induce an additional elevation in activated MAPK levels was inhibited.

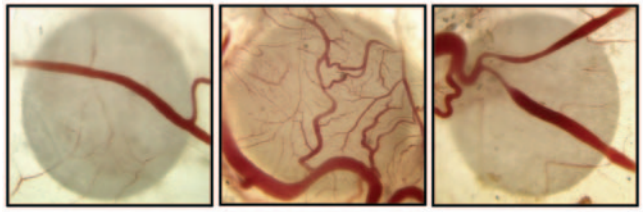
### *Hormone-induced angiogenesis is blocked by antibody to $\alpha_V\beta_3$*

Angiogenesis is stimulated in the CAM assay by application of physiological concentrations of $T_4$ (Fig. 6A; summarized in Fig. 6B). $T_4$ ($10^{-7}$ M) placed on the CAM filter disk induced blood vessel branch formation by 2.3-fold ($P < 0.001$) compared with PBS-treated membranes. We have shown previously that propylthiouracil, which prevents the conversion of $T_4$ to $T_3$, has no effect on angiogenesis caused by $T_4$ in the CAM model (30). The addition of a mAb, LM609 (10 $\mu$g/filter disk), directed against $\alpha_V\beta_3$, inhibited the proangiogenic response to $T_4$.

## Discussion

Studies from several laboratories have demonstrated the ability of thyroid hormone to activate the MAPK signal transduction cascade. These pathways typically are activated by physical and chemical signals at the cell surface. Although the kinetics and analog specificity for binding of thyroid hormone to the plasma membrane have been repeatedly reported (11, 34–36), a cell surface receptor for thyroid hormone has not been previously identified. The present studies describe an initiation site for the induction of MAPK signaling cascades in $T_4$-treated cells.

A




B

| CAM Treatment | # of Branches ± SEM | % Inhibition ± SEM |
|---|---|---|
| PBS | 73 ± 8 | - |
| $T_4$ (0.1 μM) | 170 ± 16 | 0 |
| $T_4$ + LM609 (10 μg) | 109 ± 9 | 64 ± 9 *** |

FIG. 6. Inhibitory effect of $\alpha_V\beta_3$ mAb (LM609) on $T_4$-stimulated angiogenesis in the CAM model. CAMs were exposed to filter disks treated with PBS, $T_4$ ($10^{-7}$ M), or $T_4$ plus 10 $\mu$g/ml LM609 for 3 d. A, Angiogenesis stimulated by $T_4$ was substantially inhibited by addition of the $\alpha_V\beta_3$ mAb LM609. B, Tabulation of the mean ± SEM of new branches formed from existing blood vessels during the experimental period is shown. ***, $P < 0.001$, comparing results of $T_4$/LM609-treated samples with $T_4$-treated samples in three separate experiments, each containing nine images per treatment group. Statistical analysis was performed by one-way ANOVA.

Using purified integrin, we report that a member of the heterodimeric plasma membrane integrin protein family, integrin $\alpha_V\beta_3$, binds $T_4$ and that this interaction is perturbed by $\alpha_V\beta_3$ antagonists. Radioligand binding studies revealed that purified $\alpha_V\beta_3$ binds $T_4$ with high affinity ($EC_{50}$, 371 pM) and appears to bind $T_4$ preferentially over $T_3$. This is consistent with previous reports that show MAPK activation and nuclear translocation (6, 20, 22) as well as hormone-induced angiogenesis by $T_4$ compared with $T_3$. Integrin $\alpha_V\beta_3$ antagonists inhibit binding of $T_4$ to the integrin and, importantly, prevent outside-in activation by $T_4$ of the MAPK signaling cascade. This functional consequence, MAPK activation, of hormone binding to the integrin together with inhibition of the MAPK-dependent proangiogenic action of thyroid hormone by integrin $\alpha_V\beta_3$ antagonists allow us to describe the integrin as a receptor for iodothyronine. It should be noted that 3-iodothyronamine, a thyroid hormone derivative, has recently been shown by Scanlan *et al.* (37) to bind to a trace amine receptor, but the actions of this analog, interestingly, are antithetic to those of $T_4$ and $T_3$.

The traditional ligands of integrins are proteins. That a small molecule, thyroid hormone, is also a ligand of an integrin is a novel finding. We have observed recently that another small molecule, resveratrol, a polyphenol with some estrogenic activity, binds to integrin $\alpha_V\beta_3$ with a functional cellular consequence, apoptosis, different from those that result from the binding of thyroid hormone (Lin, H.-Y., L. Lansing, P. J. Davis, unpublished observations). The exact site on the integrin at which $T_4$ binds is not yet known, but the evidence we present here suggests that the protein-ligand interaction occurs at or near the RGD binding groove (38, 39) of the heterodimeric integrin. It is possible, however, that $\alpha_V\beta_3$ binds $T_4$ elsewhere on the protein, and occupation of the RGD recognition site by tetrac or RGD-containing peptides allosterically blocks the $T_4$-binding site or causes a conformational change within the integrin that renders the $T_4$ site unavailable.

We speculate that the modulation by $T_4$ of the laminin-integrin interaction of astrocytes described by Farwell *et al.* (26) may be a consequence of binding of the hormone to the integrin. This interaction was shown by Farwell *et al.* (26) to be subject to disruption by RGD peptide. The possibility thus exists that at the cell exterior, thyroid hormone may affect the liganding by integrin $\alpha_V\beta_3$ of extracellular matrix proteins in addition to laminin.

Actions of $T_4$ that are nongenomic in mechanism have been well documented in recent years (6, 7, 10, 40). A number of these activities are MAPK mediated. We have shown that initial steps in activation of the MAPK cascade by thyroid hormone, including activation of protein kinase C, are sensitive to guanosine-5′-O-(3-thiotriphosphate) $\gamma$S and pertussis toxin; this indicates that the plasma membrane receptor for thyroid hormone is G protein sensitive (6). It should be noted that certain cellular functions mediated by integrin $\alpha_V\beta_3$ have been shown by others to be G protein modulated (41). For example, site-directed mutagenesis of the RGD binding domain abolishes the ability of the nucleotide receptor P2Y2 to activate $G_o$, whereas the activation of $G_q$ was not affected (41). Wang *et al.* (42) demonstrated that an integrin-associated protein, integrin-associated protein/CD47, induced smooth muscle cell migration via $G_i$-mediated inhibition of MAPK activation.

In addition to linking the binding of $T_4$ by integrin $\alpha_V\beta_3$ to activation of a specific intracellular signal transduction pathway, we also show that liganding of the hormone by the integrin is critical to induction by $T_4$ of MAPK-dependent angiogenesis. In the CAM model, significant vessel growth occurs after 48–72 h of $T_4$ treatment, indicating that the plasma membrane effects of $T_4$ can result in complex transcriptional changes. Thus, what is initiated as a nongenomic action of the hormone, transduction of the cell surface $T_4$ signal, interfaces with genomic effects of the hormone that culminate in neovascularization. We have previously described interfaces of nongenomic and genomic actions of thyroid hormone, *e.g.* MAPK-dependent phosphorylation at $Ser^{142}$ of TR$\beta$1 that is initiated at the cell surface by $T_4$ and that results in shedding by TR of corepressor proteins and recruitment of coactivators (43). We have also shown that $T_4$ stimulates growth of C-6 glial cells by a MAPK-dependent mechanism that is inhibited by RGD peptide (44), and that thyroid hormone causes MAPK-mediated serine phosphorylation of the nuclear estrogen receptor in MCF-7 cells (21) by a process we now know to be inhibitable by an RGD peptide (Lansing, L., and H.-Y. Lin, unpublished observations). These findings in several cell lines all support the participation of the integrin in functional responses of cells to thyroid hormone.

Identification of $\alpha_V\beta_3$ as a membrane receptor for thyroid

hormone permits speculation about clinical significance of the interaction of the integrin and the hormone and the downstream consequence of angiogenesis. For example, $\alpha_V\beta_3$ is overexpressed in many tumors, and this overexpression appears to play a role in tumor invasion and growth (45–47). Relatively constant circulating levels of thyroid hormone may facilitate tumor-associated angiogenesis. In addition to demonstrating the proangiogenic action of $T_4$ in the CAM model here and previously (30), we have recently found that human dermal microvascular endothelial cells also form new blood vessels when exposed to thyroid hormone (Mousa, S. A., F. B. Davis, and P. J. Davis, unpublished observations). Local delivery of $\alpha_V\beta_3$ antagonists or tetrac around tumor cells might inhibit thyroid hormone-stimulated angiogenesis. Although tetrac lacks many of the biological activities of thyroid hormone, it does gain access to the interior of certain cells (48). Anchoring of tetrac or specific RGD antagonists to nonimmunogenic substrates (agarose or polymers) would exclude the possibility that the compounds could cross the plasma membrane, yet retain, as shown in this study, the ability to prevent $T_4$-induced angiogenesis. Thus, agarose-$T_4$, used in previous studies (6, 22, 30), is a prototype for a new family of thyroid hormone analogs that have specific cellular effects, but do not gain access to the cell interior.

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