Pax-6 Interactions with TATA-Box-Binding Protein and Retinoblastoma Protein

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PURPOSE. To identify proteins that physically interact with Pax-6, a paired domain- and homeodomain (HD)-containing transcription factor that is a key regulator of eye development.

METHODS. Protein–protein interactions involving Pax-6, TATA-box-binding protein (TBP), and retinoblastoma protein were studied using affinity chromatography with Pax-6 as ligand, glutathione-transferase (GST) pull-down assays, and immunoprecipitations.

RESULTS. The authors have shown that Pax-6 is a sequence-specific activator of many crystallin genes, all containing a TATA box, in the lens. Others have shown that lens fiber cell differentiation, characterized by temporally and spatially regulated crystallin gene expression, depends on retinoblastoma protein. In the present study it was shown that Pax-6 interacted with the TBP, the DNA-binding subunit of general transcription complex TFIIID. GST pull-down assays indicated that this interaction was mediated by the Pax-6 HD, with a substantial role for its N-terminal arm and first two α-helices. The experiments also indicated a binding role for the C-terminal-activation domain of the protein. In addition, the present study showed that the HD of Pax-6 interacted with retinoblastoma protein. Immunoprecipitation experiments confirmed retinoblastoma protein/Pax-6 complexes in lens nuclear extracts.

CONCLUSIONS. Blending the present results with those in the literature suggests that Pax-6 and retinoblastoma protein participate in overlapping regulatory pathways controlling epithelial cell division, fiber cell elongation, and crystallin gene expression during lens development. (Invest Ophthalmol Vis Sci. 1999;40:1343–1350)

Recent progress in the genetics of vertebrate and invertebrate eye development is linked to the discoveries of genes encoding transcription factors, the functions of which are essential for normal vision. Pax-6 is a member of the paired domain (PD) family of transcription factors and has a specialized homeodomain (HD) downstream of the DNA-binding PD. Pax-6 is expressed in the developing and adult eye, central nervous system, and pancreas and has a critical regulatory role in eye morphogenesis of vertebrates and invertebrates.1–3 Especially impressive is the demonstration that ectopic expression of Pax-6 from flies, mice, squid, or ascidians generates compound eyes in the wing, antenna, or leg of Drosophila.4 In humans, heterozygous Pax-6 mutations have been linked to aniridia,5 Peter’s anomaly,6 autosomal dominant keratosis,6 and foveal hypoplasia7 and to the small-eye phenotype (Sey) in mice.8 Homozygous Pax-6 mutations in humans and mice result in brain defects and the absence of eyes and a nose and are lethal at birth.1,9 Overexpression of Pax-6 in transgenic mice results in phenotypes resembling the heterozygous Sey mice.10 Pax-6 also has been shown to regulate a number of genes for the lens crystallins9 and the Drosophila rhodopsin gene.11

Early eye development in chickens12 and mice13 is associated with gradual restrictions in Pax-6 expression, consistent with its involvement in different pathways. The complex phenotypes, developmentally regulated expression patterns, and haploinsufficiency suggest that Pax-6 has numerous target genes and interacts with other regulatory proteins that may modulate biologic functions of Pax-6, depending on the molecular context.

Experiments have shown that a number of HD proteins14,15 can interact with both the TATA-box-binding protein (TBP) of the basal transcriptional machinery16,17 and with members of the tumor-suppressor18 retinoblastoma protein (pRB).19 These interactions involve the HD region of the proteins and affect gene transcription. Thus, in view of the importance of Pax-6 for eye development it seemed reasonable to test whether it too could interact with TBP and pRB. Pax-6 interactions with TBP and/or pRB would be of particular interest for lens differentiation, because crystallin promoters contain TATA boxes2 and are activated20–25 or suppressed26 by Pax-6, and pRB-deficient mice have specific defects in lens development and crystallin gene expression.27 The fiber cells in pRB-deficient knockout27 or chimeric28–29 mice are disordered and continue to divide. The lens fiber region of pRB-null mice display p53-dependent apoptosis and reduced expression of the β- and γ-crystallins and the intrinsic membrane protein MIP26, all markers for terminal differentiation of lens fiber

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cells. Similar effects have been observed in transgenic mice expressing viral proteins that bind pRB using the mouse α-crystallin promoter exclusively in the lens.26,27

The present results show that the HD of Pax-6 interacted in vitro and in vivo with TFIID and with pRB. Based on these data, we consider the possibility that Pax-6 and pRB interact in overlapping pathways regulating lens cell differentiation.

**Methods**

**Recombinant Plasmids**

The following cDNAs were used to obtain the corresponding proteins: Pax-6 in pKW10,32 TBP in pCG,33 pGST-RB pocket A and pGST-RB pocket B,34 pRB (aa 379–792) in pGEX-2T (provided by William Kaelin, Dana Farber Cancer Institute, Boston, MA),35 GST-Bcd, Ftz, Prd, and Zen36 and GST-Msx-114 are described elsewhere. A series of Pax-6 C-terminal truncated proteins (see Fig. 3) were made by polymerase chain reaction (PCR) and cloned as BamHI– HindIII fragments in pKW10. The Pax-6 HD (aa 206 to 294) was generated by polymerase chain reaction and cloned as an EcoRI–HindIII fragment in pGEM2 (Promega, Madison, WI). Glutathione-S-transferase (GST) fusion proteins in the expression vector pGEX-5P-1 (Pharmacia, Piscataway, NJ) were generated by PCR, and cloned as BamHI–EcoRI fragments: Pax-6 HD (aa 197 to 285), Pax-6 activation domain (AD) 1 (for a truncated AD; aa 320 to 422), and Pax-6 AD2 (for a severely truncated AD; aa 345 to 422). All constructs were verified by dideoxy sequencing. A GST-Pax-6–paired and HD (GST-Pax-6 PD/HD)-expressing vector in pGEX2 (Pharmacia) was generously provided by Jonathan Epstein and Richard L. Maas (Howard Hughes Medical Institute, Harvard Medical School, Boston, MA). A GST-Pax-6(HD/AD) expression vector encoding a C. elegans Pax-6 without the PD was provided by Scott Emmons (Albert Einstein College of Medicine, Bronx, NY). A GST-Prox-1 expression vector was provided by Stanislav Tomarev (National Eye Institute, Bethesda, MD).

**Expression of Proteins in E. coli and Protein Purification**

GST-fusion proteins were expressed in E. coli strain BL21(DE3) (Novagen, Madison, WI), grown at 30°C, induced by 0.5 mM isopropyl-β-thiogalactopyranoside and harvested after 3.5 hours of cultivation, according to the standard protocol (Pharmacia), leading to purified recombinant proteins or using ammonium sulfate precipitation resulting in enriched extracts, as described elsewhere.37 Protease-inhibitor cocktail (CompleteTM; Boehringer Mannheim, Indianapolis, IN) was added to the cell lysis buffer.

**Antibodies**

Two anti-Pax-6 antibodies were used. One (serum 11) was a rabbit polyclonal antiserum against the PD of Pax-6 (a gift of Simon Saule, Institut Pasteur, Lille, France), and the other was an affinity-purified antiserum raised against the C-terminal heptapeptide of Pax-6 (a gift of Janine Davis and Randall Reed, Johns Hopkins University, Baltimore, MD).39 Antibodies to TBP (provided by Nancy Thompson, McArdle Laboratory, University of Wisconsin, Madison), TAFII250 (Santa Cruz Biotechnology), and pRB and E2F-1 (provided by Ali Fattaey, Onyx Pharmaceuticals, Richmond, CA) were also used.

**Affinity Chromatography Using a GST-Pax-6 Column**

Columns using bacterially expressed GST, GST-Pax-6, GST-Tat, and GST-p53 fusion proteins were prepared from glutathione-Sepharose-4B (Pharmacia) resin. HeLa cell nuclear extracts and partially purified TFIID (the phosphocellulose and DE52 column fractions) were prepared according to standard protocols.40 After loading the proteins and collecting the flow-through fractions, the microcolumns were extensively washed with TNE300 (50 mM Tris-HCl [pH 7.9], 300 mM NaCl, and 1 mM EDTA) containing 0.1% NP-40, and proteins were eluted with the same buffer supplemented with 1 M NaCl.41 The aliquots (25 μl) were analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The flow-through fractions and 1 M NaCl eluate (after dialysis against buffered 100 mM KCl) were used in a standard in vitro transcription assay with the adenovirus major late promoter (MLP) as a template.41

**Protein–Protein Interaction, or GST Pull-Down, Assays**

GST-TBP or pRB fusion protein (5 μg) was bound to glutathione-Sepharose-4B in 50 mM Tris (pH 7.9), 75 mM NaCl, 1 mM EDTA (TNE) and 0.1% NP-40 in a final volume of 200 μl. Pax-6 or Pax-3 coding sequences cloned in pKW10 were transcribed using SP6 RNA polymerase according to the manufacturer’s protocol (Promega). Proteins were translated in vitro using a rabbit reticulocyte lysate (TNT, Promega) in the presence of [35S]methionine (1000 Ci/mmol, Amersham). Ten microliters of extract containing [35S]Met-Pax-6 proteins were incubated at 4°C for 2 hours with beads containing immobilized TBP or pRB. The protein–protein complexes attached to the beads were washed three times with TNE buffer containing 1% NP-40, and bound proteins were released by heat denaturation, analyzed on a 4% to 20% SDS gel (Novex, Encinitas, CA), dried, and subjected to autoradiography. The percentage of bound radioactivity was calculated using PhosphoImager (Molecular Dynamics, Sunnyvale, CA). All GST fusion- and [35S]Met-Pax-6 proteins were normalized according to protein concentration, as described previously.41 Alternatively, some GST pull-down assays were performed in the presence of ethidium bromide (final concentration of 100 μg/ml) included in the binding step.42

**Results**

**Pax-6 Interaction with TFIID**

To test our hypothesis that Pax-6 physically interacts with at least one general transcription factor, we prepared affinity columns consisting of glutathione-Sepharose coupled with a GST-Pax-6 fusion protein containing the paired and HDs, GST-Tat, and GST-p53; a column containing glutathione-Sepharose coupled to GST served as a negative control. GST-Tat and GST-p53 are known to interact with TBP and were used as positive controls.41,42 Nuclear extracts prepared from HeLa cells were loaded onto these columns and flow-through (100 mM NaCl), wash (300 mM NaCl), and high-salt (1 M NaCl) fractions were collected, as described in the Methods section. HeLa cell nuclear extracts do not contain endogenous Pax-6 protein.25 Proteins eluted with 1 M NaCl were examined by
Western blot analysis. An immunoblot using an antiserum specific to TBP detected this protein in the material released from the Pax-6 immobilized on these beads (data not shown). In a parallel experiment, we detected immunoreactive bands with antisera specific to human coactivator, TAFII250 (Fig. 1B, lanes 1 through 4) consistent with Pax-6 complexing with TFIID. Finally, we supplemented HeLa whole-cell extract with full-length Pax-6 generated in a reticulocyte lysate, immunoprecipitated Pax-6 using a specific antiserum, and analyzed the immunoprecipitated proteins using antisera specific to TBP (Fig. 1C). This procedure detected a TBP/Pax-6 complex (Fig. 1C, lane 1). In addition, Pax-6/TBP complexes were confirmed using nuclear extracts prepared from cultured αTN4-1 lens cells expressing endogenous Pax-6 protein, using immunoprecipitations followed by immunoblots with antibodies against TBP and Pax-6 (Fig. 1D). Collectively, these data support the idea that Pax-6 associates in vivo with TFIID. This interaction may be mediated by TBP alone and/or by one or more TBP-associated factors.

Interaction of Pax-6 In Vitro with TBP

We next tested Pax-6 and Pax-3 for their ability to interact with TBP in vitro. Pax-3 was included in this experiment because it has a molecular structure similar to that of Pax-6 (Fig. 2A). GST pull-down assays were performed using [35S]-labeled Pax-6 or Pax-3 and GST-TBP fusion protein bound to glutathione-Sepharose beads. Both Pax-3 and Pax-6 bound to GST-TBP but not to GST alone (Fig. 2B). The presence of 100 μg/ml of the DNA intercalator, ethidium bromide, did not affect formation of the Pax-6 complex with GST-TBP (data not shown). We conclude that both Pax proteins tested formed a specific complex with TBP in the absence of DNA.

western immunoblot analysis using an antibody specific to TBP (Fig. 1A, lanes 1 through 4), the TATA-box-binding subunit of the general transcription factor TFIID. The results indicated that Pax-6 associated either directly or indirectly with some general transcription factors.

To test the possibility that Pax-6 interacts with TFIID, we incubated glutathione beads with recombinant GST-Pax-6 and partially purified human TFIID complex. The TFIID complex was isolated by two chromatographic steps that separate TFIID from SL1 (a component of the RNA polymerase I system) and TFIIB (subunit of the RNA polymerase III system). After loading the TFIID fraction on the GST-Pax-6(PD/HD) beads, followed by washing, the Pax-6-associated proteins were released and analyzed by western
Interaction of TBP with the Homeodomain and C-Terminal Region of the AD of Pax-6

To define the regions of Pax-6 that interact with TBP, we produced a series of C-terminally truncated Pax-6 proteins (Fig. 3A). The fine structure of the α-helical regions of the HD indicating the precise sequences that were deleted in the Pax-6 mutants are indicated in Figure 3B. The diagrammatic structures of the truncated Pax-6 proteins and their relative binding efficiencies to GST-TBP are approximated in Figure 3A. Approximately 5% of the input full-length Pax-6 bound to the GST-TBP, which was considered 100% binding. Each test was conducted twice; the relative binding data were averaged and grouped into four categories: 0% to 24% binding (−); 25% to 50% binding (+); 51% to 75% binding (++); and 76% to 100% binding (+++). Less than 4% of the Pax-6 truncated to residue 238, pP6D238 (which retained only the N-terminal helix I of the HD), bound GST-TBP compared with the amount bound by wild type Pax-6, pP6D253 (which contains both helix I and II of the HD; Fig. 3B) showed an approximate 15-fold increase in binding efficiency to TBP compared with that of pP6D238. Although pPax-6HD (a sequence containing only the HD flanked by an additional 10 aa on its N terminus and 15 aa on its C terminus) had reduced binding to GST-TBP. That it bound as well as it did indicates the importance of the HD for binding GST-TBP. However, pPax-6HDΔ10 (without 10 aa from the N-terminal end of the HD) did not bind GST-TBP.

The present data suggest that Pax-6 may interact with TBP through the HD and C-terminal AD. We confirmed that the Pax-6 HD alone (Fig. 4, lane 6) and with the AD (Fig. 4, lane 5) can interact with TBP in GST pull-down assays using GST-Pax-6 HD incubated with [35S]TBP. Independent involvement of the AD was tested further in GST pull-down assays using GST-Pax-6 HD.


**Interaction of Pax-6 with pRB In Vitro**

Next we investigated which domains of Pax-6 and pRB are responsible for their interaction. The GST pull-down assay showed initially that Pax-6 interacted with GST-pRB protein that contained both pockets A and B, from amino acid residue 379 to 792 (data not shown). Next, we used the same panel of truncated Pax-6 proteins that was used to test the binding to GST-TBP to test for the Pax-6 regions that are required for binding to GST-pRB (Fig. 3A). As with Pax-6/TBP binding, Pax-6 binding to pRB depended on the presence of the HD, and the HD by itself was able to bind to a limited extent (pPax-6HD).

The Pax-6 PD by itself did not bind to pRB (pP6D140). Pax-6 binding to isolated pocket domain A (aa 379 to 612) or domain B (aa 612 to 792) of GST-pRB was also tested. Pax-6 did not bind to either domain A or B by itself, suggesting that a complete pocket of pRB is essential (data not shown).

**DISCUSSION**

The present study adds Pax-6 to a growing list of proteins that bind TBP, E2F-1, and pRB. As had been found for other HD transcription factors, the HD of Pax-6 appeared critical for binding, however the AD also seemed to have a role in these interactions. We have also found in GST pull-down experiments that Drosophila HD proteins Bcd, Ftz, Prd, and Zen and chicken Prox-1 (which contains a highly divergent HD) bind to pRB (data not shown). Because Pax-6 can activate or repress crystallin promoters and pRB is involved in suppressing cell division during lens fiber cell differentiation, we propose that Pax-6 and pRB may participate in interacting pathways controlling epithelial cell division, fiber cell elongation, and crystallin gene expression during lens development.

We may speculate about some consequences of the binding of Pax-6 to TBP in the lens. First, Pax-6 contains a strong, Ser/Pro/Thr-rich AD located between the HD and the C terminus. There is 95% conservation of this domain in different species (mouse, human, quail, and zebrafish), suggesting that the AD is important for mediating the biologic functions of Pax-6. Initial dissection of this domain indicated that its full activity depends on its integrity. This is further supported by a natural human PAX-6 mutation generating a truncated pro-
tein without peptides from the C terminus.\textsuperscript{1,9} Although one recent report indicates that truncated PAX-6 produced from the mutated allele may act as a dominant negative suppressor of protein produced by the normal allele by binding more tightly to DNA than the wild-type protein containing its AB,\textsuperscript{35} our data suggest also that the molecular mechanism for the human phenotype consisting of aniridia and early-onset cataract\textsuperscript{27} may include the absence of the C-terminal part of the AD required for TFIIID binding, thereby reducing the PAX-6 transactivation potential. It is also possible that the biologic stability of PAX-6 without 70 of 106 C-terminal residues is reduced and that the in vivo concentration of these aberrant forms is much less than that of the protein produced by the normal allele.

Another possible consequence of Pax-6 binding to TBP concerns the interpretation of earlier experiments using reporter transgenes with mutations in the TATA elements of the mouse \(\alpha A\)-crystallin\textsuperscript{56} and \(\alpha B\)-crystallin\textsuperscript{57} promoters. These mutated crystallin promoters showed reduced activities and altered transcription initiation sites but retained their lens-specific expression in transgenic mice. A possible explanation suggested from the present data is that Pax-6, which binds next to the TATA elements in both promoters,\textsuperscript{21,25} facilitates formation of atypical transcription preinitiation complexes by recruiting TFIIID. It is also possible that Pax-6 represses the activity of specific promoters by binding but being blocked from making contact with TBP in the TFIIID complex. Such a situation may exist for the chicken \(\beta B1\)- and \(\beta A3/A1\)-crystallin promoters that are downregulated by binding Pax-6.\textsuperscript{56} This would bestow Pax-6 with a silencing function, even though it has no repressor domain\textsuperscript{9} such as exists in Pax-2, Pax-3, Pax-5, and Pax-8.\textsuperscript{58,59} Pax-6 may also stabilize TFIIID binding to DNA through direct protein–protein contacts,\textsuperscript{51,57} or TFIIID may act as a carrier of Pax-6 to DNA binding sites. Additional consequences of protein binding to TBP have been reviewed recently.\textsuperscript{61}

What are the possible consequences of Pax-6 binding to pRB in lens development? pRB plays multiple roles in cellular biology.\textsuperscript{18,46,62} It is a classic tumor-suppressor gene and an essential regulator of cellular differentiation in different tissues including the lens. In addition to other defects, lens fibers fail to differentiate in pRB null mice, and consequently, the normal pattern of crystallin gene expression is disrupted.\textsuperscript{22} Our immunoprecipitation and GST pull-down assays showed the presence of a Pax-6/pRB complex in embryonic lens nuclear extracts. In vitro experiments showed that the Pax-6 HD interacts with a complete pRB pocket for complex formation. Parallel experiments showed the presence of an E2F/pRB complex in lens nuclear extracts. The same electrophoretic mobility of pRB after dissociation from complexes immunoprecipitated with E2F or Pax-6 antibodies suggests that Pax-6 interacts with a hypophosphorylated form of pRB. This possibility is supported by our preliminary data showing the presence of a Pax-6/pRB complex in G1, but not in S and G2-arrested \(\alpha TN4-1\) lens cells (data not shown). G1-arrested cells contain hypophosphorylated pRB, whereas S and G2-arrested cells contain hyperphosphorylated pRB.\textsuperscript{18} By contrast, we found Pax-6/TFIIID complexes at all stages of the \(\alpha TN4-1\) cell cycle (data not shown). Although preliminary, these findings support the idea that phosphorylation of pRB modulates its ability to bind Pax-6 in a manner similar to that known for the E2F family of transcription factors. It would be interesting to compare the relative amounts of pRB complexed with E2F and Pax-6, to determine whether the Pax-6/pRB complex can interact with DNA and to elucidate the possible effect of Pax-6/pRB complexes on lens-specific transcriptional regulation.

Further insight into the roles of Pax-6 and pRB during lens development can be obtained from studies of mice with pRB inactivated by gene targeting. Heterozygous mice (pRB\textsuperscript{+/−}) are grossly normal but are highly predisposed to the development of pituitary adenocarcinomas. Homozygous mice (pRB\textsuperscript{−/−}) die at 13 to 15 days of gestation. They have specific defects during hepatic erythropoiesis, widespread neuronal cell death in the brain and spinal cord, and lens defects.\textsuperscript{57} Of interest, chimeric mice partially composed of pRB-deficient cells are relatively normal with notable histologic defects in the lens, hyperplasia of the adrenal medulla, and enlarged cells in the cerebellum and the liver.\textsuperscript{28,29} Because the lens and cerebellum are sites of Pax-6 expression,\textsuperscript{13} it is possible that the defects in these tissues in the pRB-deficient mice originate, at least in part, from reductions in Pax-6/pRB complexes. The data showing an oncogenic potential of Pax-6\textsuperscript{63} suggest that pRB may be important in inactivating the proliferation roles of Pax-6 under certain circumstances.

Whatever the exact and, no doubt, multiple consequences of Pax-6 binding to TBP and pRB, our data clearly open several research avenues for exploring lens development. In particular, these data suggest the possibility that the pathways regulating crystallin gene expression and lens fiber cell differentiation overlap by virtue of the physical interactions of Pax-6 with pRB and TBP.

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References

Pax-6 Interactions with TFII D, TBP, and pRB


