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The TRβ1 Is Essential in Mediating T3 Action on Akt Pathway in Human Pancreatic Insulinoma Cells

Cecilia Verga Falzacappa,[1,2,3†] Valentina Patriarca,[1,2†] Barbara Bucci,[2] Claudia Mangialardo,[1,2†] Simona Michienzi,[1,2†] Giulia Moriggi,[1,2†] Antonio Stigliano,[1,2†] Ercole Brunetti,[2] Vincenzo Toscano,[1†] and Silvia Misiti[1,2*,†]

[1]*II Faculty of Medicine, Sapienza University of Rome, Rome, Italy*

[2]*Centro Ricerca, Ospedale San Pietro FBF, Rome, Italy*

[3]*DEM, Fondazione per il Diabete, Endocrinologia e Metabolismo, Rome, Italy*

ABSTRACT

Thyroid hormone action, widely recognized on cell proliferation and metabolism, has recently been related to the phosphoinositide 3 kinase (PI3K), an upstream regulator of the Akt kinase and the involvement of the thyroid hormone receptor β1 has been hypothesized. The serine-threonine kinase Akt can regulate various substrates that drive cell mass proliferation and survival. Its action has also been characterized in pancreatic β-cells. We previously demonstrated that Akt activity and its activation in the insulinoma cell line hCM could be considered a specific target of the non-genomic action of T3. In this study we analyzed the molecular pathways involved in the regulation of cell proliferation, survival, size, and protein synthesis by T3 in a stable TRβ1 interfered insulinoma cell line, derived from the hCM, and evidenced a strong regulation of both physiological and molecular events by T3 mediated by the thyroid hormone receptor β1. We showed that the thyroid receptor β1 mediates the T3 regulation of the cdk4·cyc D1·$p21^{CIP1}$·$p27^{KIP1}$ complex formation and activity. In addition TRβ1 is essential for the T3 upregulation of the Akt targets β-catenin, p70S6K, and for the phosphorylation of Bad and mTOR. We demonstrated that the β1 receptor mediates the T3 upregulation of protein synthesis and cell size, together with the cell proliferation and survival, playing a crucial role in the T3 regulation of the PI3K/Akt pathway. J. Cell. Biochem. 106: 835–848, 2009.

KEY WORDS: T3; THYROID HORMONE RECEPTORS; AKT; PANCREATIC β-CELLS

The thyroid hormone T3 has diverse biological functions in growth, differentiation, and metabolism. Its effects are mediated by the thyroid receptors, which have widely been known to act as transcriptional factors on gene transcription regulation [Oetting and Yen, 2007]. Recently, many laboratories have been focused their research on the so-called "non genomic action" of T3, which is often related to some crucial signaling pathways. These non-genomic effects are characterized by the lack of dependence on nuclear TRs, rapid onset of action, occurrence in the face of transcriptional blockade, and utilization of membrane-signaling pathways. Some of these effects involve TRs, particularly TR located outside the nucleus, whereas others utilize other proteins that can bind thyroid hormone, such as the integrin, aVb3 [Bergh et al., 2005]. TR-mediated, non-transcriptional actions by thyroid hormone can take place anywhere in the cell and up to 10% of TRs are located in the cytoplasm [Baumann et al., 2001].

It has been evidenced the action of the thyroid hormone T3 on the PI3K/Akt pathway, showing that T3 can stimulate the phosphatidylinositol 3-kinase (PI3K) at the plasma membrane [Cao et al., 2005; Furuya et al., 2006; Verga Falzacappa et al., 2006]. This activation involves the binding of the thyroid receptor β1 and the subunit p85 of the PI3K; the said interaction has been proved to be both nuclear and extranuclear [Furuya et al., 2006]. The activation of the PI3K leads to events that include the triggering of the Akt kinase and its downstream mTOR. These evidences have suggested that the action of TRβ1 on the PI3K/Akt pathway might drive the increment of cell proliferation and the suppression of apoptosis.

†Chair of Endocrinology.

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*Correspondence to: Dr. Silvia Misiti, MD, PhD, Centro Ricerca, Ospedale San Pietro FBF, Via Cassia, 600, 00189 Rome, Italy. E-mail: silvia.misiti@uniroma1.it

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The PI3K/Akt pathway has recently been investigated in pancreatic β-cells and, as reviewed in Elghazi et al. [2006], it has been identified as a crucial regulator of cell proliferation, survival, and size in this cellular system. In particular Akt appears to target various substrates that control different biological signaling cascades including insulin-mediated glucose transport, protein synthesis, cell proliferation, growth, differentiation, and survival [Dickson and Rhodes, 2004; Woodgett, 2005]. Moreover the overexpression of constitutively active Akt in β-cells in transgenic mice resulted in augmented β-cell mass by increase in β-cell proliferation and cell size, together with the resistance to streptozotocin (STZ)-induced diabetes [Bernal Mizrachi et al., 2001; Tuttle et al., 2001]. At the present moment the molecules and mechanisms involved in the regulation of β-cell mass and function by Akt in β-cells are still ill defined, however some specific targets have been identified. Akt can be a convergent point in the regulation of cell proliferation, apoptosis, and size in pancreatic β-cells.

Cell cycle related molecules directly regulated by Akt have been identified in several cellular systems, and even in β-cells some targets have been identified. One of the mechanisms used for cell cycle regulation by Akt is the phosphorylation and inhibition of glycogen synthase kinase 3β (GSK3β). GSK3β inhibition abolishes phosphorylation-dependent proteolytic degradation of short-lived proteins, such as cyclin D1, c-Myc, and β-catenin. Moreover, levels and cellular location of cell cycle inhibitors, such as p21CIP and p27Kip1, are regulated by Akt via phosphorylation and the consequent cytoplasmic translocation GSK3β mediated [Chang et al., 2003].

Moreover, the Akt/PKB signaling is one of the critical pathways regulating cell survival, and its importance in β-cells has been suggested by increased apoptosis observed in *Irs2*−/− mice. Akt/PKB affects survival by directly regulating members of the Bcl-2 family. Phosphorylation of BAD inhibits the pro-apoptotic activity by releasing it from the Bcl-2/Bcl-X complex and binding to 14-3-3 proteins [Johnson et al., 2003].

Akt can also regulate cell size and protein synthesis, as reviewed in Elghazi et al. [2006]. The regulation of cell size by Akt/PKB is mediated by activation of the mammalian target of rapamycin (mTOR). The mTOR targets ribosomal S6 kinase (S6K) and eukaryote initiation factor 4E binding protein 1 (4EBP1), key regulators of protein translation, and cell size. All these events define the regulation of proliferation, mass, and apoptosis of pancreatic β-cell in an Akt-dependent manner.

We have previously showed [Verga Falzacappa et al., 2006] that the thyroid hormone T3 is able to induce cell proliferation and to trigger the PI3K survival pathway in the cells undergoing apoptosis in the insulinoma cell line hCM. We also evidenced that the thyroid receptor β1 is crucial in mediating the Akt activation by T3 in the same cell model, being able to bind the p85 subunit of PI3K at the cytoplasm and to enhance the kinase activity. In addition our data showed that the interaction between the PI3K and the TRβ1 is crucial for the activation of both the PI3K and the Akt in the hCM cells.

In this study we found TRβ1 to be essential for the T3 action on the hCM cell proliferation, survival, and size. The knocking down of TRβ1 in our system completely abolished the regulation of some central Akt targets in the regulation of proliferation, apoptosis, and size of the human CM cell line.

MATERIALS AND METHODS

CHEMICALS

3,5,3′-Triiodothyronine (T3), LY-294,002 hydrochloride, STZ, and puromycin were obtained from Sigma–Aldrich (St. Louis, MO).

ESTABLISHMENTS OF STABLE TRβ1 SILENCED CLONES

siRNA SmartPool THRB sequences were purchased from Dharmacon (Lafayette, CO). The DNA ds oligonucleotides were specifically designed to be inserted into the puro si-strike vector (Promega, Madison, WI), according manufacturer instruction. A control non-targeting construct designed to silence firefly luciferase mRNA (U47296) was inserted in the si-strike vector.

hCM cells were seeded in six multi-wells and after 24 h were lipofected (Lipofectamine reagent, Invitrogen, Carlsbad, CA) with 500 ng of DNA of the si-strike iTHRB1, si-strike iTHRB2, si-strike iTHRB3, si-strike control, and a mix of the si-strike iTHRB constructs, following the manufacturer recommendations. After 3 h the transfection medium was replaced with fresh complete medium. Cells were selected for si-strike puro transfection in the presence of puromycin (Sigma, 1 μg/ml) added every day for the first 7 days. Transfected cells were continuously maintained in culture with puromycin (0.5 μg/ml).

TRα1 SILENCING

To ascertain that the thyroid hormone receptor α1 was not implied in the effects obtained, a transient silencing of this isoform was performed too. Cells were plated onto six multi-wells and grown in complete medium, after 24 h cells were transfected with ON-TARGETplus SMARTPool, human THRA (Dharmacon). Transfection was performed by incubating cells with 200 pmol of siRNAs in 2 ml of serum free transfection medium using Lipofectamine reagent (Invitrogen). After 3 h of incubation, normal medium supplemented with serum replaced transfection medium and cells were grown for 20 h before starting T3 treatment. Subsequently cells were lysed for Western Blot analysis and samples were analyzed as described.

CELL CULTURE

Human insulinoma cell line CM was characterized from Dr. M.G. Cavallo [Cavallo et al., 1996; Baroni et al., 1999]. Cells were cultured in RPMI 1640 (ICN) containing 5% FBS; all cell culture media were supplemented with L-glutamine (2 mM) and penicillin (100 μg/ml)–Streptomycin (50 μg/ml). The puromycin antibiotic for the selection of resistant cells was added to the medium every 2 days at the final concentration of 0.5 μg/ml.

The hormone treatment was performed by adding T3 or to vehicle alone 12 h after plating cells. Every 24 h, fresh aliquots of T3 (10^{-3} M) were added to culture medium in all the experiments. In the apoptosis studies STZ (15 mM) was added only once at the last 2 h of T3 treatment.

CELL GROWTH ANALYSES

Cell growth was analyzed by determining Trypan Blue negative cell number in a Thomas's hematocytometer. LY-294,002 hydrochloride (1 μM) was added together with the hormone. At 24, 48, and 72 h of continuous exposure, viable cells were harvested and counted. Cell number was determined, and data presented as means ± SD.

FACS ANALYSES

The cell cycle was evaluated using PI staining and FCM analysis. Cells were harvested, fixed in 70% ethanol, and after removing alcoholic fixative, stained with a solution containing 50 μg/ml PI (ICN Biomedicals, Germany) and 75 KU/ml RNase (Sigma–Aldrich) in PBS 1× for 30 min at room temperature, in dark.

Samples were then measured by using a FACScan cytofluorimeter (Becton Dickinson, Sunnyvale). Twenty thousand events per sample were acquired. The percentages of the cell cycle distribution were estimated on linear PI histograms by using the MODFIT software.

Scattering light was used to denote the cell size.

COIMMUNOPRECIPITATION

Cells were lysed in a low stringency lysis buffer for 10 min on ice, then samples were sonicated and centrifuged at 12,000*g* for 20 min; the total cellular protein content was measured using Bradford method (Bio-Rad, Richmond, CA). Five hundred micrograms of cell lysate were incubated for 1 h with 30 μl A-protein (Roche Diagnostics, Basel, CH) for preclearing.

After preclearing, the extracts were incubated overnight at 4°C with rabbit anti-cdk4 (Sta. Cruz, 2 μg) and 30 μl freshly prepared A-protein. The immunoprecipitates were then electrophoresed onto a 10% SDS–polyacrylamide gel, and Western Blot analyses were performed with the mouse-anti p21, -anti p27, -anti cyclin D1, and rabbit-anti cdk4 antibodies (Sta. Cruz).

WESTERN BLOT ANALYSES

Cells were lysed and the total cellular protein content was measured using the Bradford method (Bio-Rad). Forty micrograms of total extracts per sample were loaded onto an 8-, 10-, 12% SDS–polyacrylamide gel, electrophoresed, and then blotted onto Nitrocellulose membranes (Bio-Rad). Membranes were incubated 16 h at 4°C with: TRβ1 (Sta. Cruz Biotechnology, Inc., San Diego, CA. 1:500), Akt (Sta. Cruz, 1:400), phospho Akt 1/2/3-Ser 473 (Sta. Cruz, 1:500), TRα1 (Sta.Cruz, 1:500), pRb (BD Pharmingen, San Jose, CA. 1:500), ppRb (BD Pharmingen, 1:500), pBad (Upstate, Millipore, Billerica, MA. 1:500), β-catenin (Sta. Cruz, 1:500), p-mTOR (Cell Signaling Technology Danvers, MA. 1:1,000), mTOR (CST, 1:500), p70S6k (Sta. Cruz, 1:500), and 1 h at RT with β-Actin (Sigma, 1:1,000), or vinculin (Sigma, 1:10,000). After three washes the membranes were incubated with the secondary HRP antibodies (anti-mouse, anti-rabbit, Sigma) 1:4,000 for 45′ at RT. Immunoreactivity was visualized by the ECL immunodetection system (Amersham Corp, Arlington Heights, IL) following manufacturer's instruction. The relative band intensity was evaluated by densitometric analysis (Total lab, Nonlinear dynamics, New Castle, UK) and normalized to β-actin.

IMMUNOFLUORESCENCE ANALYSES

Cells were cultured in multi-chamber slides (BD Falcon Franklin Lakes, NJ) and treated as previously described. Slides were stained with primary antibodies (mouse anti-p27, mouse anti-Bad Sta. Cruz, 1:100) for 45 min at RT in a humid chamber. After three washes in PBS 1×, slides were incubated with secondary antibodies (TRITC conjugated rabbit anti-mouse IgG, DAKO, Denmark, 1:40) for 45 min at room temperature in dark. Negative controls including omission of the primary antibody were also performed. Immunofluorescence analyses of cell slides were carried out using an inverted fluorescence microscope (Leica, Germany); images were acquired by a Canon digital camera and processed by ImageJ Software.

TUNEL ASSAY

TUNEL assay was performed by using the In situ Cell Death detection kit (Roche).

Cells were plated at a 70% confluence onto multi-chamber slides (BD Falcon) and then exposed to STZ and to the hormone. After the removal of the culture medium, the slides were washed in PBS 1× (ICN) and fixed in paraphormaldeide 4% in PBS 1× for 30 min at RT. After washes slides were incubated in Triton 0.1% in sodium citrate 0.1% for 2 min on ice and then washed. The slides were incubated with the TUNEL mixture according to manufacturer's instructions, for 1 h, at 37°C in dark; then slides were washed and counter-stained with Hoechst (1 μg/ml). TUNEL positivity was visualized with a Leica (Germany) fluorescence microscope and the images were taken by a Canon digital camera.

MTT ASSAY

Cell viability in the apoptosis studies was assessed by MTT assay (Promega). Cells were plated in 96 multi-wells and treated as previously described. A solution of a tetrazolium salt was added to the culture medium and, after 3 h, the metabolic formazan product was solubilized in an organic solution. After 1 h of solubilization, the absorbance at 570 and 630 nm were recorded by using a 96-well plate reader.

PROTEIN CONTENT QUANTIFICATION

Cells were harvested, counted, and then lysed. The total cellular protein content was measured using the Bradford method (Bio-Rad) and then normalized for the total cell number. The ratio of mg protein per million cells has been calculated.

C-PEPTIDE CONTENT

Cells were harvested, counted, and then lysed. The intracellular c-peptide content was measured using a competitive chemiluminescent immunoassay (Roche) and normalized for the mg of total protein.

STATISTICAL ANALYSIS

The data were presented as means ± SD. A comparison of the individual treatment was conducted by using Student's *t*-test or, if

there were more than two groups, by one-way ANOVA, followed by Dunnett or Tukey post hoc analyses. A P-value <0.05 was considered significant.

RESULTS

ESTABLISHMENT OF STABLE TRβ1 SILENCED HCM CELLS

We have previously demonstrated that transient silencing of TRβ1 abolished the T3 induced activation of Akt in hCM cells [Verga Falzacappa et al., 2007]. We have now analyzed the T3–TRβ1 role in the Akt mediated regulation of cell growth, apoptosis, and size by silencing, or efficiently downregulating TRβ1 expression by stable RNAi. To this aim, hCM cells were transfected with a vector containing either one of three different constructs (iTHRB1, iTHRB2, and iTHRB3), or a mix of them (iTHRBmix), expressing 19 bp double stranded DNA oligonucleotides directed against the coding region of human TRβ1. The effectiveness of interference against TRβ1 expression compared to control cells transfected with control vector (si-strike) was assessed by Western Blot analysis. As shown in Figure 1, the densitometry analyses revealed a drastic reduction of TRβ1 levels, particularly in the iTHRB1 cells (77%) and in the iTHRB2 cells (75%). Next the ability of the specific silencing of TRβ1 to abolish the T3 induced Akt activation (24 and 48 h) was demonstrated in all the obtained mixed cell population by Western Blot analyses (Fig. 1b). A good silencing of TRβ1 was obtained with all the different constructs utilized, so we decided to use the iTHRB1 and iTHRB2 cells to analyze the thyroid hormone receptor β1 role in the regulation of pancreatic β-cell proliferation, survival and size.

THE T3 ACTIVATION OF AKT IN β-CELLS DOES NOT DEPEND ON TRα1

As stated in the Discussion Section [Kenessey and Ojamaa, 2006], it has been demonstrated that even the thyroid hormone receptor alpha 1 is able to interact with the PI3Kp85α and could be a good mediator for T3 activation of this kinase signal cascade. The absence of non-specific interference of the thyroid hormone receptor TRα1 was previously verified in the iTHRB1 clone [Michienzi et al., 2007].

To confirm that the T3 activation of Akt via PI3K pathway in hCM is not due to a compensatory effect of TRα1, we analyzed if the specific silencing of TRα1 by RNAi experiments could alter the T3 ability to promote Akt phosphorylation. The cells were transfected with TRα1 siRNAs (THRA) and exposed or not to T3 treatment; total extracts were then immunoblotted for TRα1, TRβ1, and pAkt Ser 473. As shown in Figure 2, panels a and b, when TRα1 was silenced, as demonstrated by Western Blot analyses, the phosphorylation of Akt induced by T3 was still evident. The TRβ1 expression was not altered by the TRα1 silencing, as shown. The specific silencing of TRα1 and not of TRβ1 was also confirmed by RT-PCR performed with primers specific for each isoform as previously described [Misiti et al., 2005] (data not shown).

In addition the absence of the TRα1-PI3Kp85α interaction was verified by coimmunoprecipitation experiments (data not shown).

These data strongly indicated that the ability of T3 to induce Akt activation in the islet cells utilized is specifically mediated by the thyroid receptor β1 and not by the α1.

CELL PROLIFERATION

TRβ1 mediates T3 action on pancreatic β-cell growth and proliferation. Our previous data demonstrated that T3 induces cell proliferation in human hCM cells [Verga Falzacappa et al., 2006]; here at first we set out to investigate the involvement of PI3K in this T3 effect by its inhibitor LY; as shown in Figure 3a the presence of LY effectively reduced the T3 increment of cell growth in the hCM T3 treated cells. We next asked whether the induction of cell growth was specifically mediated by the thyroid hormone receptor β1 and performed cell growth analyses on the stable TRβ1 hCM cells we generated. As shown in Figure 3b the T3 effect on cell growth was completely abolished in the TRβ1 silenced cells, thus demonstrating the essentiality of this receptor for the T3 induced cell growth of human CM cells. We next sought to establish whether induced cell growth reflected an increment in the percentage of cells in the S phase and if there was a role for TRβ1 even in this case. Given the similar results obtained for the two-hCM iTHRB cells, only the iTHRB1 CM cells have been utilized for the next experiments. FACS analysis was employed to measure the DNA content (revealed by PI assay) after 24, 48, and 72 h of T3 exposure in the si-strike and the iTHRB1 CM cells. As evident (Fig. 3) only the si-strike cell percentage in the S phase was increased by 48 and 72 h T3 treatment (20%, 25%), whether iTHRB1 cells did not, thus suggesting a role for TRβ1 in the T3 effects on cell growth and proliferation in hCM cells.

Specific cell cycle-related Akt targets are differently regulated by T3 in hCM stable interfered for TRβ1 expression. We next intended to deepen the T3–TRβ1 effect on cell cycle and probe the eventual involvement of Akt by studying some cell cycle molecules status. The cdk4–cycD1 complex plays a crucial role in the G_1/S transition, and its activation is regulated by Akt activity. It has been demonstrated that under physiological conditions $p21^{CIP1}$ and $p27^{KIP1}$ can bind the Cdk4/cycD1 complex and both stabilize and induce the complex activity to trigger the transition from early G_1 to the S phase [Sherr and Roberts, 1999]. Considered these observations and the changes observed in the cell cycle distribution, we analyzed the presence and the levels of the cdk4·cycD1·p21·p27 complex formation in our hCM cells. As shown in Figure 4a, the complex formation was clearly detectable in both si-strike and iTHRB1 cells, but only in the si-strike cells a clear increment in the expression of all the complex components was detected after T3 treatment. To assess if the observed modulation concerned also the complex activity, the phosphorylation of the retinoblastoma protein pRB was analyzed by Western Blot (Fig. 4b). Even in this case, the densitometric analysis indicated a T3 induction of the hyperphosphorylated ppRb levels only in the si-strike cells, confirming a role for TRβ1 in the regulation of the cell cycle machine by T3.

The PI3K/Akt pathway has also been implicated in altering the $p27^{KIP1}$ activity; in particular it has been demonstrated [Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002] that Akt can directly phosphorylate p27, cause its retention in the cytoplasm, and thus prevention of p27 cell cycle arrest. As shown in Figure 4c, by

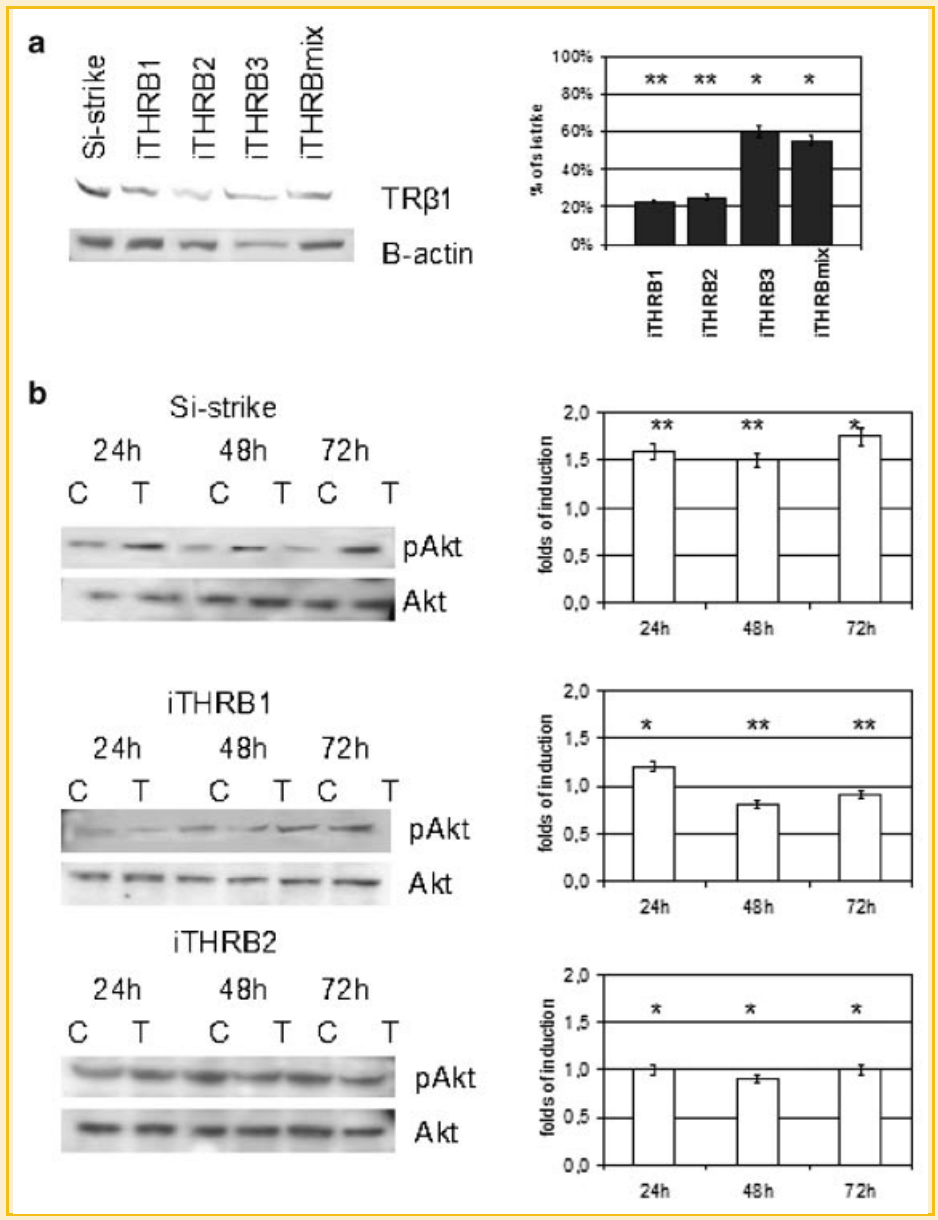


Fig. 1. hCM cells were stably transfected with si-strike vectors containing either a non-targeting construct (si-strike) or a TRβ1 interfering construct (iTHRB1, 2, 3) or a mix of the three different TRβ1 interfering construct (iTHRB mix). Panel a: Western Blot analyses for the detection of TRβ1 were performed as described in Materials and Methods Section and a specific band corresponding to thyroid receptor β1 was detected. The expression of β-actin was analyzed as a control for gel loading. At least three different experiments were performed, and a representative one is shown here. Panel b: Cells were cultured in the presence or not of the thyroid hormone T3 (10^{-7} M) for the indicated times and total extracts were analyzed by Western Blot as described in Materials and Methods Section and a specific band corresponding to phosphorylated Akt (Ser 473) was detected. The expression of unphosphorylated Akt was analyzed as a control for gel loading. At least three different experiments were performed, and a representative one is shown here. Densitometric absorbance values from three separate experiments were averaged (±SD), after they had been normalized to β-actin (panel a) or Akt (panel b) for equal loading. Data relative to each protein are presented on the right of the Western Blot panel in the histogram as percentage of si-strike (panel a) or fold of induction (panel b, y-axis), calculated as treated sample/control in Relative Densitometric Absorbance Unit. The different experimental groups are indicated on the x-axis. A comparison of the individual treatment was conducted by using Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$. A *P*-value <0.05 was considered significant.

immunofluorescence analyses we evidenced that T3 causes p27 retention in the si-strike, but not in the iTHRB1 cells, indicating a role for the thyroid receptor even at this level.

On the whole our data so far strongly suggest that the thyroid hormone receptor β1 plays a key role in mediating the T3 regulation of hCM cell proliferation both at a physiological and at a molecular level, involving some of the specific Akt targets.

CELL APOPTOSIS

hCM stable interfered for TRβ1 expression cannot counteract apoptosis when exposed to T3. The Akt/PKB signaling is one of the critical pathways regulating cell survival and apoptosis; we have previously evidenced the ability of T3 treatment to counteract an apoptotic process ongoing in insulinoma cells hCM [Verga Falzacappa et al., 2006] and demonstrated the involvement of

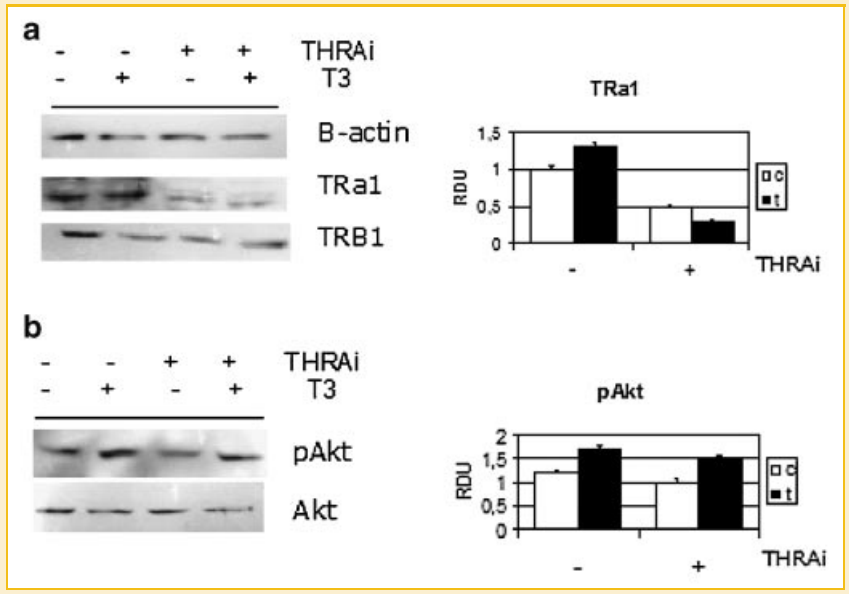


Fig. 2. Panel a: RNA interference experiments to silence TRα1 expression were performed as described in Materials and Methods Section on hCM cells exposed or not to T3 (10^{-7} M) for the indicated times. Western Blot analyses were performed as described in Materials and Methods Section and a specific band corresponding to TRα1 and TRβ1 was detected. The expression of B-actin was analyzed as a control for gel loading. At least three different experiments were performed, and a representative one is shown here. Panel b: Western Blot analyses were performed as described in Materials and Methods Section and a specific band corresponding to the phosphorylated Akt (Ser 473) was detected. The expression of Akt was analyzed as a control for gel loading. At least three different experiments were performed, and a representative one is shown here. Densitometric absorbance values from three separate experiments were averaged (±SD), after they had been normalized to β-actin (panel a) or Akt (panel b) for equal loading. Data relative to each protein are presented on the right of the Western Blot panel in the histogram as Relative Densitometric Units (y-axis). The different experimental groups are indicated on the x-axis. A comparison of the individual treatment was conducted by using Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$. A *P*-value < 0.05 was considered significant.

PI3K in this T3 action. To probe the participation of TRβ1 in this T3 action, we induced apoptosis by STZ (15 mM, 2 h) in si-strike and iTHRB1 hCM cells and concurrently exposed or not the same cells to T3 treatment (10^{-7} M, 24 h). As shown in Figure 5a, TUNEL assay was utilized to determine the effectiveness of STZ administration and revealed that T3 could counteract apoptosis only in the si-strike hCM cells, suggesting that TRβ1 is crucial in mediating the T3 survival effect.

To confirm these data an MTT assay was performed in the same conditions, as shown in Figure 5b. Although cell viability of si-strike and iTHRB1 hCM cells was affected by STZ exposure, in the si-strike cells exposed concurrently to STZ and T3 cell viability reached values as high as the control cells (not STZ treated), indicating a normal cell metabolism; on the contrary, the iTHRB1 cells exposed to both T3 and STZ were characterized by very low values of cell viability, indicating a strong impairment of cell metabolism by STZ. These data confirm the TUNEL observations and unambiguously demonstrate a role for the receptor TRβ1 in mediating the T3 survival effect in hCM cells.

TRβ1 is essential in the regulation of different apoptosis-related Akt targets in hCM cells. As a key regulator of cell survival, Akt has several anti-apoptotic substrates. To delve into the link between TRβ1 and Akt in the anti-apoptotic T3 action, Western Blot analyses have been performed. As shown in Figure 6a the phosphorylation of Bad, directly triggered by Akt to initiate the survival stimulus, resulted upregulated by T3 only in the TRβ1 expressing cells. Also β-catenin, which acts as a direct substrate of GSK3β activity, was upregulated by T3 treatment only in si-strike cells. As shown the T3 treatment was efficacious also in the cells not exposed to STZ, this in accordance with the cell proliferation induction we observed and considering the role that β-catenin exerts even at this level.

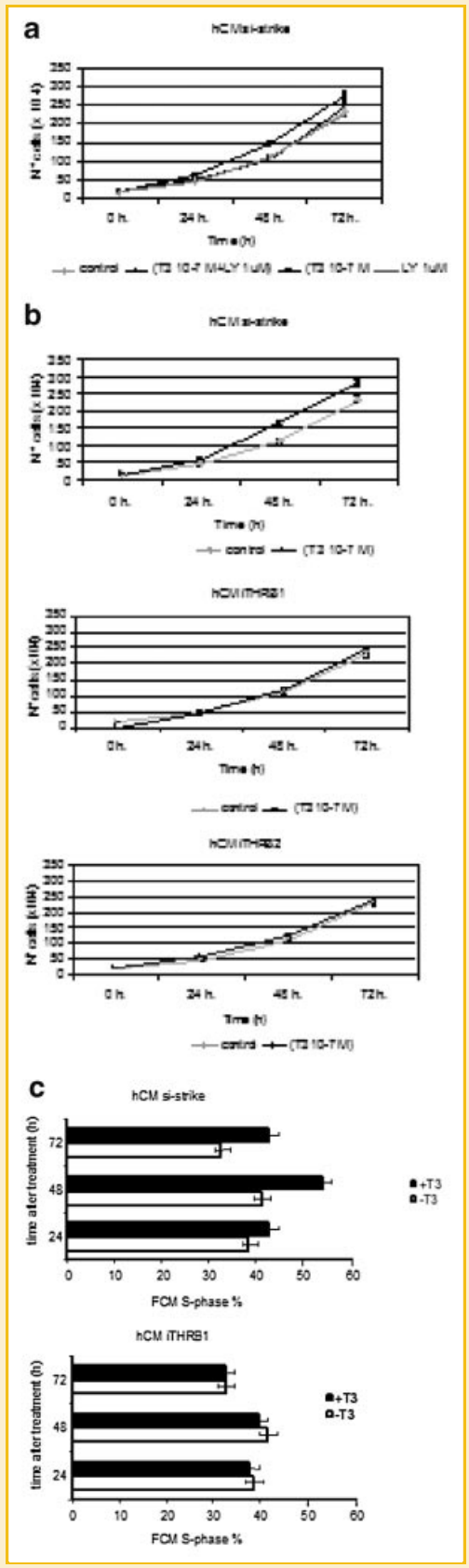
At this point, since Bad action takes place predominantly at a cytoplasmic level, immunofluorescence experiments (Fig. 6b) have been performed to verify that the increase in Bad phosphorylation was accompanied by an increase in the cytoplasmic localization of the protein. As shown, only in the si-strike cells concurrently exposed to STZ and T3, a deeper red signal was detectable in the cytoplasm, indicating that the protein is mainly located in that compartment.

Taken together these data underline the key role of the thyroid receptor β1 in the anti-apoptotic action of T3 on hCM cells, which specifically involves some of the principal Akt targets.

CELL SIZE AND PROTEIN SYNTHESIS

Thyroid receptor β1 mediates T3-induced increase of cell size and protein synthesis. In the β-cells, Akt is also involved in the regulation of cell size. To assess whether T3 could increase cell size in hCM cells and to investigate the role of TRβ1, FACS analyses of cells size has been performed.

As shown in Figure 7a, both the Forward and the Side Scatters distributions of si-strike hCM cells were altered by 5 and 7 days of hormone treatment. In particular the T3 treated cells showed higher values of Side Scatters, which indicate an increase in cell complexity and granulosity, together with an augmented Forward Scatter values, which refer to cell dimensions. The said alterations were not



detected in the iTHRB1 cells, underlying the essentiality of TRβ1 in the effects observed.

Increased cell size often correlates with an increase in general protein synthesis. To ascertain that the increase in cell size was accompanied by an increase in protein synthesis, total cellular extracts have been quantified and normalized to cell number. As shown in Figure 7b, the T3 treatment provoked a strong increase in protein synthesis already after 5 days of treatment only in the si-strike cells and not in the iTHRB1 cells, indicating once again that TRβ1 is crucial in mediating the T3 effects we observed on pancreatic β-cells.

TRβ1 mediates T3 increase of mTOR phosphorylation and p70S6K expression. Akt has several substrates that influence rates of protein synthesis, depending on their phosphorylation state. In particular the regulation of cell size by Akt is mediated by activation of the mammalian target of rapamycin (mTOR). To investigate further the molecular features of the observed phenomena, we analyzed the phosphorylation levels of mTOR in the same experimental conditions. As shown in Figure 8 the p-mTOR levels were clearly augmented only in the si-strike cells after 3,5, and 7 days of T3 treatment.

As a target of mTOR activity, p70S6K plays a role in regulating protein synthesis by Akt, too. Our experiments revealed that the p70S6K expression also increased by 3,5, and 7 days of T3 treatment, only in the si-strike cells.

β-CELL FUNCTION

TRβ1 is essential for T3 induced increase in c-peptide content. The main function of a pancreatic β-cell is the insulin secretion. The validity of the CM cells as a β-cell model has recently been discussed [Jonnakuty and Gragnoli, 2007; Cavallo et al., 2008]; in particular, concerning insulin secretion it has been demonstrated that although

Fig. 3. Panel a: Cell growth analyses: To evaluate the T3–TRB1 action on cell growth si-strike cells (non targeting control) were grown as a monolayer and exposed to thyroid hormone treatment (10^{-7} M) in the presence or not of LY-294002 hydrochloride (1 μM) to ascertain the PI3K involvement in the T3 induced proliferation. The graphic shows the effect of the T3 treatment on the cell growth determined by counting Trypan Blue negative cells. y-axis: cell number, x-axis: hours of T3 treatment. All the data are presented as means ± SD and are the results of three individual experiments at least. Panel b: Cell growth analyses: Si-strike and iTHRB1, 2 hCM cells were grown as a monolayer and exposed to thyroid hormone treatment (10^{-7} M). The graphics show the effect of the T3 treatment on the cell growth determined by counting Trypan Blue negative cells. y-axis: cell number, x-axis: hours of T3 treatment. All the data are presented as means ± SD and are the results of three individual experiments at least. A comparison of the individual treatment was conducted by using one-way ANOVA followed by Tukey post hoc test. Panel c: Cell cycle analysis: To evaluate the cell cycle distribution si-strike and iTHRB1 cells were cultured in the presence or not of T3 treatment (10^{-7} M) and then processed as described in Materials and Methods Section for PI staining. The percentages of cells in the S phase (x-axis) treated for the indicated times (y-axis) has been represented in the histogram. All the data are presented as means ± SD and are the results of three individual experiments at least. A comparison of the individual treatment was conducted by using one-way ANOVA followed by Tukey post hoc test.

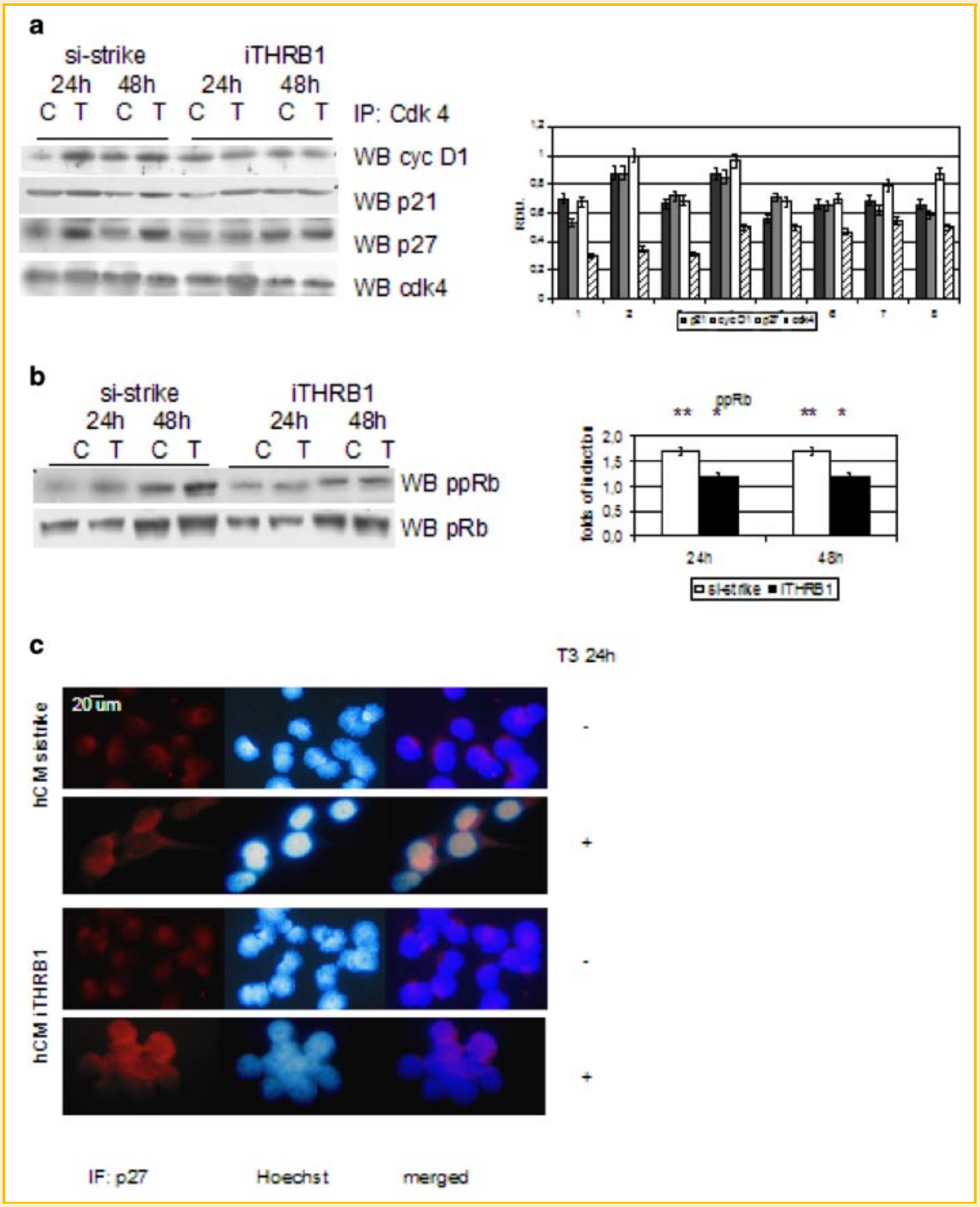


Fig. 4. Panel a: Coimmunoprecipitation experiments. To evaluate the formation of the CycD1–Cdk4–p21–p27 complex and the T3 action on it, si-strike and iTHRB1 cells were cultured in the presence of T3 (10^{-7} M) for the indicated times and immunoprecipitation experiments were performed. Cdk4 was immunoprecipitated and Western Blot analyses for cyc D1, p21, p27, and Cdk4 to analyze the presence of protein–protein interaction were performed as described in Materials and Methods Section on a single SDS gel. A representative one is showed for each factor analyzed. Densitometric absorbance values from three separate experiments were averaged (±SD), and are presented in the histogram as Relative Densitometric Absorbance (y-axis). Panel b: Western Blot analyses: To analyze the T3 effect on the pRb phosphorylation, cells were plated and exposed or not to T3 (10^{-7} M) for the indicated times and Western Blot analyses were performed as described in Materials and Methods Section. As shown a specific band corresponding to hyperphosphorylated Rb (ppRb) was detected. The expression of underphosphorylated Rb (pRb) was analyzed as a control for gel loading. A representative one is showed for each factor analyzed. Densitometric absorbance values from three separate experiments were averaged (±SD), after they had been normalized to pRb for equal loading. Data are presented in the histogram as fold of induction (y-axis), calculated as treated sample/control in Relative Densitometric Absorbance Unit. The different exposure times to T3 are indicated on the x-axis. A comparison of the individual treatment was conducted by using Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$. A *P*-value <0.05 was considered significant. Panel c: Immunofluorescence analysis: To evaluate the compartmentalization of the CDKI $p27^{KIP1}$, si-strike, and iTHRB1 cells were cultured and immunostained for $p27^{KIP1}$ (red fluorescence); nuclei were counterstained with Hoechst. The images were then merged to evidence the different localization of the two signals. The images were acquired and analyzed through fluorescence microscopy as described in Materials and Methods Section. At least ten fields per chamber and three independent cultures were examined. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

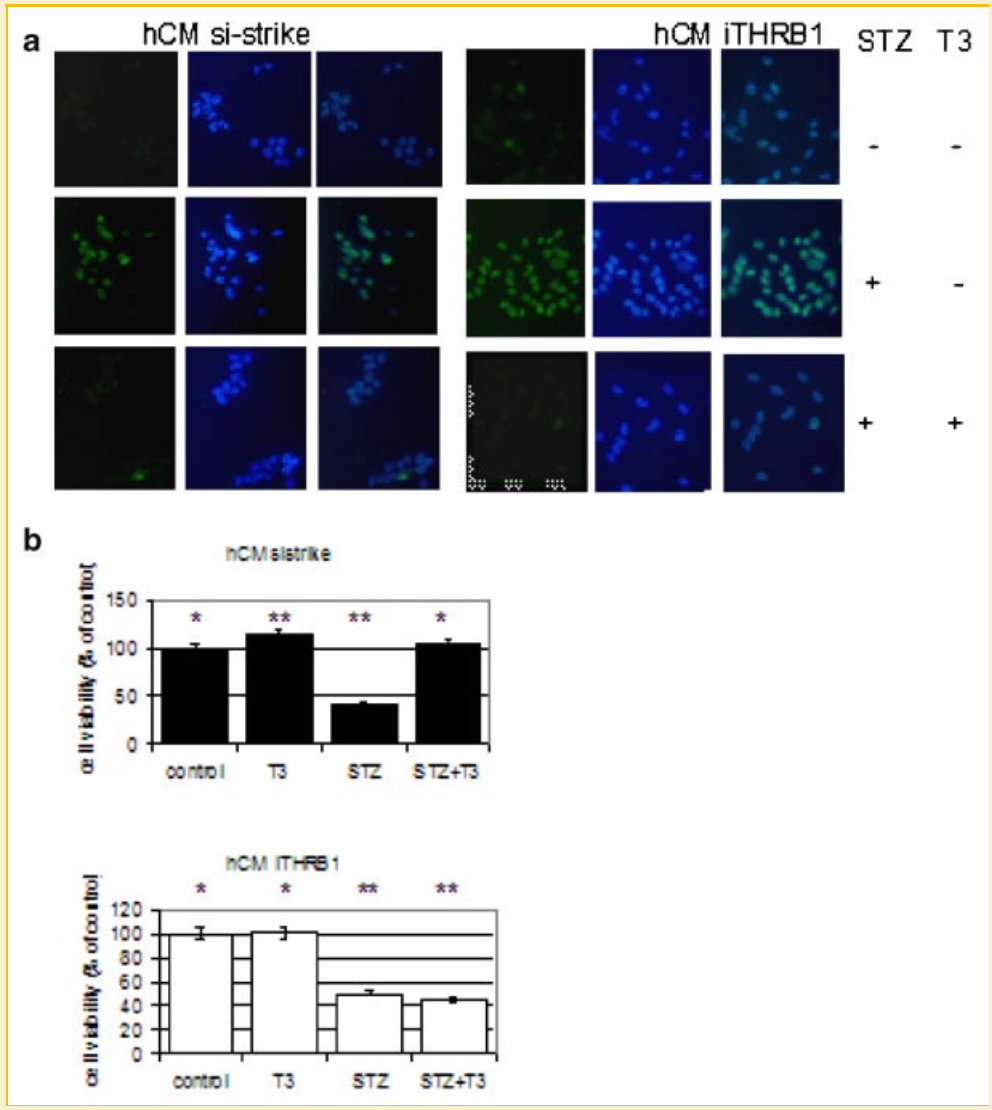


Fig. 5. Panel a: TUNEL assay: To analyze the survival action of T3/TRB1 when an apoptotic cascade is ongoing, si-strike and iTHRB1 hCM cells were cultured in the presence or the absence of T3 10^{-7} M (24 h) and exposed to STZ (15 mM, 2 h). Apoptotic nuclei were detected as TUNEL-positive only in the cells exposed to drug alone (panels on the left). Nuclei were counter-stained with Hoechst (panels on the right) and the images were then merged to show that the same view-field is considered. At least ten fields per chamber and three independent cultures were examined. Panel b: MTT assays: To analyze the metabolic rate of the si-strike and iTHRB1 CM cells cultured in the presence or in the absence of T3 (10^{-7} M), and exposed to STZ (15 mM) (indicated on the x-axis), MTT assays were performed. Data are presented as % of the OD (570 nm) control value (taken as 100%) on the y-axis, as means ± SD, and are the results of at least five independent experiments. A comparison of the individual treatment was conducted by using one-way ANOVA followed by Dunnett post hoc test. $^{*}P < 0.05$; $^{**}P < 0.01$. A P-value < 0.05 was considered significant. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

hCM cells have lost their ability to secrete insulin after a long time culture, they still produce insulin and c-peptide [Baroni et al., 1999], which are retained in the cytoplasm and can be detected in this compartment [Verga Falzacappa et al., 2006]. To assess whether T3 can induce insulin production in hCM cells and to investigate the TRβ1 role. C-peptide content was analyzed in control and T3 treated iTHRB1 and si-strike hCM cells at 24 and 48 h. As shown in Figure 9 the T3 treatment induced a strong increase in the c-peptide content already after 24 h (one, seven folds) and at 48 h (three folds) of treatment in the si-strike cells. The TRβ1 cells showed a little increment in the c-peptide content (1,3 folds at 24 h and 1,15 folds at 48 h) probably due to the small amounts of TRβ1 still present.

DISCUSSION

The present study has provided insights into the molecular mechanisms by which thyroid hormone T3 increases pancreatic β-cell function potentially through the direct activation of the kinase Akt and by the specific action of its receptor TRβ1. Evidence for these conclusions emerges from the generation of four stable hCM derived cell lines, in which the expression of TRβ1 was stably downregulated. Although it has recently been discussed the validity of the hCM cell line as a model for β-cell function, [Jonnakuty and Gragnoli, 2007], a number of studies have consistently demonstrated that, under appropriate experimental

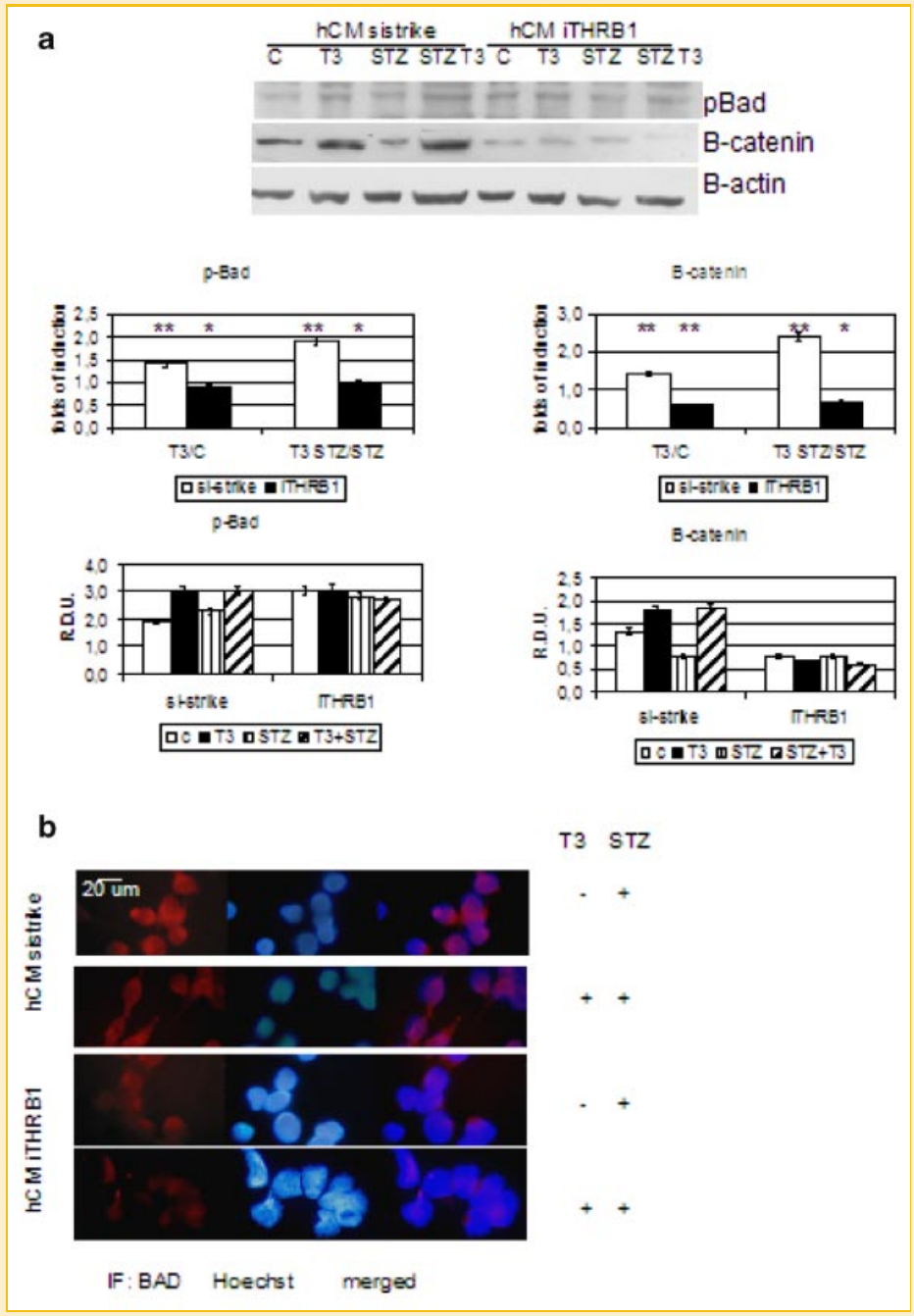


Fig. 6. Panel a: Western Blot analysis: To evaluate the T3 effect on the phosphorylation of Bad and on the β-catenin levels Si-strike and iTHRB1 cells were plated and exposed or not to T3 (10^{-7} M) and concurrently to STZ (15 mM). Western Blot analyses were performed as described in Materials and Methods Section and a specific band corresponding to pBad and β-catenin was detected. The expression of β-actin was analyzed as a control for gel loading. At least three different experiments were performed, and a representative one is shown here. Densitometric absorbance values from three separate experiments were averaged (±SD), after they had been normalized to β-actin for equal loading. Data are presented in the histogram as fold of induction (y-axis), calculated as treated sample/control in Relative Densitometric Absorbance Unit (upper panel) and as Relative Densitometric Units (lower panel). The different experimental groups are indicated on the x-axis. A comparison of the individual treatment was conducted by using Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$. A *P*-value <0.05 was considered significant. Panel b: Immunofluorescence analysis: To analyze the compartmentalization of Bad under T3 treatment, si-strike, and iTHRB1 cells were cultured in the presence or not of T3 (10^{-7} M) and STZ (15 mM) and immunostained for Bad (red fluorescence); nuclei were counterstained with Hoechst. The images were then merged to evidence the different localization of the two signals. The images were acquired and analyzed through fluorescence microscopy as described in Materials and Methods Section. At least ten fields per chamber and three independent cultures were examined. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

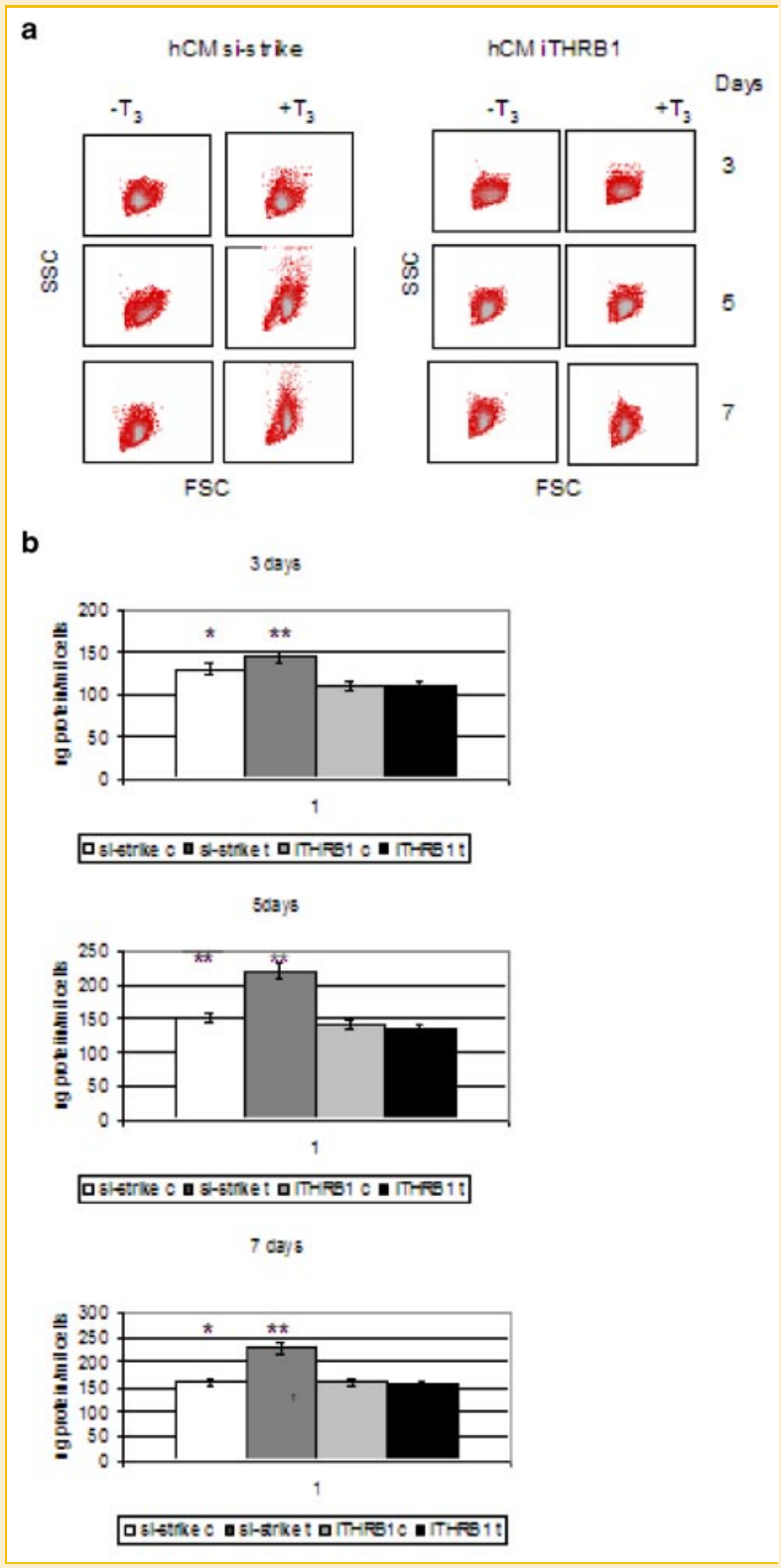


Fig. 7. Panel a: FACS analysis: To evaluate the T3/TRB1 effects on cell size, the FACS Forward and Side Scatters have been evaluated. Si-strike and iTHRB1 cells were plated and exposed or not to T3 (10^{-7} M) for 3, 5, and 7 days. FACS analysis for the determination of cell size has been performed as described in Materials and Methods Section. SSC: Side Scatter, FSC: Forward Scatter. Panel b: Protein Content: T3–TRB1 effect on protein synthesis was evaluated. Si-strike and iTHRB1 cells were plated and exposed or not to T3 (10^{-7} M) for 3, 5, and 7 days. Total protein extracts have been obtained and normalized as described in Materials and Methods Section. Data are presented as μg protein per million cells on the y-axis, as means ± SD, and are the results of at least three independent experiments. Different experimental groups are indicated. A comparison of the individual treatment was conducted by using Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$. A *P*-value <0.05 was considered significant. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

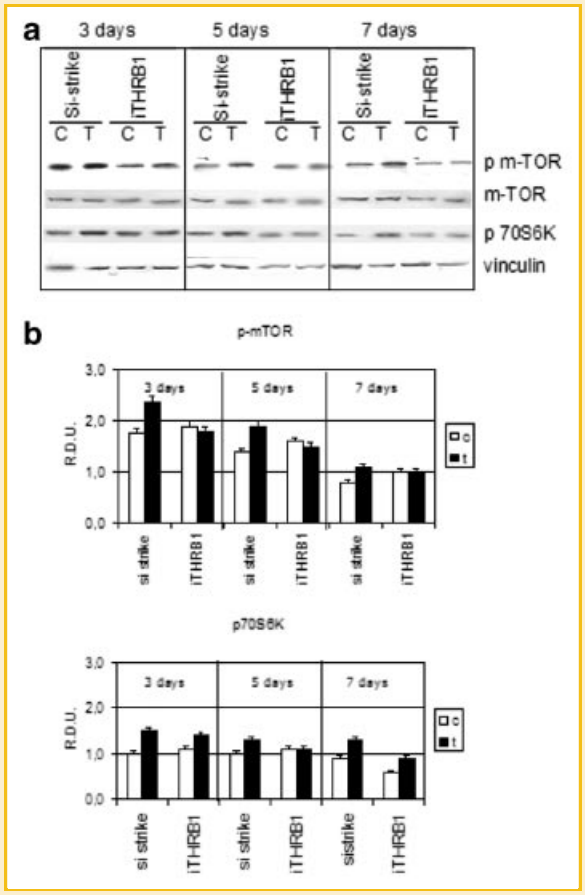


Fig. 8. Panel a: Western Blot analyses To analyze the phosphorylation of mTOR together with the expression of p70S6K under T3 treatment, si-strike, and iTHRB1 cells were plated and exposed or not to T3 (10^{-7} M) for 3, 5, and 7 days. Western Blot analyses were performed as described in Materials and Methods Section and a specific band corresponding to p-mTOR and p70S6K were detected. The expression of mTOR and vinculin was analyzed as a control for gel loading. Panel b: Densitometric analyses: Densitometric absorbance values from three separate experiments were averaged (±SD), after they had been normalized to either mTOR or vinculin for equal loading. Data are presented in the histogram as Relative Densitometric Units. The different experimental groups are indicated on the x-axis. A comparison of the individual treatment was conducted by using Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$. A *P*-value <0.05 was considered significant. At least three different experiments were performed, and a representative one is shown here.

conditions, CM cells acted as expected by insulin-producing cells [Cavallo et al., 1996]. Thus, in our opinion it represented a good in vitro model to investigate the molecular relation between the TRβ1, strongly expressed, and the Akt pathway, which is relevant concerning the islets origin of the cells.

The functional features of the stably interfered cells reveal that TRβ1 is involved in the molecular mechanisms controlling T3 regulation of β-cell function in our system, and disclose distinct aspects underlying this mechanism. First, the functional knockdown of TRβ1 expression in iTHRB transfected cells yielded the abolishment of the T3-induced activation of Akt, which plays a pivotal role in mediating a number of cellular processes that include mitogenesis, cell size, survival, and differentiation.

Thyroid receptor β1 has been recently implicated in the regulation of Akt pathway; herein we propose that thyroid hormone-induced β-cell function is mediated by activating the PI3K/Akt signaling pathway through cytosol-localized TRβ1.

β-CELL PROLIFERATION

Since one main feature of diabetes is β-cell loss, the chance to induce β-cell proliferation may provide a good device to replace insulin-secreting cells. We now demonstrate that T3–TRβ1 can induce cell proliferation in the hCM insulin producing cells. T3 participates to cell cycle control via stimulation of the G_1-cyclins [Barrera-Hernandez et al., 1999; Pibiri et al., 2001]; moreover Akt has been widely linked to the regulation of the G_1-S molecules [Barrera-Hernandez et al., 1999; Shin et al., 2002; Viglietto et al., 2002; Cozar Castellano et al., 2006]. Mitogenesis is associated with an increment in cyclin D and cyclin D–cdk4 complex levels. As deeply discussed at the moment [Olashaw et al., 2004], to its full activity the complex might need the sequestration of CDKIs, event that can be promoted by Akt through GSK3β action on cyclin D1 [Chang et al., 2003]. In this work we showed that the CDKIs-cyclin D–cdk4 complex is present and upregulated by T3; considering the strong effect that the hormone exerts on the S-phase, we could speculate that the cell proliferation regulation observed concerns quite this complex formation, suggesting an Akt activation of the CDKIs–cdk4–cyclin D. In addition T3 induced pRb phosphorylation, suggesting an increment in the complex activity, only in the TRβ1 expressing cells. The p27 ^{KIP1} cytoplasm sequestration is another direct consequence of Akt activity [Liang et al., 2002; Shin et al., 2002]. We evidenced that TRβ1 expressing cells when T3 treated show a strong cytoplasmic compartmentalization for this factor, while the TRβ1 interfered cells do not. We previously evidenced a reduction of $p27^{KIP1}$ levels in T3 treated hCM [Verga Falzacappa et al., 2006]; it is likely that in these cells the TRβ1/Akt pathway regulates $p27^{KIP1}$ localization and levels, outlining a fine regulation of cell proliferation at different levels, which could include many other different players. Our data evidenced also a strong regulation by T3 in the β-catenin expression TRβ1 dependent. β-catenin represents another crucial point of Akt pathway, being a multiple regulator of both cell cycle and apoptosis, directly targeted by GSK3β [Cozar Castellano et al., 2006; Elghazi et al., 2006]. Nonetheless it has been previously described as a T3 target involving the Wnt signaling [Miller et al., 2001], and being directly regulated in particular via TRα1 [Plateroti et al., 2006], but its regulation remained controversial. It has recently been demonstrated that TRβ1 can bind β-catenin to promote β-catenin degradation [Guigon et al., 2008]; this effect, however, is independent on GSK3β activity. In pancreatic β-cells, β-catenin phosphorylation and its consequent degradation are prevented by GSK3β. Given the evidences we previously provided [Verga Falzacappa et al., 2007] that T3 can regulate GSK3β, our consideration is that the TRβ1 mediated regulation of β catenin could imply GSK3 activity, as a target of T3 activated Akt.

β-CELL APOPTOSIS

β-cell apoptosis is another of the important process targeted by Akt activity. The data here presented showed clearly that T3–TRβ1

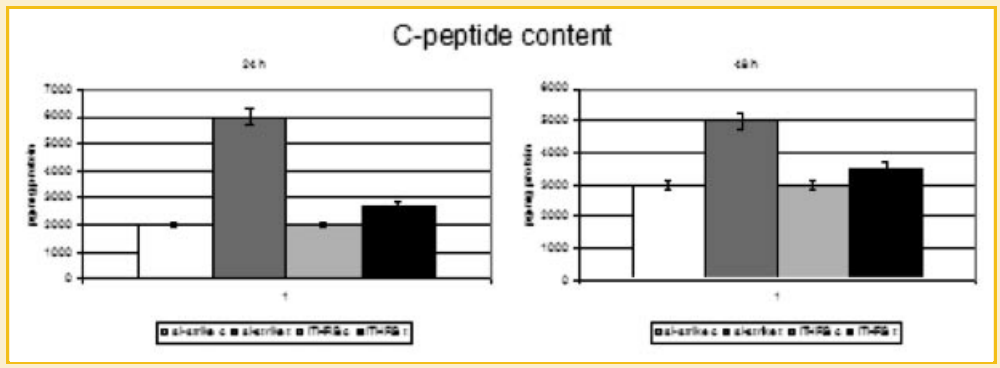


Fig. 9. To analyze the T3–TRB1 effect on insulin production, c-peptide content was measured. Si-strike and iTHRB1 cells were plated and exposed or not to T3 (10^{-7} M) for 24 and 48 h. Total protein extracts have been obtained and c-peptide content has been measured as described in Materials and Methods Section. Data are presented as pg protein per mg of protein on the y-axis, as means ± SD, and are the results of at least three independent experiments. Different experimental groups are indicated. A comparison of the individual treatment was conducted by using Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$. A *P*-value <0.05 was considered significant. At least three different experiments were performed, and a representative one is shown here.

trigger the anti-apoptotic stimulus. This ability is strictly related to the regulation of Akt targets such as β-catenin and BAD phosphorylation. Together with prevention of β-catenin degradation, the Akt has also been shown to directly phosphorylate BAD, preventing its activation and causing its cytosol sequestration, thus preventing its association with Bcl-X_L and resulting in increased cell survival [Chang et al., 2003]. We recently demonstrated [Verga Falzacappa et al., 2007] that T3 activation of Akt does not depend on protein translation. In our previous work we also evidenced that T3 treatment decreased Bad expression [Verga Falzacappa et al., 2006], thus it is possible to speculate that the observed induction of Bad phosphorylation could not involve protein synthesis regulation and depend on T3 non-genomic action on Akt.

β-CELL SIZE, PROTEIN SYNTHESIS, AND C-PEPTIDE CONTENT

One additional target of Akt contributing to β-cell function is the regulation of cell size and protein synthesis. We demonstrated that T3 provokes an increment in both these features via TRβ1. The regulation of cell size by Akt/PKB is mediated by activation of the mammalian target of rapamycin (mTOR) [Elghazi et al., 2006; Ackermann and Gannon, 2007]. The activated mTOR targets ribosomal S6 kinase (S6K), one key regulator of protein translation and cell size. In the pancreatic β-cells it has also been suggested a role for the mTOR/S6K signaling in proliferation; mice deficient in S6K1 exhibited a reduction in β-cell size [Pende et al., 2000]. In addition the increased cell size observed in Akt overexpressing animals suggests that S6K1 could relate some of the growth signals induced by Akt/PKB. Our data showed that T3 provokes the activation of mTOR together with an increase in S6K expression, this in accordance with the effects observed on cell size and protein synthesis. Moreover we can hypothesize that T3 action on Akt reflecting contemporary on cell cycle regulator modulation and on mTOR/S6K signaling could target cell proliferation via both signaling. Recent reports supported a role for the PI3K–Akt–mTOR pathway in the T3 regulation of cardiomyocytes, hypertrophy, and protein synthesis; this regulation involved a cytoplasmic thyroid receptor alpha 1 and hypothesized a non-genomic action of T3 [Kenessey and Ojamaa, 2006]. It is likely that thyroid hormone non-genomic action could target the PI3K/Akt pathway via T3 binding of alternatively TRα1 or TRβ1, maybe depending on the cellular system. The data we here presented evidenced a role for the β1 isoform of the thyroid hormone receptor in mediating a T3 action on the Akt pathway contributing to the stimulation of β-cell proliferation, survival, and size both in a rapid and a long term fashion.

Given the relevant role that these cited features play in the regulation of pancreatic β-cell function, we might hypothesize that T3 is a good factor to enhance β-cell function via Akt.

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