WBP-2, a WW domain binding protein, interacts with the thyroid-specific transcription factor Pax8

Roberto NITSCH, Tina DI PALMA, Anna MASCIA and Mariastella ZANNINI[1]

Istituto di Endocrinologia ed Oncologia Sperimentale–CNR and Dipartimento di Biologia e Patologia Cellulare e Molecolare, Universita' di Napoli Federico II, Via Pansini 5, 80131 Napoli, Italy

The *Pax* gene family encodes transcription factors that are essential in organogenesis and in the differentiation of various organs in higher eukaryotes. Pax proteins have a DNA binding domain at the N-terminus, and a transcriptional activation domain at the C-terminus. How these domains interact with the transcriptional machinery of the cell is still unclear. In the present paper, we describe the identification by means of immunological screening of the WW domain binding protein WBP-2 as a biochemical interactor of Pax8 (a WW domain is a protein-interaction domain containing two conserved tryptophan residues). Pax8 is required for the morphogenesis of the thyroid gland and for the maintenance of the thyroid differentiated cellular phenotype. WBP-2 was identified originally as a WW domain binding protein, and its function is still unknown. WBP-2 binds to Pax8 *in vitro* in pull-down assays, and *in vivo* in tissue culture cells in co-immunoprecipitation assays. Interestingly, Pax8 does not contain a WW domain. Our results point to the identification of a new protein-interacting domain that is present in the C-terminal portion of Pax8 and that is required for protein–protein interaction with WBP-2. Our results demonstrate that WBP-2 is not a transcriptional co-activator of Pax8, but rather behaves as an adaptor molecule, as suggested in other studies.

Key words: adaptor molecule, Pax protein, protein–protein interaction, thyroid, transcriptional regulation, WW domain binding protein (WBP).

INTRODUCTION

It is well known that activation of gene expression involves interplay between various sequence-specific transcription factors and transcriptional co-regulators with the basal transcriptional machinery of the cells. Sequence-specific transcription factors are considered to tether multisubunit co-regulator complexes through protein–protein interactions with the promoter. The functions of transcriptional co-activators can be divided broadly into two overlapping categories. First, co-activators can mediate chromatin modifications either through acetylating reactions mediated by histone acetyltransferases [1] or through nucleosome remodelling complexes [2]. Alternatively, some co-activators function as bridging factors that mediate complex formation between sequence-specific transcription factors and RNA polymerase II or other components of the basal transcription machinery [3,4]. Moreover, adaptor molecules are non-catalytic polypeptides that contain one or more domains that are able to bind to other proteins or non-protein ligands [5,6]. These molecules selectively control the spatial and temporal assembly of multiprotein complexes that transmit intracellular signals involved in the regulation of cell growth, differentiation, migration and survival [5,6].

The *Pax* family of genes encode DNA binding proteins which regulate the development of a variety of tissues in a range of species, such as flies, worms and humans [7–9]. *Pax* genes have also been implicated in the initiation and maintenance of the differentiated cellular phenotype. Despite their critical role during development and in human disease processes, the biochemical basis of Pax protein function within the cell nucleus and in the context of chromatin structure remains obscure. In mammals, there are nine known *Pax* genes, grouped into four different classes based on the identity within their DNA binding domain (the paired domain), on gene structure and on expression pattern [10]. All Pax proteins have a paired domain, which spans 128 amino acids near the N-terminus and consists of two helix–turn–helix motifs [11]. Sequence conservation among Pax proteins is highest in the paired domain, but can also be extended to a paired-type homeodomain and to a stretch of residues between the paired domain and the homeodomain called the octapeptide. The homeodomain and octapeptide are not present in all Pax proteins, but rather in a subset of them. The homeodomain represents a second *bona fide* DNA binding moiety in some classes of Pax proteins, and may enhance DNA target specificity in co-operation with the paired domain [12–15]. However, members of the Pax2/5/8 subclass have only a partial homeodomain, which is unlikely to participate in DNA binding [16]. Transcriptional activation and repression activities map to the C-terminus of Pax proteins [17–19].

Pax8 is a member of the murine Pax family of genes, and is expressed in the developing kidney, neural tube, and developing and adult thyroid [20]. Specifically, Pax8 has been demonstrated to be required both for morphogenesis of the thyroid gland [21] and for maintenance of the thyroid differentiated phenotype [22]. Together with another thyroid-specific transcription factor named TTF-1, Pax8 is involved in the regulation of thyroid-specific genes such as those encoding Tg (thyroglobulin), TPO (thyroperoxidase) and the NIS (sodium/iodide symporter) [23,24]. Pax8 binds to a single site on the Tg and TPO promoters and, in both cases, the functional role of its binding sites within these promoters has been studied in rat, bovine and human [25–28]. Recently, studies from our laboratory have demonstrated a

Abbreviations used: CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; LUC, luciferase; NIS, sodium/iodide symporter; RT-PCR, reverse transcription–PCR; Tg, thyroglobulin; TPO, thyroperoxidase; TTF, thyroid-specific transcription factor; TRITC, tetramethylrhodamine β-isothiocyanate; WBP, WW domain binding protein; tWBP-2, thyroid-specific transcript of WBP-2; WW domain, protein-interaction domain containing two conserved tryptophan residues.

[1] To whom correspondence should be addressed (e-mail stella@szn.it).

biochemical and functional interaction between TTF-1 and Pax8 [29]. It is likely that, depending on the promoter context within target genes, Pax8 and TTF-1 can act separately or in co-operation with various factors or cofactors to regulate gene expression. There is increasing evidence that TTF-1 functions cooperatively with a number of the other transcription factors, forming complexes on regulatory regions of target genes [30–35]. At the same time, as has already been observed for other members of the *Pax* gene family [36,37], Pax8 is likely to play a role in the recruitment to the thyroid promoters of other factors, such as co-activators.

We have focused our studies on the molecular mechanisms involved in Pax8-mediated transcriptional regulation. To identify proteins that interact with Pax8, we performed an immunological screening of a rat thyroid cDNA expression library using as a probe the fusion protein GST (glutathione S-transferase)–Pax8. We have thus identified the WW domain binding protein WBP-2 as a partner of Pax8 (a WW domain is a protein-interaction domain containing two conserved tryptophan residues). In addition, we have also characterized an alternative splicing form of WBP-2, which we have named tWBP-2, that is expressed only in the thyroid. WBP-2 is ubiquitously expressed [38], but its function is still unknown. Our results suggest that WBP-2 is not a transcriptional co-activator of Pax8, but rather behaves as an adaptor molecule.

EXPERIMENTAL

Screening of a λgt11 expression library and DNA sequencing

A rat thyroid λgt11 expression library ($> 10^8$ plaque-forming units $\cdot$ ml^{-1}) was generated in our laboratory. Recombinant phages were plated at 50 000 plaque-forming units per 90-mm plate of *Escherichia coli* Y1090, and screened with the fusion protein GST–Pax8. Briefly, in order to obtain cell infection, cells and phages were first incubated at 42 °C in NZY top-agarose medium and then plated. Plates were incubated for 4 h at 42 °C, overlaid with nitrocellulose membranes saturated with isopropyl β-D-thiogalactoside, and then incubated overnight at 37 °C. Membranes were washed extensively with PBS and blocked by incubation with Tris-buffered saline containing 0.05 % (v/v) Tween-20 and 2 % (w/v) BSA. To reduce the background, we incubated the membranes for 1 h in a solution of Tris-buffered saline containing Tween-20, 0.5 % BSA and 3 μM GSH. The same solution containing 0.3 g/ml GST–Pax8 was used to incubate membranes for 1 h at room temperature. After several washes with 150 mM NaCl, 20 mM Tris/HCl, pH 7.5, and 0.05 % Tween 20, membranes were incubated first with the antibody anti-GST (1 μg/ml), and then with iodinated ^{125}I-Protein A at a concentration of 0.5×10^6 c.p.m./ml. Visualization was by autoradiography. Positive clones were purified through three rounds of screening. Extraction of phage DNA was performed using the Qiagen kit 'lambda midi kit'. The inserts were amplified from colony-purified cDNA clones using specific λgt11 oligonucleotide primers. Amplification was performed following a general protocol consisting of 5 min of denaturation at 95 °C, and then 30 cycles of 60 s at 94 °C, 60 s primer annealing at 65 °C and 60 s extension at 72 °C. An additional 5 min incubation at 72 °C was performed to allow the termination of initiated molecules. The amplified cDNA insert was then separated by electrophoresis in a 0.8 % (w/v) agarose gel and purified using the QIAEX II gel extraction kit (Qiagen). Sequencing of cDNA inserts on both strands was performed by the Service of Molecular Biology (SBM) of the Stazione Zoologica A. Dohrn (Napoli, Italy). Sequence similarity searches were performed with the BLAST program.

Plasmids

The GST–Pax8 fusion protein was generated by PCR amplification of the Pax8 coding region and subsequent subcloning in the *Eco*RI site of the pGEX-4T3 vector. The orientation of the fragment and the correct reading frame of the fusion protein were verified by DNA sequencing. The GST–WBP-2 fusion protein was generated by PCR amplification of the WBP-2 coding region and subsequent cloning in the *Eco*RI/*Xho*I sites of the pGEX-4T3 vector. 3 × FLAG–WBP-2 was generated by PCR amplification of the WBP-2 coding region and subsequent cloning in the *Eco*RI/*Xba*I sites of the 3 × FLAG CMV-10 vector (Sigma). The Pax8 deletion mutant Δ349 was generated by PCR and subcloned in the expression vector pCMV5.

The plasmids used in transient transfection experiments have been described previously, and were as follows: Tg-CAT [39] (where CAT is chloramphenicol acetyltransferase), TPO-LUC [40] (where LUC is luciferase), NIS-LUC9 [41] and pCMV5-Pax8 [25]. CMV-CAT and CMV-LUC plasmids were used as internal controls in transfection assays. The DNA of all plasmids was prepared by Qiagen cartridges and used for cell transfection.

Cell culture and transfection

PC Cl3 rat thyroid cells were grown in Coon's modified F12 medium (EuroClone) supplemented with 5 % (v/v) calf serum and a six-hormone mixture as described by Ambesi-Impiombato and Coon [42].

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum. For transactivation experiments, cells were plated at a density of 3×10^5 cells/60-mm tissue culture dish, 12–24 h prior to transfection. Transfections were carried out using the FuGene6 reagent (Roche Diagnostics, Indianapolis, IN, U.S.A.) as suggested by the manufacturer. The DNA/FuGene ratio was 1:2 in all experiments.

Cell extracts were prepared 48 h after transfection to determine either the levels of CAT protein with a CAT ELISA (Boehringer Mannheim) or LUC activity, as described previously [25].

Transfection experiments were performed in duplicate and repeated at least three times.

In vitro and *in vivo* protein interaction assays

GST–Pax8 protein was purified from BL21 (DE) LysS bacterial cells transformed with pGEX-Pax8. At $D_{600} = 0.6$, isopropyl β-D-thiogalactoside (0.1 mM final) was added to the culture to induce the expression of the fusion protein, and cells were harvested 4 h later. Cells were resuspended in lysis buffer (1 × PBS, 0.5 mM EDTA, 1 mg/ml lysozyme, 0.5 mM dithiothreitol, 1 mM PMSF and protease inhibitors diluted 1:1000), and sonicated. Triton X-100 (1 %, v/v) was then added, and the cell extract was centrifuged at 200 000 ***g*** for 40 min at 4 °C. The supernatant was subjected to affinity chromatography using glutathione–agarose beads (Amersham-Pharmacia). After binding, beads were washed three times with washing buffer (10 mM Tris/HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, 5 mM dithiothreitol). GST–Pax8 was eluted with a buffer containing 10 mM glutathione, 50 mM Tris/HCl, pH 8.0, and 50 mM NaCl for 10 min at 4 °C. The eluted protein was stored at − 80 °C. GST–WBP-2 protein was purified using the same procedure as described above. Protein concentration was judged from Coomassie Blue staining.

Pull-down assays were performed by challenging 4 μg of GST or GST–WBP-2 purified protein bound previously to glutathione–agarose beads with affinity-purified bacterial Pax8 protein. The binding reactions were carried out for 90 min at 4 °C on a rotating

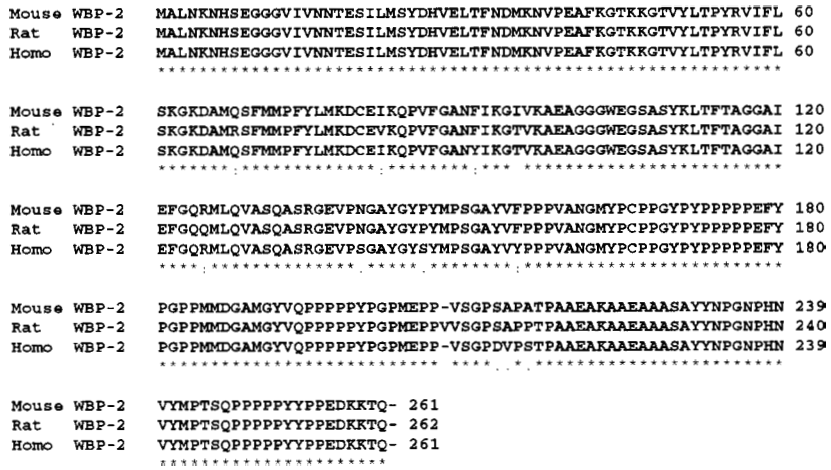

Figure 1 Alignment of the predicted amino acid sequences of mouse, rat and human WBP-2 proteins

Identities, similarities and conservative changes are indicated.

wheel, and then the beads were washed several times with a buffer containing 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.1 % Triton X-100, 10 % (v/v) glycerol, 1 mM dithiothreitol, 1mM PMSF and protease inhibitors (from Sigma). The bound protein was eluted by resuspending the beads directly in 2 × SDS/PAGE sample buffer and heating at 95 °C for 3–5 min before loading on the gel.

The co-immunoprecipitation experiment was performed by incubating 2 mg of total protein extract with 20 μl of anti-FLAG agarose affinity gel (Sigma) overnight at 4 °C on a rotating wheel. The samples were then centrifuged and the agarose gel-bound proteins were washed several times with a buffer containing 50 mM Tris/HCl, pH 7.5, 120 mM NaCl, 0.5 % Nonidet P40, 10 % (v/v) glycerol and protease inhibitors, resuspended in 2 × SDS/PAGE sample buffer and heated at 95 °C for 3–5 min before loading on the gel.

For Western blot analysis, gels were blotted on to Immobilon-P membranes. Immunodetection was performed using a specific anti-Pax8 rabbit polyclonal antibody (α-mPax8-bI) diluted 1:5000 in 150 mM NaCl, 20 mM Tris/HCl, pH 7.5, and 0.05 % Tween 20 containing 0.5 % (w/v) non-fat dry milk. Subsequently, the filters were developed using an ECL® detection method (Amersham) according to the manufacturer's directions.

Indirect immunofluorescence

Cells were grown directly on glass coverslips for 72 h, fixed in 4 % (v/v) paraformaldehyde in PBS for 20 min at room temperature, permeabilized for 7 min in 0.1 % (v/v) Triton X-100 in PBS and incubated for 10 min in 0.1 M glycine in PBS. The coverslips were subsequently incubated for 1 h with a mixture of primary antibodies diluted in 0.5 % (w/v) BSA in PBS and then, after extensive PBS washing, incubated for 20 min with a mixture of fluorescein-tagged goat anti-mouse and rhodamine-tagged goat anti-rabbit secondary antibodies diluted 1:50 in 0.5 % BSA in PBS. After final washes with PBS, the coverslips were mounted on a microscope slide using a 70 % (v/v) solution of glycerol in PBS.

Primary antibodies were rabbit polyclonal anti-Pax8 (α-mPax8-bI) and anti-c-Myc (9E10) monoclonal antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.).

Confocal scanning laser microscopy

Images were collected with a Zeiss LSM 510 confocal laser scanning microscope equipped with a 488 nm argon ion laser, a 543 nm HeNe laser and a Plan-Apochromat 63 ×/1.4 oil-immersion objective. Emitted fluorescence was detected using a BP 505-530 bandpass filter for FITC and an LP 560 longpass filter for TRITC (tetramethylrhodamine β-isothiocyanate). Pairs of images were collected simultaneously in the green and red channels.

RESULTS

Identification of a Pax8-interacting protein by immunological screening of an expression library

To identify potential Pax8 interactors, immunological screening of a rat thyroid cDNA expression library was performed, using the fusion protein GST–Pax8 as bait. Approx. 7×10^6 recombinant phage plaques were screened with GST–Pax8 protein purified from bacterial cells. After the fourth round of purification, a total of 50 clones were obtained. In order to eliminate false-positive signals, a second screening of the clones was performed utilizing the GST protein alone as bait. Of the 50 clones, 27 were confirmed as positive and characterized further. The clones were shown to contain inserts ranging from 1.2 to 2.5 kb in size, and the entire nucleotide sequence of each cDNA insert was determined. A database search revealed that a few sequences were indeed non-coding sequences, 14 sequences were unknown genes and await further investigation, whereas 11 sequences showed identity, albeit to differing extents, with known genes. One clone, denoted 1d, showed identity with the known protein WBP-2 [38], was detected twice and was selected for further study. Indeed, the comparison of the sequence of 1d protein against the databases revealed almost total identity with mouse and human WBP-2 proteins (Figure 1). In fact, clone 1d contained the full-length cDNA of rat WBP-2 (GenBank® accession number no. NM_138975), and was therefore renamed WBP-2.

Expression analysis of WBP-2 and characterization of a thyroid-specific isoform

In order to examine the pattern of expression of WBP-2 in different cell types, we performed RT-PCR (reverse transcription–PCR) analysis on total RNA prepared from thyroid and non-thyroid cells, using primers specific for WBP-2. As shown in Figure 2(A), we found WBP-2 to be expressed in all cell types analysed, as suggested by previous studies [38]. Interestingly, another

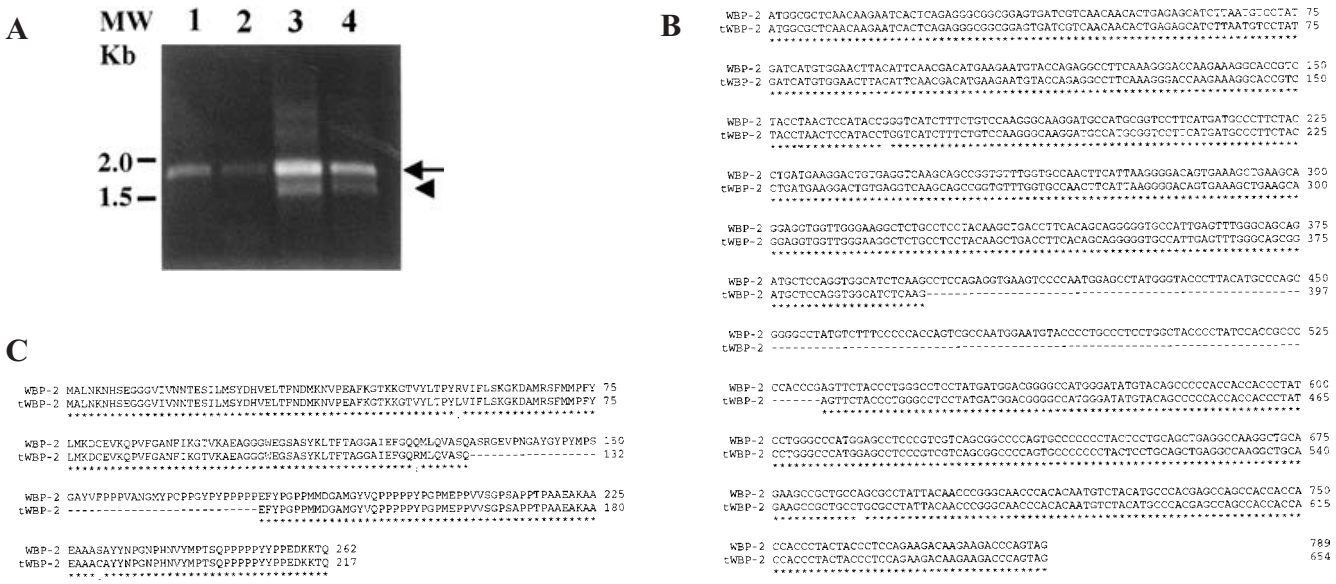

Figure 2 Two WBP-2 isoforms are expressed in thyroid cells

(**A**) RT-PCR was performed on total RNA from different cell lines: lane 1, NIH 3T3; lane 2, HeLa; lane 3, PC Cl3; lane 4, FRTL-5. PC Cl3 and FRTL-5 cells are both rat thyroid cells, while NIH 3T3 and HeLa are non-thyroid cells. The arrow and arrowhead indicate the amplified DNA fragments corresponding to WBP-2 and tWBP-2 respectively. (**B**) Alignment of the nucleotide sequences of WBP-2 and tWBP-2. (**C**) Alignment of the amino acid sequences of WBP-2 and tWBP-2.

smaller transcript of 1.7 kb was present only in the RNA of thyroid cells, suggesting the existence of an alternatively spliced isoform (Figure 2A). To verify this hypothesis, the RT-PCR product corresponding to the 1.7 kb transcript (denoted tWBP-2) was subcloned and completely sequenced. The sequencing data showed that the N-terminal and C-terminal sequences of tWBP-2 were completely identical with those of WBP-2; however, an internal deletion of 135 bp was observed (Figure 2B). The open reading frame of tWBP-2 cDNA encodes a polypeptide of 217 amino acids, whose only difference from the WBP-2 protein is the lack of 45 amino acids (Figure 2C). To understand the origin of the 135 bp deletion, a genomic DNA fragment spanning the deletion was cloned by PCR. The nucleotide sequence of 1022 bp was obtained, and the presence of one exon surrounded by two introns was detected (Figure 3B). Thus our data suggest that the shorter transcript (tWBP-2) corresponds to a thyroid-specific variant of WBP-2, generated by alternative splicing (Figure 3A). As shown in Figure 2(A), the level of expression of tWBP-2 is very low compared with that of WBP-2. This could be the reason why this isoform cannot be detected in expression studies performed by Northern blot (results not shown). Moreover, these results demonstrate that the full-length WBP-2 protein is expressed ubiquitously in all cell types, while alternatively spliced forms (one identified by us) could generate protein products responsible for cell-type-specific functions.

To obtain information on the subcellular distribution of WBP-2 and tWBP-2, we transiently transfected PC Cl3 thyroid cells with expression vectors encoding the two proteins, and analysed their localization by indirect immunofluorescence. Since specific antibodies against the WBP-2 protein are not available, PC Cl3 cells were transfected with expression vectors encoding Myc-tagged WBP-2 and tWBP-2 proteins. For immunofluorescence analysis, proteins were labelled using an anti-Myc primary antibody and a rhodamine-conjugated secondary antibody. The antibody against Pax8 is highly specific, as shown in previous studies [29]. Confocal microscopy examination of dual-labelled samples confirmed that the anti-Pax8 antibody stained only the nuclei, and did not give any staining outside the nucleus (Figures 4b and 4e). At the same time, WBP-2 and tWBP-2 proteins were detected both in the cytoplasmic and in the nuclear compartments (Figures 4a and 4d); only in the latter were they co-localized with Pax8 (Figures 4c and 4f).

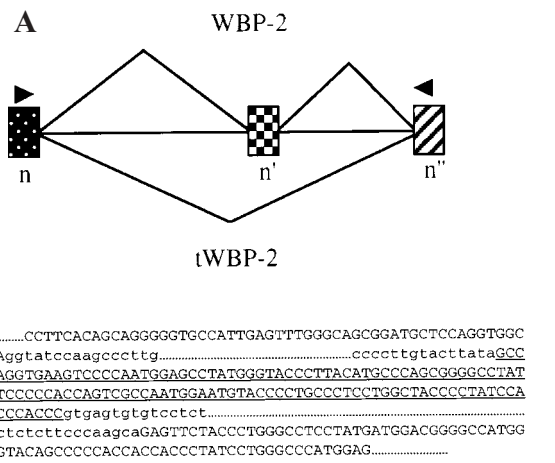

Figure 3 WBP-2 isoforms are generated by alternative splicing

(**A**) Schematic representation of the genomic organization of WBP-2 leading to alternative splicing. Approx. 1 kb of mouse genomic DNA fragment was subcloned by the PCR method. The arrowheads indicate the primers used (RN-10 and RN-11). The mRNA of tWBP-2 lacks the exon n′ sequence. (**B**) Partial nucleotide sequence of WBP-2 genomic DNA. The nucleotide sequence corresponding to the exons is in upper case. The alternatively spliced exon (n′) is underlined.

Pax8 and WBP-2 interact directly *in vitro*

To confirm the direct interaction between Pax8 and WBP-2, pull-down assays were performed using a bacterially expressed and

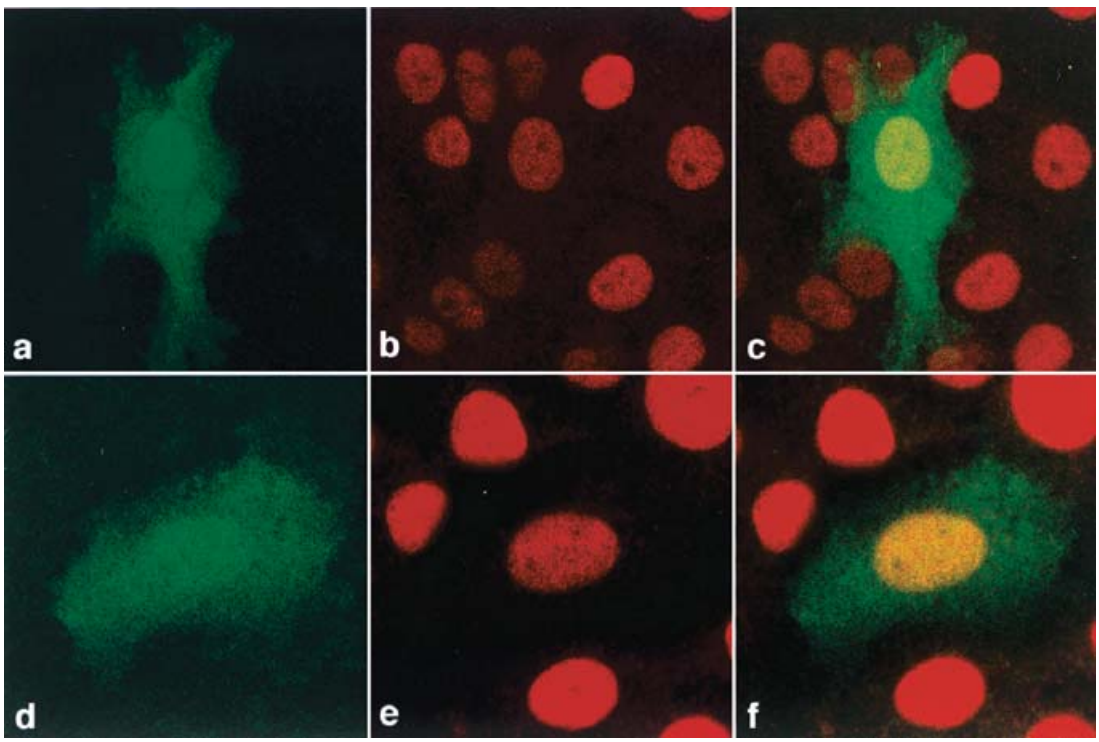

Figure 4 Localization of WBP-2 and tWBP-2 in PC Cl3 thyroid cells

PC Cl3 cells were plated on glass coverslips and subsequently transfected with 250 ng of an expression vector encoding Myc-tagged WBP-2 or tWBP-2. At 48 h after transfection, cells were double-stained by immunofluorescence with a monoclonal anti-Myc antibody (**a** and **d**) and with a polyclonal anti-Pax8 antibody (**b** and **e**). Fluorescein isothiocyanate and TRITC signals were acquired together at a confocal microscope, by line-wise scanning. The staining for WBP-2 (**a**) and tWBP-2 (**d**) proteins was detected both in the nucleus and in the cytosol of the cells. Pax8 staining (**b** and **e**) was exclusively nuclear, as described previously [29]. The overlay of the signals is shown in (**c**) and (**f**). Bar = 5 μm.

purified GST–WBP-2 fusion protein and an affinity-purified bacterial Pax8 protein. As shown in Figure 5(A) (lane 2), Pax8 bound specifically to the GST–WBP-2 fusion protein loaded on glutathione–Sepharose beads. Figure 5(A) (lane 1) shows that Pax8 was not able to bind to GST protein alone loaded on the beads. This result proves that the interaction between Pax8 and WBP-2, observed in the immunological screening, is a specific and direct interaction. The protein domain of WBP-2 involved in protein–protein interactions has been described [38]. Since that portion of the protein is not affected in the splicing event and thus is present in tWBP-2 as well as in WBP-2, the biochemical interaction between Pax8 and tWBP-2 was not investigated further.

The interaction between Pax8 and WBP-2 occurs *in vivo* and is mediated by the C-terminal region of Pax8

Our results prompted us to assess the existence of an *in vivo* interaction between Pax8 and WBP-2. To this end, we subcloned WBP-2 cDNA into an expression vector containing the 3 × FLAG epitope and used a heterologous system constituted by HeLa cells co-transfected with 3 × FLAG-WBP-2 and an expression vector encoding full-length Pax8. By using the anti-FLAG–agarose affinity gel, we immunoprecipitated the 3 × FLAG–WBP-2 protein from an extract of the co-transfected HeLa cells. Subsequently we revealed the presence of Pax8 by Western blot, utilizing a specific anti-Pax8 antibody (Figure 5B, lane 5). When HeLa cells were transfected with Pax8 only, Pax8 was not pulled down in the co-immunoprecipitation (Figure 5B, lane 4), thus demonstrating that the interaction with WBP-2 is specific. At the same time, we

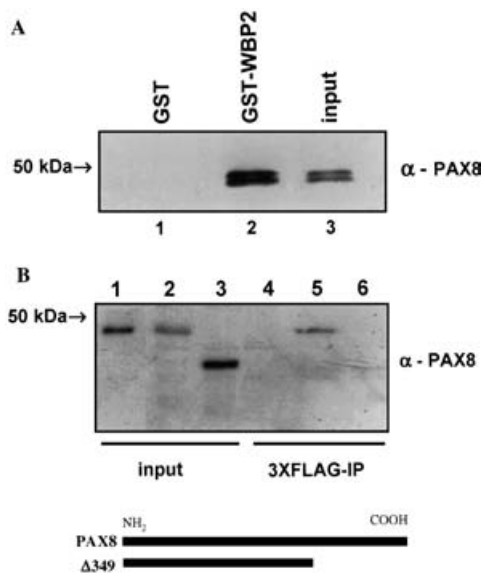

Figure 5 WBP-2 and Pax8 interact both *in vitro* and *in vivo*

(**A**) GST–WBP-2 pull-down assay performed with affinity-purified bacterial Pax8. Lane 1, Pax8 protein precipitated with GST as control; lane 2, Pax8 protein precipitated with GST–WBP-2; lane 3, purified bacterial Pax8 input. Western blot was performed using a specific polyclonal antibody against Pax8. (**B**) 3 × FLAG-WBP-2 co-immunoprecipitation (IP) from protein extracts of transiently transfected cells. HeLa cells were transiently transfected with full-length Pax8 (lane 1, input), with the 3 × FLAG-WBP-2 expression vector together with full-length Pax8 (lane 2, input), or with the 3 × FLAG-WBP-2 expression vector together with Pax8Δ349 deletion mutant (lane 3, input). Only full-length Pax8 was able to bind WBP-2 (lane 5).

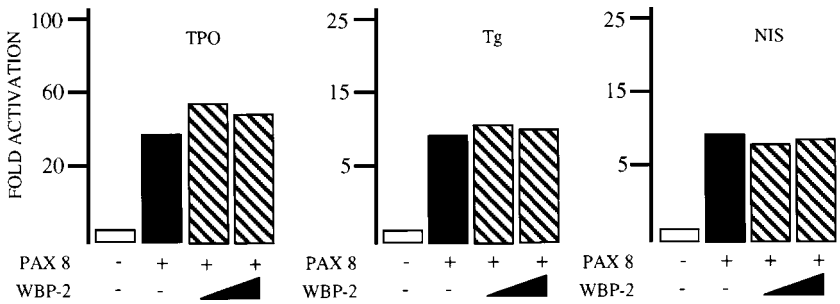

Figure 6 WBP-2 does not enhance the transcriptional activity of Pax8 on thyroid-specific promoters

HeLa cells were transiently transfected with the reporter plasmids Tg-CAT, TPO-LUC and NIS-LUC9, containing the minimal promoter region of the rat Tg gene or of the rat TPO gene, or the enhancer element of the NIS gene, respectively, together with the expression vectors encoding Pax8 and WBP-2. The cells were subsequently harvested and assayed for CAT or LUC activity. Fold activation is the ratio between values obtained with and without co-transfection of the expression vectors. Values are means of at least three independent experiments.

co-transfected HeLa cells with 3 × FLAG-WBP-2 and a truncated form of Pax8 called Δ349. Pax8-Δ349 lacks the final 108 amino acids in the C-terminal region of the protein; this domain was first identified as the transactivation domain of Pax8 and is very rich in proline residues. Co-immunoprecipitation assays showed that the final 108 amino acids in the C-terminal domain of Pax8 are essential for the interaction between WBP-2 and Pax8 (Figure 5B, lane 6). An analysis of the C-terminal domain of Pax8 revealed that not a single tryptophan residue is present, unlike in the WW module, thus leading to the conclusion that Pax8 is not a WW domain protein. Hence we can assert that the domain by which Pax8 interacts with WBP-2 is a novel domain of interaction.

Binding of WBP-2 does not influence the transcriptional activity of Pax8

Pax8 is a transcriptional activator of thyroid-specific gene promoters. In particular, Pax8 was shown to be able to efficiently activate transcription from the TPO, Tg and NIS promoters in thyroid and non-thyroid cells [25,41]. To investigate whether the physical interaction between WBP-2 and Pax8 could lead to an increase in the transcriptional activity induced by Pax8, we performed transactivation assays in HeLa cells co-transfected with the expression vectors encoding WBP-2 and Pax8, together with the reporter construct TPO-LUC. Our results demonstrated that WBP-2 does not increase the transcriptional activation of the TPO promoter by Pax8 (Figure 6). We repeated the same transactivation assays utilizing two other thyroid-specific promoters, Tg-CAT and NIS-LUC9, and again were unable to detect any effect of WBP-2 on Pax8-dependent transcriptional activation (Figure 6). Moreover, the same results were obtained using the tWBP-2 protein (results not shown). Thus our conclusion is that WBP-2 is not a transcriptional co-activator of Pax8.

DISCUSSION

In the present study we report the first experimental evidence that WBP-2 binds directly to a Pax protein, i.e. Pax8. Although initially identified in an immunological screening, the interaction between WBP-2 and Pax8 was verified by *in vitro* biochemical association assays. In addition, the same interaction was demonstrated *in vivo* by co-immunoprecipitation. Moreover, we identified by RT-PCR a splicing isoform of WBP-2, that we named tWBP-2. Since our experiments did not identify a specific function of tWBP-2, the functional relevance of this alternatively spliced isoform remains unknown. WBP-2 is a ubiquitous protein that binds to the WW domain of YAP (Yes-associated protein) [38]. The WW domain is a small module composed of 40 amino acids that plays a role in mediating protein–protein interactions via proline-rich regions [43,44].

From the analysis of transgenic and knockout mice, it has become clear that *Pax* genes are key regulators during crucial steps of the organogenesis of various tissues [7,45–47]. In fact, they encode essential developmental regulators that control the morphogenesis of complex tissues, such as the eye, the vertebral column, the central nervous system, the kidney and others. In particular, Pax8 knockout mice have a barely visible thyroid gland, which is devoid of follicular cells [21], and human patients suffering from congenital hypothyroidism have been shown to carry mutations in the *Pax8* gene [48–50]. Pax8 has been shown to be a master gene for the maintenance of a thyroid differentiated phenotype, being important for transcriptional activation of all of the thyroid differentiation markers, such as the genes encoding Tg, TPO and NIS [22]. Recently it was demonstrated that Pax8 and the homeodomain-containing transcription factor TTF-1 interact biochemically and synergistically to activate transcription from the Tg promoter [29]. Whether WBP-2 or tWBP-2 might influence transcriptional activation of thyroid-specific promoters, such as those of the NIS, Tg and TPO genes, has been examined in HeLa cells. In our experiments we did not observe an effect on Pax8 transcriptional activity when WBP-2 or tWBP-2 was co-expressed in transiently transfected HeLa cells, although our results indicate that Pax8 and WW-domain binding proteins are co-localized in the nuclei of these cells (results not shown), and can be isolated in a complex by co-immunoprecipitation. One possible explanation for this apparent lack of an effect could be the presence of endogenous WBP-2. In this case, the activity of Pax8 would already be determined by the endogenous factor, and additional WBP-2 would not induce the activity significantly. However, we favour the hypothesis that WBP-2 act as an adaptor molecule that is charged with the responsibility of bringing together various components of a complex via protein binding domains in order to participate in the co-activation of transcription. The interaction of proline-rich proteins such as WBP-2 with WW domain-containing proteins seems to play different functions in the nucleus [51]. In some cases, complexes are important for transcriptional activation [52] or for negative regulation of transcription [53], or they might modulate the activity and the degradation of RNA polymerase II [54].

Analysis of the amino acid sequence of Pax8 revealed that it does not contain a WW domain. Thus the results presented here represent the first evidence for the presence in Pax8 of a novel

domain that is able to make a physical interaction with WBP-2. By deletion analysis we have determined the region of Pax8 involved in the interaction with WBP-2, and our results showed that the C-terminal domain of Pax8 is involved in the interaction. In fact, the Pax8 deletion mutant Δ349, which lacks the final 108 amino acids of the protein, is no longer able to interact with WBP-2. This domain, like the C-terminal region of all Pax proteins, contains the transactivation domain, and it is very rich in proline residues, even though it does not contain the WW module. Our hypothesis is that Pax8 functions alone or, more often, in combination with various factors, cofactors or adaptor molecules to regulate gene expression. In future studies, it will be essential to determine whether WBP-2 is indeed a link between Pax8 and other proteins, such as chromatin remodelling proteins or transcriptional activators or repressors, in order to fully understand the functional significance of this interaction and its role in thyroid-specific gene expression.

We thank P.P. Di Fiore for the anti-GST antibody used to perform the immunological screening of the expression library, and L. Nitsch for providing great help with the confocal microscopy. This work was supported by grants from the Ministero per l'Universita' e la Ricerca Scientifica (MIUR-URST), project "Molecular mechanisms responsible for thyroid cell differentiation" and from the Istituto Superiore di Sanita', project "Basi molecolari dell'ipotiroidismo congenito: predizione, prevenzione ed intervento".

REFERENCES

1 Xu, L., Glass, C. K. and Rosenfeld, M. G. (1999) Coactivator and corepressor complexes in nuclear receptor function. Curr. Opin. Genet. Dev. **9**, 140–147
2 Muchardt, C. and Yaniv, M. (1999) ATP-dependent chromatin remodelling: SWI/SNF and Co. are on the job. J. Mol. Biol. **293**, 187–198
3 Orphanides, G., Lagrange, T. and Reinberg, D. (1996) The general transcription factors of RNA polymerase II. Genes Dev. **10**, 2657–2683
4 Green, M. R. (2000) TBP-associated factors (TAFIIs): multiple, selective transcriptional mediators in common complexes. Trends Biochem. Sci. **25**, 59–63
5 Pawson, T., Raina, M. and Nash, P. (2002) Interaction domains: from simple binding events to complex cellular behavior. FEBS Lett. **513**, 2–10
6 Dikic, I. (2002) CIN85/CMS family of adaptor molecules. FEBS Lett. **529**, 110–115
7 Dahl, E., Koseki, H. and Balling, R. (1997) Pax genes and organogenesis. Bioessays **19**, 755–765
8 Noll, M. (1993) Evolution and role of Pax genes. Curr. Opin. Genet. Dev. **3**, 595–605
9 Stuart, E. T., Kioussi, C. and Gruss, P. (1994) Mammalian Pax genes. Annu. Rev. Genet. **28**, 219–236
10 Walther, C., Guenet, J. L., Simon, D., Deutsch, U., Jostes, B., Goulding, M. D., Plachov, D., Balling, R. and Gruss, P. (1991) Pax: a murine multigene family of paired box-containing genes. Genomics **11**, 424–434
11 Xu, W., Rould, M. A., Jun, S., Desplan, C. and Pabo, C. O. (1995) Crystal structure of a paired domain-DNA complex at 2.5 A resolution reveals structural basis for Pax developmental mutations. Cell **80**, 639–650
12 Bertuccioli, C., Fasano, L., Jun, S., Wang, S., Sheng, G. and Desplan, C. (1996) In vivo requirement for the paired domain and homeodomain of the paired segmentation gene product. Development **122**, 2673–2685
13 Jun, S. and Desplan, C. (1996) Cooperative interactions between paired domain and homeodomain. Development **122**, 2639–2650
14 Miskiewicz, P., Morrissey, D., Lan, Y., Raj, L., Kessler, S., Fujioka, M., Goto, T. and Weir, M. (1996) Both the paired domain and homeodomain are required for in vivo function of Drosophila Paired. Development **122**, 2709–2718
15 Fujioka, M., Miskiewicz, P., Raj, L., Gulledge, A. A., Weir, M. and Goto, T. (1996) Drosophila Paired regulates late even-skipped expression through a composite binding site for the paired domain and the homeodomain. Development **122**, 2697–2707
16 Czerny, T., Schaffner, G. and Busslinger, M. (1993) DNA sequence recognition by Pax proteins: bipartite structure of the paired domain and its binding site. Genes Dev. **7**, 2048–2061
17 Chalepakis, G., Jones, F. S., Edelman, G. M. and Gruss, P. (1994) Pax-3 contains domains for transcription activation and transcription inhibition. Proc. Natl. Acad. Sci. U.S.A. **91**, 12745–12749
18 Dorfler, P. and Busslinger, M. (1996) C-terminal activating and inhibitory domains determine the transactivation potential of BSAP (Pax-5), Pax-2 and Pax-8. EMBO J. **15**, 1971–1982
19 Kozmik, Z., Kurzbauer, R., Dorfler, P. and Busslinger, M. (1993) Alternative splicing of Pax-8 gene transcripts is developmentally regulated and generates isoforms with different transactivation properties. Mol. Cell. Biol. **13**, 6024–6035
20 Plachov, D., Chowdhury, K., Walther, C., Simon, D., Guenet, J. L. and Gruss, P. (1990) Pax8, a murine paired box gene expressed in the developing excretory system and thyroid gland. Development **110**, 643–651
21 Mansouri, A., Chowdhury, K. and Gruss, P. (1998) Follicular cells of the thyroid gland require Pax8 gene function. Nat. Genet. **19**, 87–90
22 Pasca di Magliano, M., Di Lauro, R. and Zannini, M. (2000) Pax8 has a key role in thyroid cell differentiation. Proc. Natl. Acad. Sci. U.S.A. **97**, 13144–13149
23 Damante, G. and Di Lauro, R. (1994) Thyroid-specific gene expression. Biochim. Biophys. Acta **1218**, 255–266
24 Damante, G., Tell, G. and Di Lauro, R. (2001) A unique combination of transcription factors controls differentiation of thyroid cells. Prog. Nucleic Acid Res. Mol. Biol. **66**, 307–356
25 Zannini, M., Francis-Lang, H., Plachov, D. and Di Lauro, R. (1992) Pax-8, a paired domain-containing protein, binds to a sequence overlapping the recognition site of a homeodomain and activates transcription from two thyroid-specific promoters. Mol. Cell. Biol. **12**, 4230–4241
26 Fabbro, D., Pellizzari, L., Mercuri, F., Tell, G. and Damante, G. (1998) Pax-8 protein levels regulate thyroglobulin gene expression. J. Mol. Endocrinol. **21**, 347–354
27 Javaux, F., Bertaux, F., Donda, A., Francis-Lang, H., Vassart, G., Di Lauro, R. and Christophe, D. (1992) Functional role of TTF-1 binding sites in bovine thyroglobulin promoter. FEBS Lett. **300**, 222–226
28 Espinoza, C. R., Schmitt, T. L. and Loos, U. (2001) Thyroid transcription factor 1 and Pax8 synergistically activate the promoter of the human thyroglobulin gene. J. Mol. Endocrinol. **27**, 59–67
29 Di Palma, T., Nitsch, R., Mascia, A., Nitsch, L., Di Lauro, R. and Zannini, M. (2003) The paired domain-containing factor Pax8 and the homeodomain-containing factor TTF-1 directly interact and synergistically activate transcription. J. Biol. Chem. **278**, 3395–3402
30 Yan, C., Naltner, A., Conkright, J. and Ghaffari, M. (2001) Protein-protein interaction of retinoic acid receptor alpha and thyroid transcription factor-1 in respiratory epithelial cells. J. Biol. Chem. **276**, 21686–21691
31 Sever-Chroneos, Z., Bachurski, C. J., Yan, C. and Whitsett, J. A. (1999) Regulation of mouse SP-B gene promoter by AP-1 family members. Am. J. Physiol. **277**, L79–L88
32 Yang, Y. S., Yang, M. C., Wang, B. and Weissler, J. C. (2001) BR22, a novel protein, interacts with thyroid transcription factor-1 and activates the human surfactant protein B promoter. Am. J. Respir. Cell Mol. Biol. **24**, 30–37
33 Yi, M., Tong, G. X., Murry, B. and Mendelson, C. R. (2002) Role of CBP/p300 and SRC-1 in transcriptional regulation of the pulmonary surfactant protein-A (SP-A) gene by thyroid transcription factor-1 (TTF-1). J. Biol. Chem. **277**, 2997–3005
34 Missero, C., Pirro, M. T., Simeone, S., Pischetola, M. and Di Lauro, R. (2001) The DNA glycosylase T:G mismatch-specific thymine DNA glycosylase represses thyroid transcription factor-1-activated transcription. J. Biol. Chem. **276**, 33569–33575
35 Liu, C., Glasser, S. W., Wan, H. and Whitsett, J. A. (2002) GATA-6 and thyroid transcription factor-1 directly interact and regulate surfactant protein-C gene expression. J. Biol. Chem. **277**, 4519–4525
36 Fitzsimmons, D., Hodsdon, W., Wheat, W., Maira, S. M., Wasylyk, B. and Hagman, J. (1996) Pax-5 (BSAP) recruits Ets proto-oncogene family proteins to form functional ternary complexes on a B-cell-specific promoter. Genes Dev. **10**, 2198–2211
37 Wheat, W., Fitzsimmons, D., Lennox, H., Krautkramer, S. R., Gentile, L. N., McIntosh, L. P. and Hagman, J. (1999) The highly conserved beta-hairpin of the paired DNA-binding domain is required for assembly of Pax-Ets ternary complexes. Mol. Cell. Biol. **19**, 2231–2241
38 Chen, H. I. and Sudol, M. (1995) The WW domain of Yes-associated protein binds a proline-rich ligand that differs from the consensus established for Src homology 3-binding modules. Proc. Natl. Acad. Sci. U.S.A. **92**, 7819–7823
39 Sinclair, A. J., Lonigro, R., Civitareale, D., Ghibelli, L. and Di Lauro, R. (1990) The tissue-specific expression of the thyroglobulin gene requires interaction between thyroid-specific and ubiquitous factors. Eur. J. Biochem. **193**, 311–318
40 Francis-Lang, H., Price, M., Polycarpou-Schwarz, M. and Di Lauro, R. (1992) Cell-type-specific expression of the rat thyroperoxidase promoter indicates common mechanisms for thyroid-specific gene expression. Mol. Cell. Biol. **12**, 576–588

41 Ohno, M., Zannini, M., Levy, O., Carrasco, N. and Di Lauro, R. (1999) The paired-domain transcription factor Pax8 binds to the upstream enhancer of the rat sodium/iodide symporter gene and participates in both thyroid-specific and cyclic-AMP-dependent transcription. Mol. Cell. Biol. **19**, 2051–2060
42 Ambesi-Impiombato, F. S. and Coon, H. G. (1979) Thyroid cells in culture. Int. Rev. Cytol. **10**, 163–172
43 Bork, P. and Sudol, M. (1994) The WW domain: a signalling site in dystrophin? Trends Biochem. Sci. **19**, 531–533
44 Sudol, M., Chen, H. I., Bougeret, C., Einbond, A. and Bork, P. (1995) Characterization of a novel protein-binding module – the WW domain. FEBS Lett. **369**, 67–71
45 Mansouri, A., Goudreau, G. and Gruss, P. (1999) Pax genes and their role in organogenesis. Cancer Res **59**, 1707s–1709s (discussion 1709s–1710s)
46 Mansouri, A., Hallonet, M. and Gruss, P. (1996) Pax genes and their roles in cell differentiation and development. Curr. Opin. Cell Biol. **8**, 851–857
47 Stuart, E. T. and Gruss, P. (1996) PAX: developmental control genes in cell growth and differentiation. Cell Growth Differ. **7**, 405–412
48 Macchia, P. E., Lapi, P., Krude, H., Pirro, M. T., Missero, C., Chiovato, L., Souabni, A., Baserga, M., Tassi, V., Pinchera, A. et al. (1998) PAX8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis. Nat. Genet. **19**, 83–86
49 Vilain, C., Rydlewski, C., Duprez, L., Heinrichs, C., Abramowicz, M., Malvaux, P., Renneboog, B., Parma, J., Costagliola, S. and Vassart, G. (2001) Autosomal dominant transmission of congenital thyroid hypoplasia due to loss-of-function mutation of PAX8. J. Clin. Endocrinol. Metab. **86**, 234–238
50 Congdon, T., Nguyen, L. Q., Nogueira, C. R., Habiby, R. L., Medeiros-Neto, G. and Kopp, P. (2001) A novel mutation (Q40P) in PAX8 associated with congenital hypothyroidism and thyroid hypoplasia: evidence for phenotypic variability in mother and child. J. Clin. Endocrinol. Metab. **86**, 3962–3967
51 Sudol, M., Sliwa, K. and Russo, T. (2001) Functions of WW domains in the nucleus. FEBS Lett. **490**, 190–195
52 Yagi, R., Chen, L. F., Shigesada, K., Murakami, Y. and Ito, Y. (1999) A WW domain-containing yes-associated protein (YAP) is a novel transcriptional co-activator. EMBO J. **18**, 2551–2562
53 Waragai, M., Lammers, C. H., Takeuchi, S., Imafuku, I., Udagawa, Y., Kanazawa, I., Kawabata, M., Mouradian, M. M. and Okazawa, H. (1999) PQBP-1, a novel polyglutamine tract-binding protein, inhibits transcription activation by Brn-2 and affects cell survival. Hum. Mol. Genet. **8**, 977–987
54 Sudol, M. and Hunter, T. (2000) NeW wrinkles for an old domain. Cell **103**, 1001–1004

Received 13 August 2003/2 October 2003; accepted 7 October 2003
Published as BJ Immediate Publication 7 October 2003, DOI 10.1042/BJ20031233