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Ref-1 Controls Pax-8 DNA-Binding Activity

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Redox potential controls the DNA-binding activity of several transcription factors. In some cases, the regulation of DNA-binding activity by the redox state is mediated by the Ref-1 nuclear protein. In this study, we demonstrate that Ref-1 is able to induce *"in vitro"* the DNA-binding activity of the Pax-8 paired domain. In cotransfection experiments, Ref-1 increases the Pax-8 activating effect on thyroglobulin promoter. Moreover, immunoreactivity data suggest that, in nuclear extracts of thyroid cells, the levels of Ref-1 correlate with the amounts of reduced Pax-8. Therefore, the regulation of the Pax-8 DNA-binding activity by redox potential, that we have demonstrated occurring *"in vitro"*, could represent a means to control *"in vivo"* the function of Pax proteins. Alignment of the Paired domains sequences present in the Protein Data Bank demonstrates a strong conservation of Cys residues, suggesting that the redox regulation of the Paired domain DNA-binding activity is widely conserved along phylogenesis.

Redox potential plays an important role in the control of gene expression in a large variety of organisms [1-2]. The redox regulation chiefly occurs through post-transcriptional modifications of transcription factors. Although several molecular functions may be subjected to redox control [2], the DNA-binding function is the major target of redox regulation. Clear examples of redox-regulated DNA-binding activities in eukaryotes are those of AP-1 and NF-κB transcription factors [3].

In order to understand how redox potential controls gene expression, in addition to identify which are the transcription factors subjected to redox regulation, it is important to find the proteins mediating the redox effects. Ref-1 has been identified as a protein capable of either apurinic/apyrimidinic endonuclease DNA repair activity and nuclear redox activity, being able to induce AP-1 DNA-binding activity [4]. Biochemical studies indicate that Ref-1 is able to directly interact, in the nucleus, with thioredoxin (TRX) to regulate AP-1 transcriptional activity [5].

Pax proteins are transcriptional regulators that play a developmental role in a wide variety of species [6]. Pax-8 is extremely important for the correct development of the thyroid gland; inactivation of the Pax-8 gene causes absence of follicular cells and, therefore, absence of thyroid hormone [7]. Pax proteins bind to specific DNA sequences through an evolutionarily conserved domain called paired domain (Prd domain). This is a 128-amino acid long structure, composed of two structurally independent subdomains, called PAI and RED [8]. Recently, we have demonstrated that the Pax-8 DNA-binding activity is, "*in vitro*", controlled by redox potential [9]. Since thyrotropin (TSH), the major regulator of the thyroid follicular cells, up-regulates Ref-1 expression [10], we addressed the question whether Ref-1 may be involved in the redox control of Pax-8. We demonstrate that the Pax-8 Prd domain DNA-binding activity is subjected to Ref-1 regulation. This effect could play a role in the "*in vivo*" regulation of Pax-8 activity, since we also demonstrate that Ref-1 regulates the activating effect of Pax-8 on Tg promoter.

MATERIALS AND METHODS

Expression vectors. The plasmid pT7.7Pax8-Prd, was obtained by subcloning the DNA sequence encoding for the Pax8 Prd domain into the plasmid pT7.7 [11]. Its construction is described in Ref. [9].

Recombinant Ref-1 His-tagged expressing plasmid pDS56Ref-1 was kindly provided by Dr. T. Curran.

Eukaryotic expression vectors for Pax-8 and Ref-1 were provided by Dr. P. Gruss and Dr. T. Curran, respectively. In both plasmids the expression of the protein is driven by CMV promoter.

Plasmid pTACAT3 contains the wild-type Tg promoter linked to the Chloramphenicol acetyltransferase (CAT) gene and is described in [12]. Plasmid CMV-Luc contains the Luciferase (Luc) gene controlled by CMV promoter.

Protein expression and purification. pT7.7Pax-8 Prd plasmid was used to transform BL21 bacterial strain. Transformed cells were grown at 37°C to OD_{600} 0.6-0.7 and then induced by 1 mM IPTG for 3 hours. Cells were harvested by centrifugation and resuspended in lysis buffer (50 mM sodium phosphate, 0.25 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 2 mg/ml leupeptin, 2 mg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.0) in a volume of 10

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ml/g of bacterial pellet. After cell lysis by sonication, the cell debris was removed by centrifugation. DNA was removed by addition of 0.3 mg/ml of protamine sulfate to the supernatant and the precipitate removed by centrifugation. The supernatant was loaded onto a Mono-S column (Pharmacia) pre-equilibrated with lysis buffer. The Pax-8 Prd domain was purified using a linear gradient of 0.25-0.7 M NaCl in 50 mM phosphate buffer at pH 7.0, containing 1 mM DTT. The purified protein gave a single band on an overloaded SDS-PAGE. Fractions containing purified proteins were dialysed against water and then stored at −85°C. The Pax-8 Prd domain concentration was determined using a molar absorption coefficient of 9890 M^{-1} cm^{-1}, calculated as previously described [13].

Recombinant Ref-1 expression and purification were made as previously reported [14]. Briefly, *E. coli* growth and induction were made as already mentioned before. Then, the pelleted cells were lysed in PBS containing 1 mg · ml^{-1} lysozyme (Sigma), 10 mM β-mercaptoethanol, 1 mM PMSF and 0.8 mM imidazol and sonicated, and was clarified by centrifugation. The supernatant was then loaded onto a Ni^{2+}-NTA (nitrilotri-acetic acid) agarose column. The column was washed and the fusion protein was eluted with a linear gradient of PBS containing 80 mM imidazol. The eluted protein was dialysed against 50 mM phosphate buffer, pH7.3, 50 mM NaCl, 5mM $MgCl_2$, 1mM EDTA, 5% vol/vol glicerol, 0.5 mM DTT.

Electrophoretic mobility shift assay (EMSA) analysis. Double-stranded oligodeoxynucleotides, labelled at the 5′ end with ^{32}P, were used as probes in gel retardation assays. The C site is a 24mer whose upper strand is 5′-CACTGCCCAGTCAAGTGTTCTTGA-3′. The gel retardation assay was performed by incubating protein and DNA in a buffer containing 20 mM Tris-HCl, 75 mM KCl, 0.25 mg/ml bovine serum albumin (BSA), pH7.6, 10% glicerol with or without calf thymus DNA (50 mg/ml), for 30 min at room temperature. Protein-bound DNA and free DNA were separated on a native polyacrylamide gel run in 0.5× TBE for 1.5 hr at 4°C. The gel was dried and then exposed to an X-ray film at −80 °C.

Cell cultures and transfections. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal-calf serum (FCS). For transient transfections cells were plated at 0.8×10^6 cells/60mm culture dish. After 4-6 hours, transfections were carried out by the calcium phosphate co-precipitation procedures [15]. The following amounts of plasmid were transfected to each 60 mm dish: pTACAT3, 1.5 μg; CMVPax8, 0.5 μg; CMVRef-1, 2.25 μg; CMVLuc, 0.5 μg. After exposure to the calcium phosphate/DNA precipitate for 12-14 hours, cells were washed with PBS and grown for 48 hours. CAT [16] and Luc [17] activities were measured as described in the cited references.

FRTL-5 cells were grown as previously described [18] except that TSH was used at a final concentration of 1 mU/ml.

Nuclear extracts and Western blot. Cell nuclear extracts were prepared as previously described [19]. Briefly, 10^7 cells were washed once with PBS and resuspended in 500 μl of hypotonic lysis buffer A (10 mM Hepes, 10 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM PMSF, pH 7.9). After 10 min, cells were homogenized by ten strokes with a loose-fitting Dounce homogenizer. Nuclei were collected by centrifugation for 5 min at 500 × g at 4°C in a microcentrifuge. Then, the nuclear proteins were extracted with 100 μl of buffer B (10 mM Hepes, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM PMSF, pH 7.9). After incubating for 20 min at 4°C, samples were centrifuged at 12000 × g at 4°C for 15 min. The nuclear extracts were then quantitated for protein levels according to the Bradford method [20] and used immediately for EMSA analysis or kept at −80°C.

Extracts of FRTL-5 cells, prepared as described previously [19], were boiled in Laemmli sample buffer (with or without β-mercaptoethanol as reducing agent, as indicated) and resolved on a 12% SDS polyacrylamide gel electrophoresis (PAGE). After electroblotting onto nitrocellulose membrane (MSI, Westboro, MA, USA), Ref-1 was detected by a Rabbit polyclonal specific antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) and Pax-8 by the rabbit polyclonal antibody αPax8-187 kindly provided by R. Di Lauro using an ECL detection kit (Amersham Corp.).

RESULTS AND DISCUSSION

Recombinant Ref-1 Activates Pax-8 Prd DNA-Binding

In a recent study we demonstrated that the DNA-binding activity of the Pax-8 Prd domain is redox regulated [9]. In the case of AP-1 and p53 transcription factors, the regulation of DNA-binding activity by the redox state is mediated by the Ref-1 nuclear protein [5, 21]. In order to demonstrate whether Ref-1 is able to directly induce the DNA-binding activity of the isolated Pax-8 Prd domain, we performed an EMSA analysis with the oxidate form of the Prd domain and the recombinant Ref-1 protein. Recombinant Pax-8 Prd domain was obtained by over-expression in *E. coli* and purified as previously described [9]. The purified protein was then oxidised by prolonged air exposure and the oxidation state was monitored by MALDI-MS spectroscopy as previously reported [9]. Recombinant Ref-1 protein (rRef-1) was obtained as hexaistidine-tag fusion protein from over-expression in *E. coli* and then purified by nickel-chelate chromatography from bacterial extracts and treated as previously described [14]. As previously demonstrated [9], the oxidate form of Pax-8 Prd domain was unable to show any kind of DNA-binding activity (Fig. 1, lane 2). The addition of increasing amounts of rRef-1 protein considerably enhanced the DNA-binding activity (Fig. 1, lanes 3, 4) also compared to the addition of the same volumes of the dialysis buffer used to dialyse the rRef-1 protein after purification (data not shown). As it is evident from Fig. 1, interaction between the Prd domain and Ref-1 is likely to be of a transient nature because there is no change in mobility of Prd-DNA complexes either in samples stimulated with the reducing agent dithiothreitol (DTT) (Fig. 1, lane 5) or with Ref-1. Contrarily to what shown by Xanthoudakis *et al.* in the case of Jun [22], we were not able to detect a stable interaction between Prd domain and Ref-1 "*in vitro*" (data not shown).

Ref-1 Increases the Pax-8-Induced Activity of Tg Promoter

In order to test whether the stimulatory effect of Ref-1 on the Pax-8 Prd DNA-binding activity could have a relevance "*in vivo*", a cell transfection approach was used (Fig. 2). In fact, the rat Tg promoter is not functional when transfected in non-thyroid cell lines and its activity can be reconstituted by the forced expression of Pax-8 [23]. We asked whether the co-transfection of a Ref-1 expression vector is able to modify the Pax-8 effect on Tg promoter. Results are

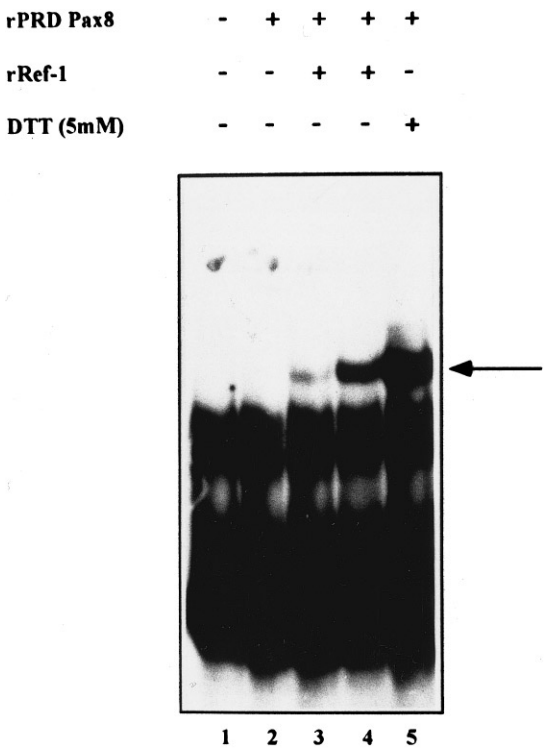

FIG. 1. Recombinant Ref-1 (rRef-1) is a stimulator of Pax-8 Prd domain DNA-binding activity. DNA binding by 50 ng of recombinant Pax-8 Prd domain, in the absence (lane 2) or presence of 100 (lane 3) and 300 ng (lane 4) of rRef-1 or the reducing agent DTT 5 mM (lane 5), was analysed by EMSA. Lane 1 contains probe alone. The oxidised form of the recombinant Prd domain of Pax-8 was obtained by prolonged air exposure and tested by MALDI-MS spectroscopy according to Ref. [9]. The arrow indicates the position of the protein-DNA complex.

shown in Fig. 2. As expected, Tg promoter is inactive in HeLa cells and the expression of Pax-8 is able to activate it. The co-transfection of a Ref-1 expression vector increases of two fold the Pax-8-induced activation of Tg promoter (Panel A). The Ref-1 effect is specific, since the activity of the CMV promoter is not modified by the co-transfection of the Ref-1 expression vector (Panel B). Moreover, in the absence of Pax-8, Ref-1 reduces the basal level of Tg promoter activity of about 50% (data not shown), excluding activating effects of Ref-1 on the basal transcriptional machinery. Although we cannot exclude a stimulatory effect of Ref-1 on the transcriptional activating function of Pax-8 [24], these data, together with those shown in Fig. 1, strongly suggest that the stimulatory effect of Ref-1 on Tg promoter activation is due to the increase of the Pax-8 DNA-binding activity. Therefore, the regulation of the DNA-binding activity of Pax-8 by redox potential, that we have demonstrated occurring "*in vitro*", could represent a system to control "*in vivo*" the function of Pax proteins.

Ref-1 Expression Levels Correlate with the Reduced Form of Pax-8 in FRTL-5 Thyroid Cells

It has been demonstrated that, in the case of p53, the redox modulation of the sequence-specific DNA-binding activity occurs through modifications of the protein conformation [25]. The authors tested the con-

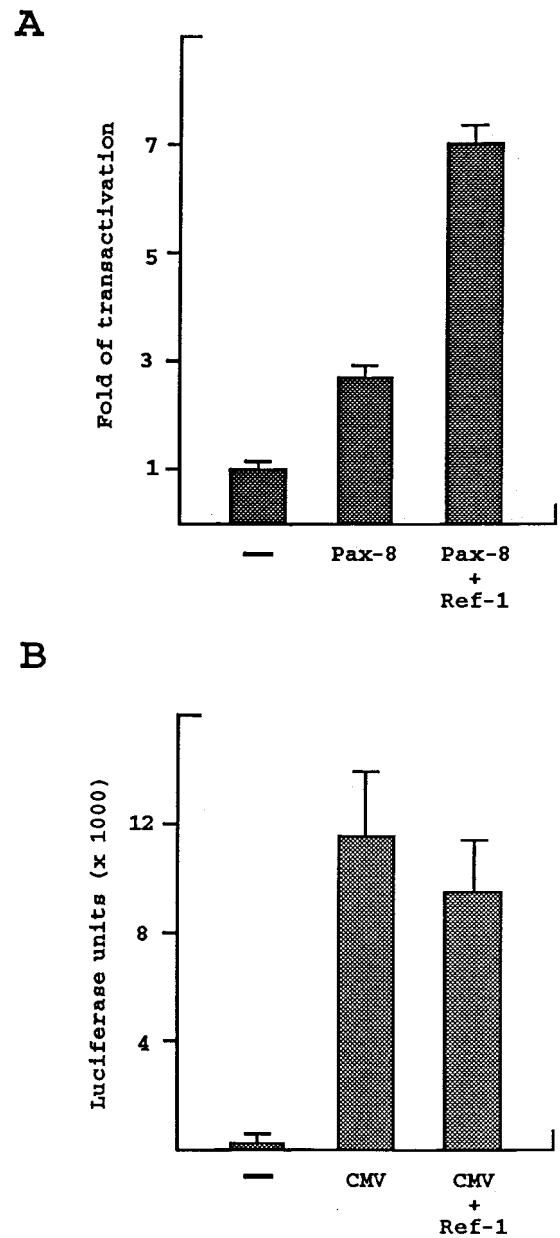

FIG. 2. Effect of Ref-1 on the activity of Tg and CMV promoters. Plasmids were transfected in HeLa cells at the concentrations indicated in Materials and Methods. 48 hours after transfections, cells were harvested and CAT and Luc activities were measured. Panel A: effect of Ref-1 on the Pax-8-induced activity of Tg promoter. Panel B: effect of Ref-1 on the CMV promoter. In both panels, bars indicate the mean value ± SD of at least three independent experiments.

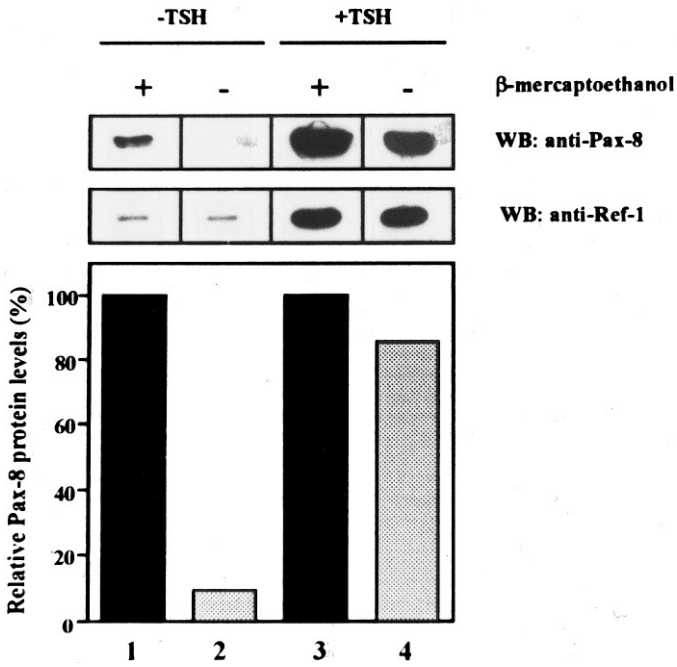

FIG. 3. Ref-1 expression levels correlate with the reduced form of Pax-8 in FRTL-5 thyroid cells. 20 μg of nuclear extracts from FRTL-5 cells, treated or not with TSH, were separated on SDS-PAGE 12% in reducing (for the presence of β-mercaptoethanol) and non reducing conditions, as indicated. Endogenous Pax-8 and Ref-1 were detected by Western blot analysis. Bars represent densitometric data of Pax-8 bands relative to the protein level detected in reducing conditions.

formational modifications of p53 by using a panel of antibodies raised against different parts of the molecule. In order to demonstrate the occurrence of a redox regulation of the endogenous Pax-8 present in thyroid cells, we also used an immunological approach (Fig. 3). FRTL-5 cells were grown for 6 days either in the absence (−TSH) or the presence (+TSH) of TSH (1mU/ml). Nuclear extracts were separated on SDS-PAGE 12% in reducing or non-reducing conditions (+ and − β-mercaptoethanol, respectively). Pax-8 and Ref-1 were detected by Western blot analysis. Pax-8 was detected by using the antibody αPax8-187, raised against a 14 residues long peptide situated just downstream at the C-term of the Prd domain (residues 157-170). Ref-1 was detected by using a commercial antibody (Santa Cruz Biotech.). Both Pax-8 and Ref-1 protein levels are increased by TSH, confirming previous results [10, 26]. However, whereas Ref-1 shows the same immunological reactivity in reducing and non-reducing conditions, Pax-8 was more immunologically reactive when run in reducing conditions. The difference in immunoreactivity was maximally detected in nuclear extracts of cells cultured in the absence of TSH; the presence of TSH in the culture medium greatly reduces the difference of Pax-8 immunoreactivity between reducing and non-reducing conditions (compare bars 1-2 and 3-4). These results are compatible with the existence, in thyroid cells, of two conformationally different forms of Pax-8: one is insensitive to the presence of β-mercaptoethanol in the gel (therefore reduced in its "native state") and strongly immunoreactive, the second one is sensitive to the presence of β-mercaptoethanol in the gel (therefore oxidised in its "native state") and poorly immunoreactive. This latter is prominent when cells are cultured in the absence of TSH; on the contrary, when the hormone is present, most of the protein is in the "reduced" form. A positive correlation is present between Ref-1 levels and the 'β-mercaptoethanol-insensitive form' of Pax-8. Therefore, the amount of nuclear Ref-1 appears to control the redox state of Pax-8.

Cysteine Residues of the Paired Domain Are Extremely Conserved along Phylogenesis

DNA-binding regulation by redox potential is achieved through reduction/oxidation of cysteine residues [2]. Hence, the conservation of the Prd domain Cys residues along phylogenesis would suggest the conservation of the redox regulation of Prd domain-containing proteins through evolution. Alignment of 20 paired domain sequences present in the EMBL Protein Data Bank (available at the site http://srs.ebi.ac.uk:5000) underlines the importance of Cys residues in the context of the paired domain either in vertebrates (Pax-1/Pax-9, upper part of Fig. 4) and invertebrates (lower part of Fig. 4). Cys37 is present in all proteins with the exception of Pax-A of Hydra. However, in this protein, a Cys residue is present at position 32. Cys49 is conserved in all Prd domains and Cys109 is present in all Prd domains with the exception of Gsbn of Drosophila. Though decreasing the sequence homology degree (from 93.8 to 60.6%) between the Prd domains of vertebrates and invertebrates using Pax-8 as a reference, the Cys residues result very well conserved thus supporting their importance in the context of Prd domains. This observation is more striking when focusing on small stretches (11 residues) of amino acids surrounding the Cys residues, especially the Cys109. In this case, the sequence homology decreases to 36% (Gsb, Pax-B, Poxn) while the Cys residue is always conserved.

It has been demonstrated that the reactivity of a cysteine residue is enhanced by the neighbouring basic amino acids through the formation of a thiol-anion base pair [27]. We tested the theoretical reactivity, in terms of susceptibility to oxidation, of each Cys residue of different paired domains by calculating its isoelectrical local point. Interestingly, both Cys37 and Cys49 have a highly basic local environment (pI ranging from 8.9 to 10.7, data not shown) therefore representing an ideal target for redox regulation. On the contrary,

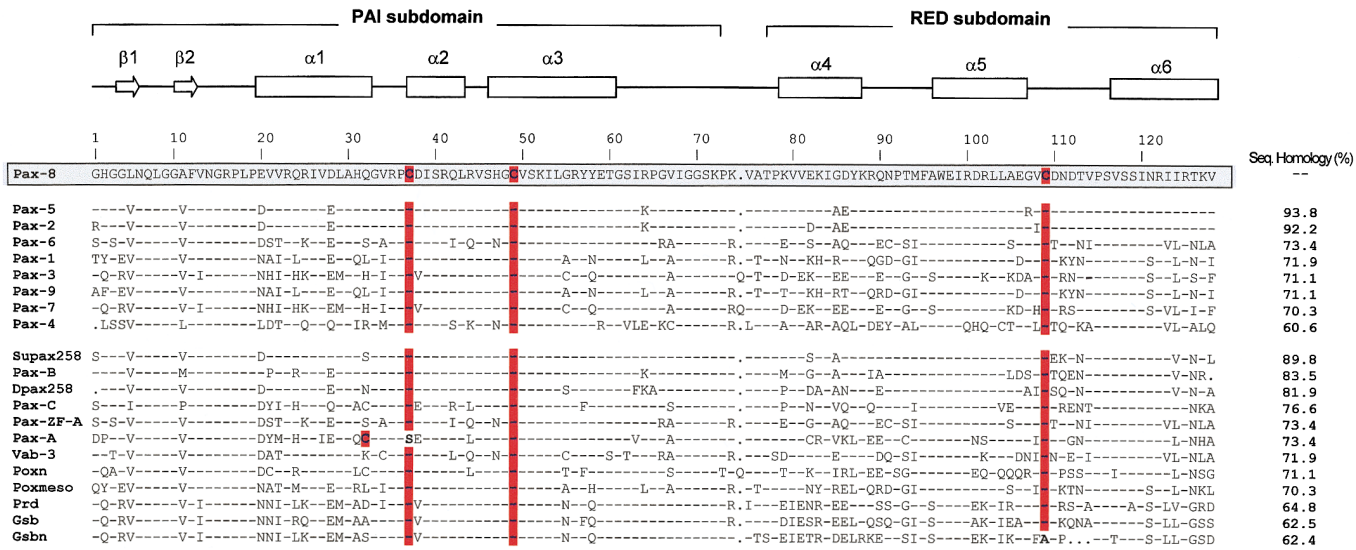

FIG. 4. Alignment of the paired domain amino acid sequences and sequence homologies relative to the Pax-8 paired domain. At the top of the figure, a secondary structure of the Paired domain is shown [29]. The arrows represent a β sheet (β-1) and a β sheet+turn (β-2), empty boxes indicate α helical regions. Conserved Cys residues are indicated by red shading. Amino acids identical to the consensus sequence are indicated by dashes and insertions/deletions are indicated by dots. The EMBL accession numbers for the sequences are: mouse Pax-8, X57487; mouse Pax-5, M97013; mouse Pax-2, X55781; mouse Pax-6, X63963; mouse Pax-1, M69222/M20978; mouse Pax-3, X59358; mouse Pax-9, X87000/X73037; mouse Pax-7, U20792; mouse Pax-4, P32115 (Swiss-Prot database); *Paracentrotus lividus* Supax258, AF016884; *Drosophila melanogaster* Gsb, M14944/2, *Drosophila melanogaster* Gsbn, M14943; *Hydra littoralis* Pax-A, U96193; *Chrysaora quinquecirrha* Pax-B, U96197; *Acropora millepora*, Pax-C, AF053459; *Brachydanio rerio* Pax-ZF-A, X61389; *Drosophila melanogaster* Poxmeso, X16992; *Drosophila melanogaster* Poxn, X58917; *Drosophila melanogaster* Prd, M14548; *Caenorhabditis elegans* Vab-3, U31537; *Drosophila melanogaster* Dpax258, AF016888.

Cys109 results very poorly reactive (local pI ranging from 2.8 to 5.6, data not shown). These data suggest that evolutive pressure has been working leading to a high reactivity for Cys37 and Cys49 associated to a low reactivity for Cys109.

To sum up, the redox regulation of Prd domain-containing proteins could exist in a wide range of organisms. The protein Ref-1 plays a role in this type of regulation. Therefore, the comprehension of how Ref-1 is functionally regulated, may help us to understand how Pax proteins can control development.

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REFERENCES

1. Storz, G., Tartaglia, L. A., and Ames, B. N. (1991) *Science* **248,** 189–194.
2. Nakamura, H., Nakamura, K., and Yodoi, J. (1997) *Annu. Rev. Immunol.* **15,** 351–369.
3. Schreck, R., Rieber, P., and Baeuerle, P. A. (1991) *EMBO J.* **10,** 2247–2258.
4. Xanthoudakis, S., Miao, G., Wang, F., Pan, Y. E., and Curran, T. (1992) *EMBO J.* **11,** 3323–3335.
5. Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K., and Yodoi, J. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 3633–3638.
6. Mansouri, A., Hallonet, M., and Gruss, P. (1996) *Curr. Opin. Cell Biol.* **8,** 851–857.
7. Mansouri, A., Chowdhury, K., and Gruss, P. (1998) *Nature Genet.* **19,** 87–90.
8. Jun, S., and Desplan, C. (1996) *Development* **122,** 2639–2650.
9. Tell, G., Scaloni, A., Pellizzari, L., Formisano, S., Pucillo, C., and Damante, G. (1998) *J. Biol. Chem.* in press.
10. Asai, T., Kambe, F., Kikumori, T., and Seo, H. (1997) *Biochem Biophys. Res. Commun.* **236,** 71–74.
11. Studer, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1991) *Methods Enzymol.* **185,** 60–89.
12. Sinclair, A. J., Lonigro, R., Civitareale, D., Ghibelli, L., and Di Lauro, R. (1990) *Eur. J. Biochem.* **193,** 311–318.
13. Gill, S. C., and von Hippel, P. H. (1989) *Anal. Biochem.* **182,** 319–326.
14. Abate, C., Rauscher, F. J. III., Gentz, R., and Curran, T. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1032–1036.
15. Grahm, F. L., and van der Eb, A. J. (1973) *Virology* **52,** 456–467.
16. Gorman, C. M., Moffat, L. M., and Howard, B. H. (1982) *Mol. Cell. Biol.* **2,** 1044–1051.
17. de Wet, J. R., Wood, K. V., Deluca, M., Helinski, D. R., and Subramani, S. (1987) *Mol. Cell. Biol.* **7,** 725–737.

18. Ambesi-Impiombato, F. S., Parks, L. A., and Coon, H. G. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 3455–3459.
19. Altomonte, M., Pucillo, C., Damante, G., and Maio, M. (1993) *J. Immunol.* **151,** 5115–5122.
20. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
21. Jayaraman, L., Murthy, K. G. K., Zhu, C., Curran, T., Xanthoudakis, S., and Prives, C. (1997) *Genes Dev.* **11,** 558–570.
22. Xanthoudakis, S., Miao, G. G., and Curran, T. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 23–27.
23. Zannini, M. S., Francis-Lang, H., Plachov, D., and Di Lauro, R. (1992) *Mol. Cell. Biol.* **12,** 4230–4241.
24. Poleev, A., Okladnova, O., Musti, A., Schneider, S., Royer-Pokora, B., and Plachov, D. (1997) *Eur. J. Biochem.* **247,** 860–869.
25. Hainaut, P., and Milner, J. (1993) *Cancer Res.* **53,** 4469–4473.
26. Fabbro, D., Pellizzari, L., Mercuri, F., Tell, G., and Damante, G. (1998) *Mol. Endocrinol.*, in press.
27. Kallis, G. B., and Holmgren, A. (1980) *J. Biol. Chem.* **255,** 10261–10265.
28. Xu, W., Rould, M. A., Jun, S., Desplan, C., and Pabo, C. (1995) *Cell* **80,** 639–650.