Identification and characterization of RanBPM, a novel coactivator of thyroid hormone receptors

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Abstract

Thyroid hormone receptors (TRs) are transcription factor members of the nuclear receptor superfamily. The transcriptional activity of TRs is controlled by thyroid hormones and cell-specific coregulators. Using the yeast two-hybrid system, we identified RanBPM as a new protein partner for TRs. RanBPM was initially discovered as an interacting partner for Ran, and was also shown to be a protein partner and coactivator of the androgen receptor. The novel interaction between RanBPM and TR isoforms was addressed by glutathione-S-transferase (GST) pull-down assays and co-immunoprecipitation in intact mammalian cells, where RanBPM was shown to bind TRs in a ligand-independent fashion. We also studied the regions implicated in the interaction with deletion mutants: the principal interacting region of RanBPM is comprised within its carboxyl-terminal end and the TR DNA-binding domain is sufficient to mediate the interaction. To investigate the potential role of RanBPM in thyroid hormone action, transient transfections with luciferase reporter genes were performed in CV-1 cells. We found that the over-expression of RanBPM increases the activation of TRETk- and DR+4-positive thyroid hormone response elements. Interestingly, over-expression of the truncated protein RanBPM55, which lacks the N-terminal polyglutaminated region but binds TRs, decreased the fold activation by almost 80%. Furthermore, we performed competition assays using transient transfection of RanBPM and increasing amounts of RanBPM55. This revealed that the stimulating effect on TR transactivation by the full-length protein is inhibited in a dose-dependent fashion by RanBPM55. This suggests that although the polyglutaminated region of RanBPM is not required for the binding to TRs, it is required for the stimulation of TR transactivation. Taken together, our results provide evidence that RanBPM is a potent novel coactivator for thyroid hormone receptors.

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Introduction

Thyroid hormones (THs) play a critical role in development, growth and metabolism. In addition, they are required for the normal function of most tissues, and have major effects on oxygen consumption and metabolic rate. Thyroid hormone receptors (TR) are very well conserved members of the nuclear receptor (NR) superfamily of ligand-dependent transcription factors. The transcriptional regulation of TH target genes is mediated via the binding of TRs to thyroid hormone response elements (TREs) in their promoter region (Yen 2001). Unlike steroid hormone receptors, such as the glucocorticoid and androgen receptors that are inactive in the absence of their ligand and found in the cytoplasm bound to heat-shock proteins, TRs are present in the nucleus both in the absence and presence of tri-iodothyronine (T_3) and modulate gene expression in both states.

The effects of coactivator and corepressor proteins have been more extensively studied on positively regulated TREs. In the absence of ligand, TRs are

associated with corepressors, such as the nuclear receptor corepressor (NCoR), creating a template for the recruitment of histone deacetylases (HDACs) and inhibiting the transcription of the upstream gene (Chen & Evans 1995, Horlein & Naar 1995). On the other hand, upon binding of their ligand, conformational changes in TRs release corepressor complexes and allow the recruitment of coactivator proteins that loosen the DNA–chromatin structure (McKenna *et al.* 1999). Chromatin remodelling is a defining step in the transcriptional initiation process essential for gene transactivation. This feature is not inherent to coactivators, such as the p160 family that mediates histone acetylation, but it is an important event required for maximal activation of T_3 -responsive genes (Lee *et al.* 2003). Another characterized group of chromatin-modifying complexes interacting with TRs is the ATP-dependent SWI/SNF complex, which disrupts the association of histones with DNA via nucleosomal modifications (Huang *et al.* 2003). Other coactivators enhance the transcription of target genes via their ability to recruit components of the basal transcriptional

Figure 1 Schematic representation of (A) the domain structure of TR isoforms, including the amino-terminus, DNA-binding (DBD), hinge (H) and ligand-binding domains (LBD) and identification of the bait used in the yeast two-hybrid screen. (B) Representation of the TR mutants used in this study: TR β 2 1–120 Δ , ØDBD and DBD-only. (C) Structure of RanBPM and RanBPM55 that contain polyglutaminated, SPRY, LisH and CTLH domains. (D) Representation of RanBPM constructions used in the present study: RanBPM 409–730 and 231–409.

machinery and function as an interface between sequence-specific transcription factors and the general transcription apparatus (Fondell *et al.* 1999).

Although most coregulators identified to date interact with TRs in a ligand-dependent fashion, the interaction of some coregulators, such as GT198 and polypyrimidine tract-binding protein-associated splicing factor (PSF), is not modified by T₃ (Ko *et al.* 2001, Mathur *et al.* 2001). Ligand-dependent transcriptional regulation by TRs is thus a complex process involving the recruitment of various coregulators to the promoter of target genes. In order to better understand TH action, further identification and characterization of transcriptional coregulators is warranted.

Using the yeast two-hybrid (YTH) system, we identified the Ran binding protein in microtubules (RanBPM) as a novel ligand-independent interacting partner for TRs. RanBPM, was first discovered to be an interacting partner of Ran, but its physiological role remains unclear (Nakamura *et al.* 1998). RanBPM was recently shown to interact with other NRs – androgen receptor (AR) and glucocorticoid receptor (GR) (Rao *et al.* 2002). In this report, we describe the interaction of RanBPM with TRs, and determine its function as a coactivator for thyroid hormone receptors.

Materials and methods

Plasmid constructions

TR and RanBPM constructions are schematically represented in Fig. 1. TR isoforms and the TR β 2

mutants previously described (Langlois *et al.* 1997, Poirier *et al.* 2005) were cloned in frame in the pGEX-4T1 or -4T2 vectors (Amersham Biosciences Inc., Baie D'Urfe, Qc, Canada) by restriction enzyme digestion or PCR. Other GST-human (h) TR β 2 mutants, ØDNA-binding domain (ØDBD) (amino acids (a.a.) 89–116 deleted) and DBD-only (a.a. 119–221) were produced by PCR amplification of hTR β 2 and inserted into pGEX-4T1 (Fig. 1B). The RanBPM55 cDNA was recovered by YTH screening of a human fetal cDNA library inserted into the pACT-2 vector (Clontech, Palo Alto, CA, USA) and subcloned in pSG5 by restriction enzyme digestion (Stratagene, La Jolla, CA, USA) (Breathnach & Harris 1983). The full-length cDNA of the human RanBPM protein inserted into pcDEBA was kindly provided by Dr T Nishimoto from Kyushu University, Fukuoka, Japan (Nishitani *et al.* 2001). GST-fusion protein vectors used for production of RanBPM mutant constructions were made by restriction enzyme digestion, as shown in Fig. 1D and inserted in pGEX-4T1/2. The expression vector used for the *in vitro* transcription/translation, RanBPM55-pTracer (Invitrogen Canada Inc, Burlington, On, Canada), was originated by PCR amplification and cloned by insertion at KpnI and NotI. In the transient transfection experiments, the mammalian expression plasmids were pTracer-RanBPM55, pcDEBA-RanBPM90, pSG5-hTR isoforms and the corresponding empty vectors as controls.

The TRETK reporter contains two copies of an idealized positive TRE (pTRE) arranged as a palindrome, upstream of a minimal thymidine kinase

promoter and fused to the luciferase gene in the PA3 vector (Chatterjee *et al.* 1989). The pTRE DR+4 (direct repeat) was also incorporated in the PA3-Luc vector. All insert sequences were confirmed by DNA sequencing.

Yeast two-hybrid screening

A cDNA fragment coding for amino acids 89–220 of the human TR β 2 isoform (Fig. 1A) was cloned into the pAS2–1 vector for expression of a fusion protein with the GAL4 DNA-binding domain (MatchMaker Two-hybrid System-2; BD Biosciences-Clontech, Mississauga, On, Canada). In this system, a human fetal brain cDNA library fused to the GAL4 transactivation domain in the pACT2 expression vector was screened. These constructions were transformed into the Y190 yeast strain. Transformants were selected for growth on minimal medium agar lacking tryptophan, histidine and leucine in the presence of 3-amino-1,2,3-triazole. Positive clones were tested by β -galactosidase assays, according to the manufacturer's instructions. Further selection of positive clones was based on their β -galactosidase scores by colony filter-lift after retransformation in yeast. Transformants with the highest scores were then sequenced (sequencing service, University of Ottawa, On, Canada) and nucleotide comparisons were performed using the GenBank database and the Basic Local Alignment Search Tool (BLAST) program (National Center for Biotechnology Information; NCBI). Clone number 83 (Fig. 1C) represented a cDNA sequence identical to amino acids 146–683 of RanBPM.

RNA expression analysis

RNA isolation analysis from CV-1 (*Cercopithecus aethiops*, ATCC #CCL-70), HeLa (*Homo sapiens*, ATCC #CCL-105), HEK-293 (*Homo sapiens*, ATCC #CRL-1573), JEG-3 (*Homo sapiens*, ATCC #HTB-36) and PC12wt (*Rattus norvegicus*, ATCC #CRL-1721) cells was performed according to standard procedures using the TRIzol Reagent (Sigma-Aldrich Canada Ltd, Oakville, On, Canada). For each cell type analyzed by northern blot, 20 μ g total RNA were loaded on a MOPS-based gel and transferred on a nitrocellulose membrane (Bio-Rad Laboratories, Indianapolis, IN, USA). The radiolabelled probe comprising 953 base pairs of the C-terminal end of RanBPM was generated using the Rediprime Random Prime Labelling System (Amersham Biosciences Inc.) and free [^{32} P]dCTP was discarded with ProbeQuant MicroColumns (Amersham Biosciences Inc.). Northern blots were then hybridized at 65 °C overnight with the ^{32} P-labelled cDNA probe and revealed by autoradiography. The blot was then stripped and hybridized with a probe against the human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) as a loading control. The Gel-Pro Analyser program,

version 4 (MediaCybernetics, Carlsbad, CA, USA) was used for gel analysis.

The expression pattern of RanBPM was also studied using a commercial nylon membrane containing normalized loadings of polyA⁺ RNA of human tissues (MTN blot; BD Biosciences-Clontech). The same RanBPM probe was used following the manufacturer's instructions. The membrane was then stripped and hybridized with a control probe against human ubiquitin cDNA supplied by the manufacturer and resulted in consistent signals for all polyA⁺ RNA dots (data not shown). The Gel-Pro Analyser program, version 4 was used for gel analysis.

Glutathione-S-transferase pull-down assays

GST-coupled proteins were produced and purified as previously described (Smith & Johnson 1988). Purified GST-fusion protein (1–5 μ g) was incubated with 5 μ l *in vitro* translated ^{35} S-labelled protein (TNT kit; Promega, Nepean, On, Canada). The binding reaction was performed at room temperature with moderate shaking during 2 h in HEMG buffer (40 mM Hepes pH 7.8, 40 mM KCl, 0.2 mM EDTA, 5 mM MgCl₂, 0.5% Triton X-100, 10% glycerol, 1.5 mM dithiothreitol), supplemented with 10 mg/ml BSA and protease inhibitors. The reactions were washed five times in non-supplemented HEMG buffer. The bound proteins were subjected to SDS-PAGE analysis and detected by autoradiography. Results shown are representative of at least three independent experiments. Autoradiograms were analyzed with the ImageQuant 5.0 Build 050 software (Molecular Dynamics, Sunnyvale, CA, USA) or the Gel-Pro Analyser program, version 4 in order to evaluate the interactions. The statistical analysis (one-way ANOVA) was performed using the SigmaStat Statistical Software version 2.03 (SYSTAT Software, Inc, Point Richmond, CA, USA).

Immunoprecipitation and immunoblotting

HeLa cells, grown in 75-mm² Petri dishes supplemented with T₃ (10 nM) or the vehicle alone (–T₃) until they reached 80% of confluence, were lysed in a 50 mM Hepes solution containing 1% Triton X-100 and protease inhibitors. After 30 min incubation on ice followed by centrifugation at 3000 $\times g$ for 15 min, supernatants were collected and placed at –20 °C overnight to complete lysis. Total cell extract concentrations were measured using a standard Bradford assay (Bio-Rad Laboratories). Immunoprecipitation was performed using a rabbit polyclonal IgG antibody raised against chicken TR α 1 but recognizing all human TR isoforms (FL-408; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (Pellizas *et al.* 2002). The formation of

immuno-complexes was made in the presence or absence of T_3 ; the positive control represents 50 μ g HeLa whole cell extracts. After overnight incubation of the pre-cleared sample with FL-408 antibody at 4 °C with rocking with T_3 (10 nM) or the vehicle alone ($-T_3$), protein G-Sepharose (Amersham Biosciences Inc.) was added in the dilution buffer (0.1% Triton X-100, 0.1% BSA in Tris saline azide (TSA) buffer (0.1 M Tris-HCl, 0.14 M NaCl, 0.025% (w/v) NaN_3)), and samples were rocked for 2 h at room temperature. After five washes, proteins were resolved on a 10% SDS-polyacrylamide gel and transferred on a polyvinylene difluoride membrane (Roche, Laval, Qc, Canada). The membrane was blocked with 5% skim milk, 0.05% Tween-20 in Tris-Buffered Saline (TBS) buffer, pH 7.5 and incubated for 2 h at room temperature with the 5 M anti-RanBPM antibody of rabbit origin provided by Dr T Nishimoto from Kyushu University (Nishitani *et al.* 2001). An anti-I κ B- α antibody (sc-371, Santa Cruz Biotechnology, Inc.) was also used as a control for the specificity of the Co-immunoprecipitation (Co-IP) experiment (data not shown). Detection was accomplished using horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham Biosciences Inc.) and enhanced by the BM chemiluminescence Blotting Substrate (ECL, Roche).

Cell culture and transient transfections

The CV-1 cells were maintained in phenol red Dulbecco's Modified Eagle's Medium media supplemented with 10% fetal bovine serum (FBS), with a mix of antibiotics and antimycotics (Life Technologies, Burlington, On, Canada). The day before transfection, cells were seeded in 6-well plates at approximately 10 000 cells per 35 mm² well. Cells were transfected as described before using the calcium phosphate precipitate technique (Speciality Media, Boston, MA, USA) (Langlois *et al.* 1997, Laflamme *et al.* 2002, Poirier *et al.* 2005). For each plate, 100 ng or 500 ng TR-pSG5, 3 μ g RanBPM55-pTracer, RanBPM90-pcDEBA or the corresponding empty vectors, and 10 μ g of the luciferase reporter gene were used unless otherwise stated. Sixteen hours after transfection, cells were fed with fresh media supplemented with charcoal and resin-stripped FBS with the addition of T_3 (10 nM) or the vehicle alone ($-T_3$). Cells were harvested 24–36 h following the hormonal treatment and processed for luciferase assay; β -galactosidase assays were used initially to assure efficiency of transfection, as previously described (Langlois *et al.* 1997, Laflamme *et al.* 2002). Luciferase activity was measured using an EG&G Berthold lumat LB 9507 luminometer. The results from at least three independent experiments each performed in triplicate are displayed as means $\pm$ S.E.M. Statistical analysis

(Student's *t*-test) was performed using the SigmaStat Statistical software version 2.03.

Results

Identification of RanBPM as an interacting partner for TRs

In order to identify novel proteins interacting with TRs, a fragment corresponding to the amino acids 89–220 of TR β 2 (Fig. 1A) was used as a bait to screen a human fetal brain cDNA library which contained approximately 3.5×10^6 clones to be screened. Following the YTH screening, five different positive clones were isolated, among which one corresponded to RanBPM. RanBPM, first described as an interacting protein for Ran (Nakamura *et al.* 1998, Nishitani *et al.* 2001), was identified as a strong positive clone on β -galactosidase assays (data not shown). The RanBPM clone corresponded to amino acids 146–683 of RanBPM (RanBPM55) (GenBank accession number: AB008515), which is schematically represented in Fig. 1C. This novel interaction with TRs was reconfirmed in yeast cotransformed with the bait using β -galactosidase assays (data not shown). The RanBPM protein has multiple domains: its amino-terminal end contains a long stretch of prolines and glutamines referred to as the polyglutaminated region, followed by repeats in the splA and Ryr domain (SPRY) (Ponting *et al.* 1997), a lissencephaly type-I-like homology motif (LisH) and a carboxy-terminal to LisH motif domain (CTLH) domain. The SPRY, LisH and CTLH domains have unknown functions but have previously been associated with protein–protein interactions (Ponting *et al.* 1997, Adams 2003).

RanBPM is expressed ubiquitously

The RanBPM sequence in humans is highly homologous in rodents and is conserved in other mammals (Nishitani *et al.* 2001). In order to establish the presence of endogenous RanBPM in different cell lines, we conducted a northern blot analysis (Fig. 2A). Blot analysis shows that RanBPM transcripts are widely expressed in eukaryotic cells lines, as was also established by others (Nakamura *et al.* 1998, Nishitani *et al.* 2001). Accordingly, it shows RanBPM mRNA expression in every cell line tested (Fig. 2A). Within the cell lines we used, the highest levels of RanBPM transcript were seen in HEK, JEG-3 and HeLa cells. In CV-1 and PC12wt cells we found the lowest level of RanBPM transcripts (Fig. 2B).

The expression pattern of RanBPM in different human tissues was also studied using a commercial dot blot membrane of human tissues revealing ubiquitous

Figure 2 Expression pattern of RanBPM mRNAs in cell lines and human tissues; analysis of the expression pattern of RanBPM in different carcinoma cell lines and human tissues. (A) Northern blot was hybridized with a ^{32}P -labelled cDNA probe representing the C-terminal end of RanBPM followed by autoradiography. The membranes were then stripped and rehybridized with hGAPDH probe as a loading control. (B) Relative expression ratios of RanBPM transcripts were calculated as the percentage of hGAPDH binding after analysis of the blot with the GelPro software. (C) A commercial dot blot membrane of human tissues mRNA hybridized with a radiolabelled probe. RanBPM is ubiquitously expressed in adult and fetal human tissues and cell lines.

expression (Fig. 2C). RanBPM transcripts were predominantly found in the testis (blot position F8) and oesophagus (A5). Recently, RanBPM was shown to be expressed in different neuronal cell types (Bai *et al.* 2003, Menon *et al.* 2004, Brunkhorst *et al.* 2005, Cheng *et al.* 2005). Accordingly, our dot blot shows that the RanBPM transcript is found throughout the central nervous system (columns 1, 2 and 3).

Characterization of the interaction between TRs and RanBPM

Next, we proceeded to the characterization of the regions implicated in the binding of RanBPM to TRs. Since RanBPM was obtained using a portion of TR β 2 as bait, this determination was first accomplished using different constructs of this isoform, represented in Fig. 1B. Figure 3A shows that RanBPM interacts with the

wild-type TR β 2 and all studied TR constructs, and that this interaction is specific since GST alone is not able significantly to precipitate TRs. However, the interaction is decreased by the deletion of a portion of the N-terminus (1–120 Δ) or the DNA-binding domain (Δ DBD). This suggests multiple interacting sites are present within TR β 2. Also, the DBD-only construction was sufficient to precipitate the radiolabelled RanBPM protein. Taken together, these results suggest that the DNA-binding domain of TRs is sufficient to bind RanBPM and is an important domain implicated in the interaction.

In order to determine the interacting region within RanBPM, we used different GST-RanBPM constructions (Fig. 1D). The C-Term mutant, corresponding to amino acids 409–730, interacts with even more affinity than RanBPM55 (Fig. 3B). The 231–409 RanBPM construct interacts very weakly with TR β 2. The SPRY

Figure 3 *In vitro* interaction between thyroid hormone receptors and RanBPM. Analysis of the interaction between different TR and RanBPM constructions using GST pull-down assays; representative pull-down analysis of GST fusion proteins and ³⁵S-labelled *in vitro*-translated proteins. Percentage of input is included for reference (lane 1) and GST alone was used as a negative control (lane 2). (A) Determination of the regions responsible for the interaction of TRβ2 with RanBPM. RanBPM interacts with the wild-type TRβ2 and all studied TR constructs, and this interaction is specific since GST alone is not able significantly to precipitate TRs. However, the interaction is decreased by the deletion of a portion of the N-terminus (1–120Δ) or the DNA-binding domain (ØDBD), suggesting multiple points of interaction. (B) Study of the regions of RanBPM important for TR-binding. The C-terminal mutant (409–730; C-Term) binds more strongly to TRβ2, and the Lish/SPRY domains (231–409) are not necessary for the interaction. (C) All ³⁵S-labelled TR isoforms, TRα1, TRβ1 and TRβ2, interact directly with GST-C-terminal of RanBPM (C-Term). The band intensity was measured and a graphical representation of the percentage of binding compared with the input ([sample – GST]/input × 100) was made. *P ≤ 0.05. (D) *In vitro* interaction between GST-RanBPM55 and radiolabelled TRβ2 is not affected by the presence of the ligand: increasing concentrations of T₃ did not significantly affect the binding of the two proteins.

domain was previously described to be important for the interaction between RanBPM and other protein partners (Wang *et al.* 2002). However, our data suggest that the SPRY and Lish domains are not required for TR-binding that occurs in the carboxy-terminus of RanBPM.

Next, we wanted to investigate the binding properties of each TR-isoform with RanBPM. To do this, we performed GST pull-down assays with the C-Term RanBPM, which shows the strongest interaction, and with radiolabelled TR isoforms. Figure 3C shows that RanBPM and all TR isoforms interact *in vitro*. The relative intensity of the interaction was quantified and the percentage of binding ([sample – GST]/input × 100) is graphically represented in the lower panel of Fig. 3C.

We found a significantly lower level of binding of TR-α1 compared with the β isoforms.

Since it is well known that many coregulatory molecules interact with TRs in a ligand-dependent fashion (Ko *et al.* 2002), we tested whether the interaction between RanBPM and TRs was affected by the presence of the ligand. Accordingly, GST pull-down assays were carried out in the presence of increasing concentrations of thyroxine (T₄) and T₃. Results show that RanBPM is associated with both the free and ligand-bound form of the receptor, in physiological and supra-physiological concentrations (Fig. 3D).

RanBPM interacts strongly with the DBD of TRs, a region that shares the highest homology among NRs.

Figure 4 RanBPM interacts with other nuclear receptors. Representative pull-down analysis of GST fusion proteins and ^{35}S -labelled *in vitro*-translated proteins is shown. Percentage of input is included for reference (lane 1) and GST alone was used as a negative control (lane 2). GST pull-down analysis of the interactions between GST-RanBPM55 and ^{35}S -labelled members of the nuclear receptor superfamily: hAR, hER α , hRAR α and hRXR α is shown. The assays reconfirm the interaction of RanBPM with hAR, and show that RanBPM interacts with hER α and only weakly with hRAR and hRXR α .

We thus studied the interaction between RanBPM and other human NRs. In Fig. 4, we reconfirmed the interaction with AR that has previously been reported (Rao *et al.* 2002), and we demonstrated that RanBPM interacts with the oestrogen receptor (hER). However, only a weak interaction is present with the retinoic acid receptor (hRAR) and the retinoic X receptor (hRXR α).

RanBPM interacts with TRs in intact cells

In order to confirm the presence of this novel interaction *in vivo*, co-immunoprecipitation in intact mammalian cells was accomplished. Lysates from HeLa cells were used, since they endogenously express TR β s (Weinberger *et al.* 1986, Doulabi *et al.* 2002, Poirier *et al.* 2005) and the RanBPM protein (Nishitani *et al.* 2001). To raise protein complexes, we used a polyclonal antibody recognizing all TR isoforms (FL-408) or normal rabbit IgG as a negative control. The membranes were also blotted using an antibody against an irrelevant protein, I κ B- α , and no band was observed in the Co-IP lanes (data not shown) assuring the specificity of the Co-IP experiment. We demonstrated that the complexes formed by precipitating TRs in living cells contain the RanBPM protein (Fig. 5). Furthermore, physiological (10 nM) and supra-physiological (100 nM, data not shown) concentrations of T $_3$ did not influence

the interaction between RanBPM and TRs, in concordance with *in vitro* GST pull-down assays. Therefore, we conclude that the interaction between RanBPM and TRs is ligand-independent in intact mammalian cells.

RanBPM is a novel coactivator for thyroid hormone receptors

To help determine the physiological function of this novel interaction in the regulation of TR-responsive genes, we have accomplished transient transfections using luciferase reporter genes. CV-1 cells were chosen since they contain very low levels of TRs (Lin *et al.* 1997, Castillo *et al.* 2004). In Fig. 6 the relative luciferase activity and the fold activation on the TRETK pTRE are shown. Over-expression of RanBPM caused an important increase in hormone-dependent transactivation. The effect of RanBPM on the transactivation of the reporter gene depended on the TR isoforms studied: the increase in fold activation was 30, 70 and 190% for TR α 1, TR β 1 and TR β 2 respectively. There was no significant effect on the transcription level in the absence of ligand for all TR isoforms. In Fig. 7 the results obtained with over-expression of the truncated protein, RanBPM55, which also interacts with TRs but lacks the polyglutaminated region are shown. When over-expressed, RanBPM55 dramatically inhibits the

Figure 5 RanBPM and thyroid hormone receptors interact *in vivo* in mammalian cells.

Co-immunoprecipitation of RanBPM with an anti-TR antibody (FL408) showing interaction in intact mammalian cells. Immunoprecipitation with normal rabbit IgG (Pi) was used as a negative control and the positive control represents 50 µg HeLa whole cell extract. The immunoblot was revealed using 5 M anti-RanBPM antibody and also with an anti-IκB-α antibody as a Co-IP control (data not shown). HeLa cells were supplemented with T₃ (10 nM) or the vehicle alone (-T₃) for 24 h before cell lysis and during the co-immunoprecipitation study. RanBPM co-immunoprecipitates with TR in HeLa cells and this interaction is not influenced by the presence of T₃.

transcription of the reporter gene, and the fold activation diminishes by almost 80%. Similar results with RanBPM and RanBPM55 were also observed with the DR+4pTRE (data not shown). Therefore, even if the N-terminal region of RanBPM is not required for the interaction with TR to occur, it is essential for TR-mediated transactivation.

We thus hypothesized that the inhibitory effect of RanBPM55 was due to competition with endogenous RanBPM present in CV-1 cells. To explore this possibility, we designed a competition experiment. In Fig. 8 the dominant-negative effect of RanBPM55 is shown: increasing amounts of the truncated protein, to the point where it induces repression, gradually abolishes the stimulating effect of RanBPM on TR-transactivation.

Discussion

In the present study, RanBPM was identified as an interacting partner and coactivator for thyroid hormone receptors. This novel interaction was first confirmed in yeast, followed by GST pull-down studies. We demonstrated that the C-terminal portion of RanBPM is able to bind to the DNA-binding domain of TRs. We have also shown that this interaction occurs *in vivo* by Co-IP in intact HeLa cells. Since RanBPM was recently found to be a coactivator for AR and GR (Rao *et al.* 2002), we reconfirmed its interaction with AR. We also found that RanBPM can bind to ER, but only weakly interacts with RARα and RXRα. The effect of the ligand on the recruitment of RanBPM was tested *in vitro* and *in vivo*, and in both systems there was no significant modification of the interaction in the presence of T₃. Therefore, RanBPM is a putative protein partner for TRs.

Investigating the transcriptional effect of this interaction, we performed transient transfections with positive response elements coupled to the luciferase reporter gene. The over-expression of RanBPM, which is already present in CV-1 cells, increased the fold activation in the presence of physiological amounts of T₃ by up to 190%. The stimulating effect of RanBPM on transcriptional activity is weaker for TRα1 compared with the β isoforms; this could be due to a decreased binding affinity of this isoform, as shown in GST pull-down studies.

In addition, over-expression of a truncated construct, RanBPM55, diminishes by 80% T₃-induced gene activation. RanBPM55 can bind TRs but it is not acting as an enhancer of transactivation; our hypothesis is that the polyglutaminated region and a complete SPRY domain are needed for the activation of transcription. Additionally, the effect of RanBPM on gene transactivation can be abolished by increasing amounts of RanBPM55, suggesting a competition for TR-binding and a dominant-negative effect of the truncated protein.

In this paper we have shown that RanBPM is able significantly to enhance TR-dependent transcription. Moreover, the coactivator effect of RanBPM uncovered here on TR-transactivation has a similar magnitude to that already established for nuclear receptor coactivators. Steroid hormone receptor-1 (SRC-1) is a ligand-dependent coactivator, first isolated following a YTH experiment using the hinge and ligand-binding domain of the human progesterone receptor (PR) and was shown to stimulate PR, ER and TR transcription (Onate *et al.* 1995). SRC-1 was later identified as a member of the p160 family of nuclear coactivators (Xu & Li 2003), increasing transactivation of pTREs by three- to fivefold when over-expressed in CV-1 cells. The amplitude of the stimulating effect of SRC-1 on transcriptional activation is dependent on the nuclear receptor and varies among NR isoforms. Thus, it is able to mediate a

Figure 6 Determination of the effect of RanBPM on TRET-K-luciferase gene reporter level of expression in mammalian cells. CV-1 cells were transfected with RanBPM, the different TR isoforms, TRET-K-LUC reporter gene, and their respective empty vectors. Cells were treated for 24 h with 10 nM T₃ or the vehicle alone (-T₃) before harvesting for luciferase assays. (A) RanBPM can enhance the activity of the reporter gene as represented in relative luciferase units (RLU). (B) RanBPM action on TRET-K-LUC represented in fold activation (RLU in the presence of T₃/RLU in the absence of T₃). Results are representative of at least three independent experiments performed in triplicate $\pm$ S.E.M. ** $P \leq 0.001$ in the presence of RanBPM (pcDEBA-RanBPM) compared with the empty vector (pcDEBA).

stronger activation, of approximately 100-fold, on ER responsive genes (Takeshita *et al.* 1997, 1998). Additionally, like SRC-1, RanBPM has an isoform-variable potency and can enhance the transactivation of other NRs (Rao *et al.* 2002), suggesting broader implication for this novel coregulator.

Others have previously demonstrated that RanBPM interacts with ARs and is able to transactivate their response on different androgen response elements (Rao

et al. 2002). However, the interaction between RanBPM and AR is different from that which we have found for TRs. The complete SPRY domain was required for AR binding *in vitro*. Furthermore, the luciferase assay results show that the AR-mediated gene activation does not require the polyglutaminated region of RanBPM. The findings that we report here thus contribute further to the understanding of the physiological role of RanBPM, but the mechanisms of the coactivator effect of RanBPM remain unknown.

RanBPM, a 90-kDa Ran-binding protein shares great homology between different species (Nishitani *et al.* 2001). We have shown that RanBPM is ubiquitously expressed in the cell lines and human tissues we tested. The cellular localization of RanBPM is perinuclear and nuclear and thus is compatible with a transcriptional coregulator function (Nishitani *et al.* 2001). The physiological functions of this protein remain unclear; it was first reported as interacting with Ran, a small G protein implicated in nucleocytoplasmic shuttling of cargo proteins through the nuclear pore complex (Nigg 1997, Nakamura *et al.* 1998). Ran has many protein partners such as RanBP1 and RanBP2, but unlike these proteins, no specific role is yet associated with RanBPM in nucleocytoplasmic transport (Nigg 1997). One possible mechanism explaining the increase in transactivation in the presence of RanBPM could thus be the modulation of the nucleocytoplasmic shuttling of TRs, which could be explored further in future studies (Li *et al.* 2003). Recent observations have shown that TRs are mainly nuclear, but are also found in the cytoplasm and in the perinuclear region (Hager *et al.* 2000). RanBPM could thus modify TR distribution by retention or recycling in the nucleus via binding to the Ran protein, allowing increased nuclear localization and transactivation of TRs. Compatible with this hypothesis is the fact that the DBD of TRs interacts with RanBPM; this region was recently shown to be important for TR export and recycling to the nucleus (Black *et al.* 2001). An increasing number of coregulatory proteins, such as general receptor for phosphoinositides 1 (GRP-1), GT198, P300/CBP associated factor (P/CAF) and PSF, have now been shown to bind to the DBD of TRs and new functions have been described for this region of the receptor (Zechel *et al.* 1994, Nagaya *et al.* 1996, Yang *et al.* 1996, Black *et al.* 2001, Ko *et al.* 2002, Wardell *et al.* 2002, Poirier *et al.* 2005). In addition to its important role in the binding and recognition of the hormone response elements (HREs), the DBD has now been found to be implicated in many new aspects of NR actions, and needs to be more thoroughly studied.

Our results demonstrate that RanBPM is a novel coactivator of TRs. RanBPM could mediate this effect through different pathways. In addition to a participation in nucleocytoplasmic shuttling, it could act either as a classical coactivator, either via an interaction

Figure 7 RanBPM55 inhibits T₃-transactivation. CV-1 cells were transfected with RanBPM55-pTracer, the different TR isoforms, TRETk-LUC reporter gene, and their respective empty vectors. Cells were treated for 24 h with 10 nM T₃ or the vehicle alone (-T₃) before harvesting for luciferase assays. (A) RanBPM55 inhibits the activity of the reporter gene as represented in relative luciferase units (RLU). (B) RanBPM55 action on the TRETk-LUC represented in fold activation (RLU in the presence of T₃/RLU in the absence of T₃). Results are representative of at least three independent experiments performed in triplicate ±S.E.M., *P≤0.05, **P≤0.001 in the presence of RanBPM (pTracer-RanBPM55) compared with the empty vector (pTracer).

with the basal transcriptional machinery or with complexes responsible for chromatin remodelling. Furthermore, it is interesting to note that Ran is able to bind to chromatin *in vivo* and interacts with histones H3 and H4 *in vitro* (Hayashi *et al.* 1995). However, sequence alignment research has not identified the presence of the LxxLLx motif, found in many cofactors, to be present in

RanBPM, and no homology has been found with known NR coactivators.

In conclusion, we have identified a new coactivator for TRs and established the regions responsible for the interaction. We believe that RanBPM should be added to the list of NR coregulators, and that it contributes to the isoform-specific and tissue-specific responses to

Figure 8 RanBPM55 has a dominant-negative effect on TR-transactivation. CV-1 cells were transfected with RanBPM-pcDEBA and increasing amounts of RanBPM55-pTracer competed with its empty vector (500 ng, 1 µg, 2 µg and 3 µg). TRETk-LUC (5 µg) was used as the reporter gene and TRβ1 isoform as the receptor. Cells were treated for 24 h with 10 nM T₃ or the vehicle alone (-T₃) before harvesting for luciferase assays. (A) Results expressed in relative luciferase units (RLU) show the stimulating effect of RanBPM (second set of bars) compared with TRβ1 alone (first set of bars). Transfection of increasing amounts of RanBPM55 inhibits the activity of the reporter gene in a dose-dependent fashion and produces a dominant-negative effect. (B) Results expressed as fold activation (RLU in the presence of T₃/RLU in the absence of T₃) showing the inhibitory dominant-negative effect of RanBPM. Results are representative of at least three independent experiments performed in triplicate ±S.E.M., **P≤0.001.

thyroid hormone. Moreover, the fact that RanBPM does not interact in the same fashion with AR and TRs, and that it has differential binding to TR isoforms, raises an important issue about receptor specificity. Further investigation needs to be pursued to determine the exact mechanism of action of RanBPM as a coregulator of nuclear receptors.

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