Poly(ADP-ribose) polymerase 1 binds to Pax8 and inhibits its transcriptional activity

Tina Di Palma, Tiziana de Cristofaro, Chiara D'Ambrosio¹, Dolores Del Prete, Andrea Scaloni¹ and Mariastella Zannini

Department di Biologia e Patologia Cellulare e Molecolare, Istituto di Endocrinologia e Oncologia Sperimentale 'G. Salvatore'-CNR, Università di Napoli Federico II, Via S. Pansini 5, 80131 Napoli, Italy

¹Proteomics and Mass Spectrometry Laboratory, ISPAAM-CNR, 80147, Napoli, Italy

(Correspondence should be addressed to M Zannini; Email: s.zannini@ieos.cnr.it)

Abstract

Pax8 is a transcription factor that plays an important role in the regulation of genes that are exclusively expressed in differentiated thyroid cells. In the thyroid cell environment, evidence exists that Pax8 is part of a multiprotein complex in which its transcriptional activity may be modulated by specific co-factors. In an attempt to identify proteins that interact with Pax8, we performed pull-down experiments challenging the GST–Pax8 fusion protein with protein extracts prepared from the thyroid differentiated cell line PC Cl3. By this approach, we isolated a 113-kDa protein that is able to associate with Pax8, which was further identified by mass fingerprint experiments as poly(ADP-ribose) polymerase 1 (PARP1). To further confirm this interaction, we also showed that PARP1 can be co-immunoprecipitated with Pax8 *in vivo* from a thyroid cell extract. Gel shifts experiments demonstrated that PARP1 binding to Pax8 significantly inhibits Pax8 binding to DNA. Accordingly, we provide evidence that the functional outcome of such an interaction is a significant downregulation of Pax8 transcriptional activity. In the context of thyroid-specific gene transcription, our results suggest that PARP1 behaves as an important negative co-factor involved in the regulation of Pax8-dependent gene expression.

Journal of Molecular Endocrinology (2008) **41**, 379–388

Introduction

Transcription factors act in a combinatorial fashion with an intricate array of co-regulators and with the general transcriptional machinery to allow tissue-specific gene expression and gene activation pathways. The interplay between sequence-specific transcription factors and co-regulator complexes determine the access to DNA either through acetylating reactions mediated by histone acetyltransferases (Xu *et al.* 1999) or through nucleosome-remodeling complexes (Muchardt & Yaniv 1999). Precise spatial and temporal switches in patterns of gene activation/repression are critical for the regulation of cell growth, differentiation and proper organ development. The differentiation program of thyroid follicular cells (TFCs) is completed only when the gland reaches its final location in front of the trachea and it depends on the crosstalk between transcription factors and transcriptional co-regulators with the basal transcriptional machinery of the cells. Differentiated TFCs are responsible for thyroid hormone synthesis and are characterized by the expression of a specific set of genes such as thyroglobulin (Tg) and thyroperoxidase, which are exclusively expressed in this cell type (Damante & Di Lauro 1994, Damante *et al.* 2001) and by the expression of genes expressed only in few tissues

other than the thyroid, such as the thyrotropin-stimulating hormone receptor and the sodium/iodide symporter.

Pax8 is one of the transcription factors involved in thyroid-specific gene expression (Damante & Di Lauro 1994, De Felice & Di Lauro 2004). It is a member of the *Pax* family of genes that are characterized by the presence of a 128-amino acid DNA binding domain, the paired domain (PD; Plachov *et al.* 1990). These genes encode for DNA-binding proteins that are involved in the regulation of the development of a variety of tissues in different species. In particular, Pax8 has been shown to be required for both the morphogenesis of the thyroid gland (Mansouri *et al.* 1998) and for the maintenance of the thyroid-differentiated phenotype (Pasca di Magliano *et al.* 2000). Interestingly, in Pax8 knockout mice the thyroid gland is barely visible and lacks the follicular cells (Mansouri *et al.* 1998). Recently, we have demonstrated that Pax8 interacts biochemically and functionally with TIF1/NKX2.1 (NK2 homeobox 1), another transcription factor involved together with Pax8 in the regulation of thyroid-specific genes (Di Palma *et al.* 2003).

Given the critical role played by Pax8 in thyrocyte differentiation, we focused our attention on the molecular mechanisms by which Pax8 modulates thyroid gene expression. Our hypothesis was that Pax8,

similarly to other members of the *Pax* gene family (Fitzsimmons *et al.* 1996, Wheat *et al.* 1999), could play a role in the recruitment of other factors and/or co-factors to the gene target promoters by specific protein–protein interactions. In this study, we report on a nuclear protein, namely poly(ADP-ribose) polymerase 1 (PARP1), which was isolated during GST-pull down experiments with thyroid cell lysates using a bait consisting of Pax8 fused to GST. Further experiments confirmed that PARP1 directly interacts with Pax8 *in vitro* and *in vivo*, although it does not poly(ADP-ribosyl)ate it. Transactivation assays finally demonstrated that PARP1 behaves as a Pax8 repressor.

PARP1 is a nuclear enzyme activated by DNA strand breakage that catalyzes the transfer of ADP-ribose units from NAD⁺ to numerous nuclear proteins (de Murcia & Menissier de Murcia 1994, D'Amours *et al.* 1999). It participates in several cellular catalytic activities, including anti-recombination and genomic stability (Ding & Smulson 1994), DNA replication (Simbulan-Rosenthal *et al.* 1998), apoptosis regulation and DNA repair (Jeggo 1998, Pieper *et al.* 1999). Recent studies have also underlined its role in the regulation of gene transcription (D'Amours *et al.* 1999, Chiarugi 2002, Kraus & Lis 2003) and as a partner of several transcription factors and (co)-factors, including NF-κB (Hassa *et al.* 2003, Nakajima *et al.* 2004), AP-2 (Kannan *et al.* 1999), B-MYB (Cervellera & Sala 2000), E2F-1 (Simbulan-Rosenthal *et al.* 2003), RAR/RXR (Miyamoto *et al.* 1999, Pavri *et al.* 2005) and p53 (Kumari *et al.* 1998). Recently, another member of the PARP family, namely PARP2, and PARP1 were identified as interacting proteins of the transcription factor TIF1/NKX2-1 (Maeda *et al.* 2006).

Accordingly, the results here reported suggest that the interaction between PARP1 and Pax8 may significantly affect a series of molecular events related to Pax8-dependent gene expression, thus disclosing important scenarios on mechanisms playing a central role in the regulation of thyroid-specific transcription.

Materials and methods

Reporter constructs and expression plasmids

The plasmids used have been previously described and are as follows: Tg-CAT (Sinclair *et al.* 1990), CP5-CAT (Missero *et al.* 1998), GST-Pax8 (Di Palma *et al.* 2003), GST-PD (pGEX-Pax8PB; Zannini *et al.* 1992), pCMV5-Pax8 (Zannini *et al.* 1992), and pPARP31 (Van Gool *et al.* 1997). The latter was kindly provided by Dr Kannan (MetroHealth Medical Center, Cleveland, OH, USA).

Protein identification by mass spectrometry

GST and GST-Pax8 proteins were purified from BL21 (DE) LysS bacterial cells as previously described (Zannini *et al.* 1992, Di Palma *et al.* 2003). The purified proteins were loaded onto glutathione–Sephadex beads (GE Healthcare, Waukesha, WI, USA) at a concentration of 1 µg/µl packed beads for 20 min at 4 °C.

A total of 5 × 10⁸ PC Cl3 cells were lysed; after a pre-clearing step on of GST-coated beads, the total extract was adsorbed with GST and GST-Pax8 beads. After washing, the bound proteins were resolved by SDS-PAGE, and visualized by ammoniacal silver staining according to Shevchenko's protocol (Shevchenko *et al.* 1996). For identification, protein bands were excised from the gel, triturated, *in-gel* Salkylated and digested with trypsin as previously reported (Caratu *et al.* 2007, Vascotto *et al.* 2007). Gel particles were extracted with 25 mM NH₄HCO₃/acetonitrile (1:1 v/v) by sonication. Samples were desalted using microZipTipC18 pipette tips (Millipore, Bedford, MA, USA) before MALDI-TOF-MS analysis. Peptide mixtures were loaded on the MALDI target together with α-cyano-4-hydroxycinnamic acid as matrix, using the dried droplet technique. Samples were analyzed with a Voyager-DE PRO spectrometer (Applied, Foster City, CA, USA; Caratu *et al.* 2007, Vascotto *et al.* 2007). Mass spectra were acquired in reflectron mode; internal mass calibration was performed with peptides from trypsin autolysis. Data were elaborated using the DataExplorer 5.1 software (Applied, USA). ProFound software (Zhang & Chait 2000) was used to identify protein bands from an NCBI non-redundant database using peptide mass fingerprint data. Candidates with program Est'd Z scores more than 2 were further evaluated by comparison with Mr experimental values obtained from SDS-PAGE. Eventual occurrence of protein mixtures was ascertained by sequential searches for additional protein components using unmatched peptide masses.

Protein extracts and immunoblotting

For reporter assays, cells were washed twice with ice-cold PBS and lysed in a buffer containing 10 mM HEPES pH 7.9, 400 mM NaCl, 0.1 mM EGTA pH 7.8, 5% v/v glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF). For GST-pull down assays, PC Cl3 cells were washed twice with PBS and lysed in whole cell extract (WCE) buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, 0.1% Triton X100, 10% v/v glycerol, 1 mM DTT, 1 mM PMSF and protease inhibitors (Sigma). For immunoprecipitation experiments, cells were washed twice with PBS and lysed in EBC buffer containing 50 mM Tris pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1 mM DTT, 1 mM PMSF and protease inhibitors (Sigma).

Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories Inc). For western blot analysis, proteins were separated by SDS-PAGE and the gels were blotted onto Immobilon P (Millipore) for 2 h; membranes were blocked in 5% nonfat dry milk in Tris-buffered saline for 2 h or overnight before the addition of antibody for 1 h. The primary antibodies used were anti-PARP1 (46D11; Cell Signaling, Boston, MA, USA), anti-Pax8 (kindly provided by Di Lauro), anti-PAR (96-10-04; Alexis, San Diego, CA, USA) and anti-FLAG-M2 (Sigma).

Pull down assays and co-immunoprecipitation

GST-PD and GST-Pax8 proteins were purified from BL21 (DE) LysS bacterial cells as previously described (Zannini *et al.* 1992, Di Palma *et al.* 2003).

GST, GST-Pax8 or GST-PD bound to glutathione beads were incubated with total protein extract prepared from PC Cl3 cells, or with 25 ng recombinant human PARP1 (ALX-201-063; Alexis). The binding reactions were carried out for 90 min at 4 °C on a rotating wheel. After extensive washing with WCE buffer, bound proteins were detected by western blot.

Co-immunoprecipitation experiments were performed by incubating 3 mg total protein extract with 40 µl anti-FLAG agarose affinity gel (Sigma), overnight, at 4 °C, on a rotating wheel. Bound proteins were washed several times with EBC buffer, resuspended in SDS-PAGE sample buffer and heated at 95 °C for 5 min, before loading on the gel. Alternatively, 3 mg total protein extracts were pre-cleared with 100 µl Protein A-Sepharose (GE Healthcare) and immunoprecipitated with PARP1 polyclonal antibody (46D11, Cell Signaling) overnight at 4 °C on a rotating wheel in EBC buffer. Hundred microliters protein A-Sepharose were added, and the samples were rocked for 1 h at 4 °C. After several washes with EBC buffer the bound protein were resuspended in 2X SDS-PAGE sample buffer and heated at 95 °C for 5 min before loading on the gel.

Poly(ADP-ribosyl)ation *in vitro*

6His-Pax8 purified recombinant protein (50, 100, and 150 ng) and recombinant human PARP1 (Alexis; 25 ng) were incubated for 10 min at 37 °C in binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM DTT) plus 10 µg/ml of sonicated DNA. In some reaction mixtures, 10 mM 3-aminobenzamide (3-AB; Sigma) was included. The reactions were started by adding 5 µCi (0.25 µM) ³²P-labeled NAD⁺ (GE Healthcare), and incubated at 37 °C for 10 additional minutes. After terminating the reactions with SDS sample buffer, the proteins were fractionated by SDS-PAGE through a

4–15% gradient gel. Poly(ADP-ribosyl)ated proteins were visualized by autoradiography.

For pull down experiments with purified proteins, recombinant human PARP1 (Alexis; 25 ng) was incubated for 10 min at 37 °C in a mixture (20 µl) containing 10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM DTT, plus 10 µg/ml of sonicated DNA. In this case, the reaction was started by adding 200 µM βNAD⁺ (Sigma) and further incubated for another 15 min. The SDS sample buffer was added to stop the reaction that was diluted 10-fold in WCE buffer and then used in the pull down assay.

Electrophoretic mobility shift assay (EMSA)

Double-stranded oligodeoxynucleotide C (5'-CACTGC-CCAGTCAAGTGTTCCTTGA-3') derived from the Tg promoter and containing one Pax8 binding site was labeled with γ-³²P ATP (GE Healthcare) and T4 polynucleotide kinase. Commercially available human PARP1, GST-Pax8, and GST-PD purified proteins were incubated for 20 min, at room temperature, in a binding buffer containing 10 mM HEPES (pH 7.9), 10% v/v glycerol, 0.1 mM EDTA, 8 mM MgCl, 1 mM DTT, 0.15 µg/ml of poly (dI-dC). In some reaction mixtures, βNAD⁺ (Sigma), 3-AB (Sigma), or both were included. Thus, ³²P-labeled oligodeoxynucleotide C (0.8 ng) was added, and the mixtures (20 µl) were incubated for additional 20 min. DNA-protein complexes were resolved on a 5% non-denaturing polyacrylamide gel and visualized by autoradiography.

Cell culture and transient transfection assays

PC Cl3 cells (Fusco *et al.* 1987, Berlingieri *et al.* 1988) were grown in Coon's modified F-12 medium (Euroclone) supplemented with 5% v/v calf serum and a six-hormone mixture (6H), as described by Ambesi-Impombato & Coon (1979).

HeLa cells were grown in Dulbecco's modified Eagle's medium (Euroclone) supplemented with 10% v/v fetal calf serum (Hyclone). For transient transfection experiments, cells were plated at 3 × 10⁵ cells/60-mm tissue culture dish 5–8 h prior to transfection, whereas PC Cl3 cells were plated at a density of 5 × 10⁵ cells/60-mm tissue culture dish 18 h prior to transfection. All transfections were carried out with the FuGENE6 reagent (Roche Diagnostics) according to the manufacturer's directions. The DNA/FuGENE ratio was 1:2 in all the experiments. The plasmid CMV-LUC was used as internal control in the transfection assays. Cells extracts were prepared 48 h after transfection to determine either the levels of the CAT protein with a CAT ELISA kit (Roche Diagnostics) or the LUC activity by a Luciferase assay, as previously described (de Wet *et al.* 1987). Transfection experiments were done in duplicate

and repeated at least three times. CAT activity values on the graph are the means of all experiments ($\pm$) s.d. Statistical analysis uses *t*-test ($P < 0.002$).

Results

A nuclear protein of 113 kDa binds to the transcription factor Pax8

To identify proteins that are able to interact with the transcription factor Pax8, we used the GST–Pax8 fusion protein in pull down assays with a total protein extract from 5×10^8 PC Cl3 thyroid cells. The extract was pre-cleared onto GST-loaded beads and then was divided and incubated with GST and GST–Pax8 loaded beads. Bound proteins were eluted, analyzed by SDS-PAGE and visualized by ammoniacal silver staining. Several proteins bound specifically to the bait (Fig. 1). Peptide mass fingerprint analysis identified the protein migrating at 113 kDa, which was specifically purified via GST–Pax8, as the PARP1 nuclear protein (SwissProt code P27008; Fig. 1). The experiment was performed twice, verifying in the mass spectra the occurrence of 11 and 10 peptides, corresponding to sequence coverage of 19 and 14%, respectively. Other bands of lower molecular weight were identified as GST–Pax8 degradation products (data not shown).

PARP1 interacts with Pax8 *in vitro* and *in vivo*

To confirm PARP1 as a Pax8-interacting protein, we performed small-scale GST-pull down assays incubating the recombinant GST–Pax8 protein with total protein

Figure 1 Pax8 interacts with PARP1. To isolate cellular proteins interacting with the GST–Pax8, glutathione-Sepharose beads loaded with GST–Pax8 or GST were incubated with total cell lysate obtained from PC Cl3, as described under Materials and Methods. After binding, the eluted proteins were separated on 4–15% SDS-PAGE and visualized by silver staining. Lane 1, proteins eluted from GST beads. Lane 2, proteins eluted from GST–Pax8 beads. The arrow indicates the protein band identified by MALDI-TOF peptide mass fingerprint analysis as PARP1. Brackets indicate fragments of the GST–Pax8 bait. Molecular mass markers are indicated on the left.

extract from 1×10^6 thyroid cells. The eluted material was loaded onto an SDS-PAGE mini-gel and subjected to western blot with a PARP1 specific polyclonal antibody. As shown in Fig. 2A, only the eluate from GST–Pax8 contained PARP1. To further investigate the hypothesis that Pax8 and PARP1 are able to interact directly, we performed pull down experiments using the fusion protein GST–Pax8 and purified human PARP1 protein. The beads loaded with GST–Pax8 were indeed able to co-precipitate the purified PARP1 protein, while the beads loaded with the GST protein did not co-precipitate any protein (Fig. 2B). These results demonstrated that the interaction between Pax8 and PARP1, already observed in pull down assays with total protein extracts, is indeed a direct protein–protein interaction.

We then investigated whether the interaction described above could also be observed *in vivo*. To this end, cell lysates obtained from PC Cl3 (mock transfected) and from a stable clone PC-3xFLAG-Pax8 (Di Palma *et al.* 2003) were immunoprecipitated with an anti-FLAG-agarose affinity gel and then analyzed by western blot with a monoclonal antibody against PARP1. As shown in Fig. 2C, PARP1 was specifically co-immunoprecipitated by anti-FLAG-agarose affinity gel only in the extract prepared from the clone PC-3xFLAG-Pax8, thus demonstrating that Pax8 and PARP1 co-exist in a complex *in vivo*. Consistent with this result, a PARP1 polyclonal antibody was able to co-immunoprecipitate the 3xFLAG-Pax8 protein (Fig. 2D).

Poly(ADP-ribosyl)ation of purified Pax8 protein

PARP1 is a nuclear protein that binds to DNA strand breaks and catalyzes ADP-ribosylation of itself and other nuclear protein, using NAD^+ as a co-factor. In order to examine whether Pax8 is directly modified by PARP1, we incubated purified human PARP1 and 6His-Pax8 purified protein in a reconstituted poly(ADP-ribosyl)ating enzyme system containing $^{32}\text{PNAD}^+$ to label the modified proteins. The reaction products were then analyzed by SDS-PAGE. As shown in Fig. 3, PARP1 was significantly auto-poly(ADP-ribosyl)ated, as indicated by the radioactive smear visible at a molecular weight higher than 113 kDa. The addition of an inhibitor of poly(ADP-ribosyl)ation, namely 3-aminobenzamide, abolished the automodification reaction of PARP1 (Fig. 3, lane 3), as already described. The presence of increasing amount of Pax8 did not exert a positive effect on the catalytic activity of PARP1 (Fig. 3, lanes 4–6). No poly(ADP-ribosyl)ation signals were instead detected at Pax8 molecular weight. Re-probing of the filter with antibodies against PARP1 and Pax8 confirmed the presence of the proteins in all the reactions and allowed us to conclude that while PARP1 is modified, Pax8 remains unmodified by poly(ADP-ribosyl)ation (Fig. 3).

Figure 2 Pax8 and PARP1 interact *in vitro* and *in vivo*. GST-pull down assays were performed with GST-Pax8 immobilized on glutathione-Sepharose beads. (A) protein extract prepared from PC Cl3 cells was incubated with GST-Pax8 and with GST alone as control; (B) purified human PARP1 was incubated with GST-Pax8 and with GST alone as control. After the binding reaction, the eluted proteins were loaded onto 10% SDS-PAGE, and analyzed by western blot using a PARP1-specific polyclonal antibody. The co-immunoprecipitation experiments were performed as follows: (C) three milligrams PC Cl3 and PC-3xFLAG-Pax8 total protein extract were incubated with anti-FLAG-agarose affinity gel. The asterisk indicates the band corresponding to the PARP1 protein; (D) three milligrams PC Cl3 and PC-3xFLAG-Pax8 total protein extract were incubated with the anti-PARP1 antibody and Protein A Sepharose. The bound protein (IP) and total lysate cells (Input) were separated on 10% SDS-PAGE and analyzed by western blot using a PARP1-specific polyclonal antibody and an anti-FLAG monoclonal antibody.

PARP1 binds the PD of Pax8 and inhibits its DNA-binding activity

To investigate the effect of the interaction with PARP1 on the Pax8 DNA-binding activity, EMSA assays were performed with bacterially expressed and purified proteins. The results reported in Fig. 4A show that the addition of increasing amounts of PARP1 to Pax8 caused a strong reduction of Pax8 binding to the double-stranded oligonucleotide containing its consensus sequence. At the same time, no retarded bands were observed in the reaction with PARP1 protein alone, thus suggesting that PARP1 cannot bind to the same oligonucleotide. We considered the possibility of explaining the results reported above on the basis of a putative interaction of PARP1 with Pax8 DNA-binding

domain, the PD. To test this hypothesis, we performed GST-pull down assays using the GST-PD (GST-PD) fusion protein and purified PARP1 protein. Results of the binding reactions demonstrated that GST-PD was indeed able to co-precipitate PARP1 protein with the same efficiency as GST-Pax8 (Fig. 4B). In order to elucidate whether poly(ADP-ribosyl)ated PARP1 retained an equal ability to interact with Pax8, PARP1 extensively modified *in vitro* was incubated with matrix-bound GST or GST-Pax8. Results of the binding reactions showed that poly(ADP-ribosyl)ated PARP1 interacts with Pax8 to the same extent as unmodified PARP1 (Figs 2A and 4C). The same result was obtained when GST-PD was used in place of GST-Pax8 (data not shown). Altogether, these data demonstrated that the

Figure 3 Susceptibility of Pax8 to covalent poly(ADP-ribosylation) *in vitro*. Twenty five nanograms of PARP1 were incubated in the absence (lanes 1 and 2) or presence of 50 ng (lanes 3 and 4) 100 ng (lane 5) and 150 ng (lane 6) of 6His-Pax8, of 0.25 μ M ³²P-labeled NAD⁺, 10 μ g/ml of sonicated DNA, 10 mM of 3-aminobenzamide (lane 3), at room temperature. Reactions were started by adding of ³²P-NAD and stopped by the addition of Laemmli buffer. Proteins were fractionated by SDS-PAGE through 4–15% gradient gel and subjected to western blot. The poly(ADP-ribosyl)ated proteins were visualized by autoradiography. The same filter was probed with anti-PARP1 and anti-Pax8 antibodies. The molecular weight markers are shown on the left.

interaction between Pax8 and PARP1 occurs via Pax8 PD and it is not affected by PARP1 automodification.

To further confirm the results reported above, we carried out EMSA assays with bacterially produced and purified GST-PD and modified or unmodified PARP1 protein. As shown in Fig. 4D, the addition of PARP1 inhibited GST-PD binding to DNA both in the absence or presence of β NAD⁺ (lanes 3 and 4). Along the same line, the inhibition of the auto-poly(ADP-ribosylation) reaction of PARP1 with 3-AB led to the same inhibition of Pax8-PD binding to DNA (lane 5).

In conclusion, these data consistently suggested that, independent of the poly(ADP-ribosylation) reaction, the PARP1 interaction with Pax8 negatively influences the DNA-binding activity of the transcription factor.

PARP1 behaves as a transcriptional repressor for Pax8

Since we demonstrated that PARP1 binds to Pax8 DNA-binding domain, we hypothesized that the interaction between the two proteins could affect Pax8 transactivating properties. To elucidate whether this physical interaction between Pax8 and PARP1 could affect Pax8 transcriptional activity, we performed transactivation

assays in HeLa cells co-transfecting the reporter construct CP5-CAT, an artificial promoter responsive only to Pax8, together with the expression vectors encoding for Pax8 and PARP1, separately or in combination. As expected, the transfection of Pax8 alone led to a significant activation of the CP5-CAT promoter, while the transfection of PARP1 alone decreased by the basal activity of the promoter only a little, due to its suppressive activity on RNA polymerase (Oei *et al.* 1998; Fig. 5A). Interestingly, the co-expression of Pax8 and PARP1 led to an inhibition of Pax8-dependent transcription (Fig. 5A). The same effect was observed in the thyroid differentiated cell line PC Cl3. Also in this case, over-expression of PARP1 produced a significant reduction of the transcriptional activity of endogenously expressed Pax8 on the transfected CP5-CAT promoter (Fig. 5B). To further investigate whether PARP1 could inhibit Pax8 transcriptional activity not only on the synthetic promoter CP5-CAT, but also in a more physiological context represented by the Tg promoter, we repeated the same transactivation assays described before, this time using the reporter Tg-CAT.

Therefore, we co-transfected the expression vector encoding for PARP1 in PC Cl3 cells together with the

reporter construct Tg-CAT. Also in these experiments, we observed that PARP1 decreased the transcriptional activity of endogenous Pax8 on the Tg-CAT promoter in an analogous manner to that observed on the CP5-CAT promoter (Fig. 5C).

Figure 4 Independently of its automodification PARP1 interacts with Pax8-PD and inhibits its specific DNA binding. (A) EMSA was performed as described under experimental procedures. Increasing amounts of PARP1 (range of 0 (–), 0.2 (+), 0.5 (++) , 1 (+++) μ g) were incubated with GST-Pax8 (0 (–), 0.05 (+) μ g) for 20 min, at room temperature. After electrophoresis on native 5% polyacrylamide gels, Pax8-oligonucleotide complexes were visualized by autoradiography. Lane 1, DNA probe control; lane 2, GST-Pax8 control; lanes 3–5, effect of PARP1 on Pax8 mobility shift; lane 6, PARP1 control. (B) Bacterially produced GST-PD, GST-Pax8 or GST alone was mixed with glutathione-Sepharose beads and incubated with an equivalent amount of purified PARP1. The eluted proteins were loaded onto a 10% SDS-PAGE, and analyzed by western blot using a PARP1-specific monoclonal antibody. Lane 1, purified PARP1. Lane 2, PARP1 protein precipitated with GST for control. Lane 3, PARP1 protein precipitated with GST-PD. Lane 4, PARP1 protein precipitated with GST-Pax8. (C) *In vitro* modified PARP1 as described under experimental procedures was incubated with GST-Pax8 or GST as control. Both GST and GST-Pax8 beads were washed several times before being loaded onto a 4% to 15% gradient gel, and analyzed by western blot with anti-PAR, a polyclonal antibody to Poly(ADP-ribose). (D) EMSA was performed as described above. PARP1 (0 (–), 0.5 (+) μ g) and GST-PD (0 (–), 0.015 (+) μ g) were co-incubated in the absence (–) or presence (+) of 200 μ M β NAD⁺. Lane 5, shows the effect of 10 mM 3-aminobenzamide. After electrophoresis on native 5% polyacrylamide gels, PD-oligonucleotide complexes were visualized by autoradiography. Lane 1, DNA probe control; lane 2, GST-PD control; lanes 3–5, effect of native or modified PARP1 on PD mobility shift; lane 6, PARP1 control; lane 7, modified PARP1 control.

Discussion

It is well known that the activity of transcription factors is regulated by the physical interaction with coactivators and co-repressor molecules. This crosstalk plays a pivotal role in the determination and maintenance of the cellular differentiated phenotype. Among the transcription factors involved in thyroid-specific gene expression, Pax8, a member of the *Pax* gene family, plays a critical role in the expression of all the thyroid-specific genes that are considered markers of the differentiated phenotype (Pasca di Magliano *et al.* 2000). The analysis of knockout mice confirmed that Pax8 has a key role in the development and differentiation of the thyroid gland. In fact, Pax8 knockout mice have a very small thyroid gland, which is deprived of follicular cells (Mansouri *et al.* 1998). Recently, it has been demonstrated that Pax8 cooperates with the transcription factor NKX2-1 (Espinoza *et al.* 2001, Miccadei *et al.* 2002), and our laboratory specifically demonstrated that Pax8 and NKX2-1 form a functional heterocomplex responsible for the synergistic transcriptional activation of the Tg promoter in differentiated thyroid cells (Di Palma *et al.* 2003). The relevance of this interaction has been further confirmed by the analysis of the *in vivo* mouse model bearing a partial deficiency of *Tcf1* and *Pax8* genes. The double-heterozygous null mice show thyroid dysgenesis, a phenotype that is completely absent in either of the single heterozygous mice (Amendola *et al.* 2005). To identify proteins interacting with Pax8 that could modulate its transcriptional activity, a protein lysate obtained from the thyroid differentiated cell line PC Cl3 was incubated with the GST-Pax8 fusion protein, and proteins able to bind to GST-Pax8 were identified. Several proteins bound to the bait, and the PARP1 protein was characterized as a Pax8 interactor by multiple independent experiments. PARP1 is the founding member of the human PARP family, whose members (18 different genes) all display a conserved catalytic domain (Ame *et al.* 2004). PARP1 is a highly conserved nuclear enzyme that binds to single and double-stranded DNA breaks and it is thought to function as a sensor of DNA damage (de Murcia & Menissier de Murcia 1994, Dantzer *et al.* 1999).

In this study, we provide evidence of a physical and functional interaction of PARP1 with the transcription factor Pax8. GST-pull down and co-immunoprecipitation assays indicated that PARP1 binds to Pax8 *in vitro* and *in vivo*, and *in vitro* binding studies with purified proteins showed that the interaction between Pax8 and PARP1 is direct. The identification of PARP1 as a partner of Pax8 is consistent with many reports that suggest the role of PARP1 in the control of gene expression (Oei *et al.* 1998, D'Amours *et al.* 1999, Kraus & Lis 2003). Data reported in the literature propose that PARP1 influences gene transcription modifying directly histones and structural proteins of the chromatin (Poirier *et al.* 1982, D'Amours

et al. 1999, Huang *et al.* 2006) and/or regulating transcription by directly interacting with other DNA binding factors and co-activators (D'Amours *et al.* 1999, Hassa & Hottiger 2002). It has been suggested that PARP1 can behave both as an inhibitor or an activator of the bound transcription factors. In fact, PARP1 binds to and inactivates the DNA binding ability of p53 by covalent poly(ADP-ribosyl)ation (Kumari *et al.* 1998, Mendoza-Alvarez & Alvarez-Gonzalez 2001), and it functions as a suppressor of the RAR/RXR signaling (Miyamoto *et al.* 1999) and of the RNA polymerase II (Oei *et al.* 1998). On the other hand PARP1 has been reported to act as a transcriptional co-activator for NF- κ B (Hassa & Hottiger 1999, 2002, Hassa *et al.* 2001, Nakajima *et al.* 2004), AP-2 (Kannan *et al.* 1999) and B-MYB (Cervellera & Sala 2000). Depending on the different situation, its enzymatic activity has been shown to be necessary as well as unnecessary to regulate gene expression (Poirier *et al.* 1982, Meisterernst *et al.* 1997, Oei *et al.* 1998, Miyamoto *et al.* 1999, Cervellera & Sala 2000, Hassa *et al.* 2003). Our findings indicate that PARP1 acts as a potent inhibitor of Pax8 transcriptional activity by interfering with the formation of the Pax8-DNA complex. In particular, our results suggest that PARP1 elicits its repressor activity on Pax8 independently of its automodification.

DNA repair and transcription are apparently closely related; in fact, association of DNA repair factors with the RNA polymerase II complex has been reported (Bardwell *et al.* 1994, Roy *et al.* 1994). Data obtained from mice lacking the PARP1 gene demonstrated its role in DNA repair (Wang *et al.* 1995, de Murcia *et al.* 1997) and *in vitro* assays with the purified enzyme and cellular extracts indicated the effect of poly(ADP-ribosyl)ation on transcriptional repression (Oei *et al.* 1998). Furthermore, it has been reported that not only PARP1 activation but also its over-expression correlates with various diseases such as malignant melanomas, hepatocellular carcinoma, colorectal carcinoma, and patho-physiological cardiac hypertrophy (Shimizu *et al.* 2004, Pillai *et al.* 2005, Staibano *et al.* 2005, Noshio *et al.* 2006). Taken together, all these observations indicate

Figure 5 PARP1 strongly inhibits Pax8 transcriptional activity. (A) HeLa cells were transiently transfected with the reporter plasmid CP5-CAT (2 μ g) with or without the expression vectors encoding for Pax8 (3 ng) and PARP1 (pPARP-31 (see materials and methods) range of (0.1 μ g), (0.2 μ g), (0.3 μ g)). PC C13 cells were transiently transfected with the reporter constructs CP5-CAT (0.1 μ g; B), Tg-CAT (0.2 μ g; C), in the absence or in the presence of increasing concentration of expression vectors encoding for PARP1 (pPARP-31 (see materials and methods) range of (0.1 μ g), (0.2 μ g), (0.5 μ g), (1.0 μ g)) (B and C). The cells were subsequently harvested and assayed for CAT activity. Folds of activation are considered as ratio between values obtained with and without co-transfection of the expression vectors. CMV-LUC was added as internal reference, and CAT values were normalized to the LUC activity. CAT values are the means $\pm$ s.d. for at least three independent experiments.

that cell stressor, not necessarily associated with DNA damage, could modify the expression level of PARP1 and, in this way, regulate transcription by both a specific and/or a general mechanism.

In this work, we propose that PARP1 acts a co-factor of Pax8, which is essential to preserving the physiological thyroid context and to allow the fine-tuning of the differentiated thyroid phenotype. Nevertheless, the observed interaction between PARP1 and Pax8 calls for additional studies to define the exact role of PARP1 in the transcriptional regulation of thyroid-expressed genes. It will be of interest to investigate the effect of PARP1 over-expression with respect to proliferation and differentiation of thyroid cells, and to analyze the effect of PARP1 absence, both *in vitro* by RNAi in differentiated thyroid cells and *in vivo* by studying the thyroid phenotype of PARP1 knockout mice.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work has been supported by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC) to M Z and from the Ministry of Instruction, University and Research (MIUR FIRB 2001 cod. RBAU01PRLA) to A S T. Di P has been supported by a fellowship from the Federazione Italiana per la Ricerca sul Cancro (FIRC).

Acknowledgements

The authors gratefully acknowledge Dr P Kannan for providing reagents and the Service of Molecular Biology (SBM) of the Stazione Zoologica A Dohrn of Naples for their technical assistance.

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Received in final form 30 July 2008

Accepted 3 September 2008

Made available online as an Accepted Preprint 3 September 2008