Cellular retinaldehyde-binding protein (CRALBP) is a direct downstream target of transcription factor Pax6

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Abstract

Pax6 is a transcription factor with key functional roles in embryonic development. In order to identify downstream effectors of Pax6 in the developing cerebral cortex we performed microarray analysis. We compared gene expression profiles of cortical tissues isolated from wild type and Pax6$^{-/-}$ mouse embryos. Cellular retinaldehyde-binding protein (CRALBP) was identified as a gene significantly down regulated in Pax6$^{-/-}$ mutant embryos. Pax6 and CRALBP were co-expressed in the same cells of wild type embryos. Pax6 protein was detected on CRALBP promoter region in Chromatin IP experiments and was able to activate CRALBP promoter *in vitro*. All our data suggest that CRALBP is a direct immediate target of Pax6. Our data provide insights into molecular cascade acting down-stream of Pax6 during development.

1. Introduction

Pax6 belongs to the Pax family of transcription factors and contains two highly conserved DNA-binding domains- a bipartite paired domain (PRD) and a homeodomain (HD), and a C-terminal region that functions in transcriptional activation .

Pax6 is one of the key transcription factors involved in the control of various aspects of embryonic development. It is a key regulator of vertebrate eye development and upon misexpression, is sufficient to induce ectopic eyes . Mice heterozygous for Pax6 show small eye (Sey) phenotype whereas homozygote Pax6 mutants completely lack the entire eye structure .

Pax6 is also important for normal development of pancreas, forebrain and spinal cord. In the pancreas it controls differentiation of beta-cells . In the forebrain it controls patterning of the telencephalon, cell type specification, regionalization and arealization of the cerebral cortex. . In the diencephalon and spinal cord certain subpopulations of neurons are affected by Pax6 mutation .

Cellular retinaldehyde- binding protein (CRALBP) is a water-soluble retinoid-binding protein that belongs to the CRAL- Trio family of proteins. It is expressed in cornea, pineal gland, optic nerve, and abundantly in Muller cells and retinal pigment epithelial (RPE) cells of the eye . Originally CRALBP was identified as a carrier of 11-*cis*-retinol and 11-*cis*-retinaldehyde, that are known to function in vision . In CRALBP mutant mice, isomerization of all-*trans*- to 11-*cis*-retinol in the visual cycle is substantially impaired . Mutations in the human gene encoding CRALBP cause autosomal recessive retinitis pigmentosa (arRP), a condition characterized by progressive photoreceptor degeneration and night blindness .

Here we identified CRALBP as a downstream target of Pax6 transcription factor. CRALBP expression was abolished in Pax6$^{-/-}$ mouse mutants. We could show that CRALBP and Pax6 are co-expressed in the same cells during embryonic development. Pax6 protein binds CRALBP promoter in vivo and can activate it in vitro. Our data demonstrate that Pax6 directly controls expression of CRALBP during embryonic development.

2. Methods and materials

2.1. Chromatin Immunoprecipitation (ChIP) Assay

Mouse embryonic cortex (E14.5 or E 15.5) was used as a tissue source of chromatin. Cortex tissues were homogenized in 1x phosphate-buffered saline (10ml for 10-14 hemispheres of cortex tissue) containing protease inhibitors (Roche Applied Science). Protein-DNA complexes were cross-linked in 1% formaldehyde for 10 min at 37 °C in a water bath incubator. Cross-linking of protein-DNA complexes was terminated with three washes in 1x phosphate-buffered saline. Samples were then processed using a ChIP assay kit, essentially as described by the manufacturer (Upstate biotechnology, Lake Placid, NY). In brief, cells were lysed in SDS lysis buffer with protease inhibitors, then sonicated using a water bath sonicator, super RK 103H from Schütt labortechnik (Goettingen, Germany) to shear DNA to fragments with a length of 100–1000 bp. To reduce nonspecific background, the cell lysates were precleared by incubation with salmon sperm DNA/protein A-agarose slurry. The agarose beads were pretreated with 2% BSA before the preclearing step as suggested by the manufacturer. Supernatants from the preclearing step were incubated with (1: 500) rabbit anti-Pax6 polyclonal IgG (Covance),

at 4 °C overnight. Cortex from Pax6 mutant mice were used as a negative control. Chromatin-antibody complexes were precipitated by incubation with Protein A-agarose beads. Chromatin was eluted from the beads after washes in several buffers provided with the kit. The DNA-protein cross-links in all samples were reversed by incubation for 4 h at 65 °C with 0.2M NaCl followed by proteinase K treatment for 1 h at 45 °C. DNA was isolated by phenol/chloroform extraction and ethanol precipitation. PCR was performed with primers that flank known and putative Pax6 binding sites.

As a positive control, the following primers were used against Pax6 promoter region (~300 bp)

5'- CCCACCCTCAGGCTTCCA- 3' (forward)

5'-CTCCCGAGCCTGCCCATT- 3' (reverse).

Five primer pairs flanking putative Pax6 consensus sequences upstream of exon1 of CRALBP gene were designed as follows:

1. -2009 to -1671 bp upstream (338 bp fragment)

      5'-GACACACTAATCCCAGCTTG- 3' (forward)

      5'-GAGTATTTGATCACCTGCCAT- 3' (reverse);

2. -2998 to -2727 bp upstream (271 bp fragment)

5'-TGTGGCTGGATTTCAGAGAA3' (forward)

      5' -CAGGCTAAGGGAATGCATC- 3' (reverse);

3. -3453 to -3128 bp upstream (325 bp fragment)

      5' -AGGTAGGTGGCCAACAGTA- 3' (forward)

      5' –TATGCCCAAAGAACCTCCCT- 3' (reverse);

4. -4660 to -4336 bp upstream (324 bp fragment)

 5'-TCTGATACCTCTGCACCAG 3' (forward)

 5'-CTTTCTTGACTGCGATTTCCC3' (reverse);

5. -5713 to -5441 bp upstream (272 bp fragment)

 5'-TGCTAAGGTGTTAACAGGCC- 3' (forward)

 5' -CTGGCACTGGGATTACAAATG- 3' (reverse);

PCR conditions were as follows: 2min at 94 °C followed by 34 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C.

All PCR products were separated by 1.5% agarose gel electrophoresis in TAE buffer. DNA was loaded using OrangeG buffer, and 100 bp DNA markers (Invitrogen) was used at a concentration of 50ng/μl.

2.2. *In situ* Hybridization

Wild-type and Pax6 mutant brain and spinal cord of E14.5 mice were isolated, fixed in 4% PFA, dehydrated in increasing concentrations of ethanols and fixed in paraffin blocks. 10μm thick sections were cut and *In situ* hybridization with RNA probes was performed as stated earlier .

2.3. Immunohistochemistry

Immunohistochemistry was performed on 10μm thick cryosections of E14.5 wild-type brain and eye. Freshly isolated brains were fixed in 4% PFA for 2-4 hrs at 4°C, soaked in 30% sucrose overnight and cryopreserved in tissue-tek (Sakura Finetek Zoeterwoude, NL). Immunohistochemistry was carried out with rabbit anti-CRALBP (kindly provided

by Prof. John Saari, University of Washington, Seattle; 1:500) and mouse monoclonal Pax6 (Developmental Studies Hybridoma Bank, Iowa; 1:100) antibodies. Sections were blocked in 1% BSA and 0.5% Tween in PBS for 1hr, rinsed in PBS and incubated for 2 hrs with primary antibodies at RT. Primary antibodies were washed in PBS and detected using fluorescent secondary antibody (1:1000) for 1 hr. Sections were rinsed in PBS and visualized using a fluorescent microscope after mounting with Prolong Gold antifade reagent with DAPI (Invitrogen).

2.4. Plasmid constructs and luciferase reporter assay

Dual-luciferase Reporter Assay System was used to measure luciferase activity (Promega, UK). Chinese hamster ovarian (CHO) cells were cultured in neurobasal medium in 10% FCS and 1% Pen/Strep. These cells were plated in triplicates into 96 well plate at a density of 3×10^4 cells/well. 12 hrs later, they were transfected with Pax6 cDNA plasmid and luciferase cDNA plasmid under the CRALBP promoter , along with renilla luciferase vectors as internal controls. Lipofectamine 2000 reagent (Invitrogen), was used to increase the efficiency of transfection according to the manufacturer's protocol. 24 hrs after transfection, cells were washed twice in cold PBS and lysed using lysis buffer. The firefly and renilla luciferase activities were measured using a Microlumat Plus LB 96V luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbag, Germany). Readings obtained for firefly luciferase were divided with readings obtained for renilla luciferase to normalize the levels of the experimental reporter activity.

2.5. Histology

Freshly isolated brains were dehydrated with a series of ethanol wash steps (30%, 50%, 70%, 80%, 90%, 95% and 100%) for atleast 2 hrs each, transferred to toluol for 6 hrs, soaked in fresh paraplast, twice, overnight and then embedded in paraffin blocks for sectioning.

2.6. Image acquisition

Bright and dark field images were obtained with a light microscope (Olympus). To obtain fluorescent images, a Zeiss confocal microscope was used (Zeiss LSM 510 META). Images obtained were edited using Adobe Photoshop.

3. Results

3.1. CRALBP expression is down-regulated by Pax6 deletion

In order to identify Pax6 downstream targets in the developing neocortex we carried out

microarray analysis of Pax6$^{-/-}$ mutant and wild type mice (Supplementary data table 1;

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32271). Pax6 is highly

expressed in the mouse cerebral cortex at embryonic day E14.5, therefore we selected

this stage in order to compare gene expression profiles between wild type and Pax6$^{-/-}$

homozigous cortici. One of the genes that was strongly down regulated in the mutant

turned out to be CRALBP. This gene was also independently identified in similar

experiments by Götz and co-authors . In order to verify extent of down-regulation of

CRALBP in Pax6 mutant embryos we performed *in situ* hybridization (ISH) and

immunohistochemistry (IHC). Neither ISH nor IHC produced any detectable CRALBP

signal in Pax6 mutant embryos.

3.2. Pax6 and CRALBP are coexpressed in several regions of the developing embryo in the same cells

To investigate whether Pax6 and CRALBP are expressed in the same regions, we carried

out comparative expression analysis by *in situ* hybridization. The expression pattern of

CRALBP RNA in E14.5 wt brains was almost identical to that of Pax6. CRALBP was

expressed in the neocortical VZ, cortical hem and in the hypothalamus (Fig: 1c). In Pax6

mutant cortex the expression of CRALBP was abolished in all these regions (Fig: 1d).

Since Pax6 is also expressed in the spinal cord, we investigated the expression of

CRALBP in the spinal cord using *in situ* hybridization. In wt spinal cord, Pax6 and

CRALBP are expressed in the VZ (Fig: 1f and g) of the spinal cord. No expression of CRALBP was detected in the spinal cord in Pax6 mutants (Fig: 1h).

Next, we addressed whether Pax6 and CRALBP are expressed in the same cells. To study protein expression, a double immunohistochemistry (IHC) was performed on E14.5 wt and Pax6 mutant brains. In the wt brain, CRALBP was expressed in all Pax6 expressing regions. These regions included pallial-subpallial boundary (PSPB) of the cortex and regions around the third ventricle in the thalamus (Fig: 2). In all these regions CRALBP showed sharp boundaries of expression that coincided with Pax6. Expression of Pax6 and CRALBP was excluded from the cortical hem (Fig: 2a-c). We did not find any CRALBP expressing cells that did not express Pax6. In the neocortex, expression of CRALBP protein was not only detected in the ventricular zone but also in the cortical plate (Fig: 2e), while Pax6 expression was confined to the VZ. This can be explained by the fact that VZ is populated by radial glia cells that reside in the VZ but extent their processes towards pial surface. Therefore, Pax6 as a nuclear protein is detected in the VZ (region where all cell nuclei are located), on the other hand, CRALBP is a cytoplasmic protein and is more likely to be detected in the radial processes. The complete abolishment of CRALBP mRNA expression in the cortex and spinal cord of Pax6 mutant mice suggested that *CRALBP* could be a direct downstream target of Pax6 (Fig: 1d and h).

Interestingly, CRALBP and Pax6 protein do not seem to co-express in the developing eye. At E 14.5, Pax6 is expressed in the neuroretina (NR) whereas CRALBP is expressed in the retinal pigment epithelium (RPE) that surrounds the neuroretina (Fig: 2m and n). Our results indicate that Pax6 and CRALBP are not co-localized in the eye (Fig: 2o). It

was not possible to study expression of CRALBP in the eye of Pax6 mutants because they fail to develop an eye.

3.3. Chromatin immunoprecipitation assay (ChIP) reveals Pax6 binding sites on CRALBP promoter

Our expression data (Fig: 1 and 2) suggested that Pax6 directly regulates the expression of CRALBP. Since putative Pax6 binding sites are known , we inspected the 5' upstream region of exon1 of the mouse *CRALBP* gene. We were able to identify 6 putative Pax6 binding sites within 6 kb upstream of the first exon. The consensus sequence "CACGTG" was located at 5.7 kb, 4.6 kb and 2 kb upstream (Fig: 3a), and the consensus sequence "GGTGGAA" was located at 3.4 kb, 2.9 kb and 2 kb (Fig: 3a) upstream of CRALBP exon1. In order to perform Chromatin immunoprecipitation assay (ChIP), we chose a time point during development where Pax6 is known to be highly active. By E14.5, expression of Pax6 can be detected throughout the anterior- posterior axis of the dorsal telencephalon in the ventricular zone and the subventricular zone . DNA-protein complexes isolated from E14.5 cortex were precipitated with Pax6 antibody and amplified using primers against sequences flanking Pax6 binding sites in *CRALBP* upstream genomic region. Pax6 antibody precipitated two DNA fragments from *CRALBP* 5´ region, at 2kb and 2.9kb upstream of exon1 (Fig. 3c). Similar experiment was also performed by precipitating DNA-protein complexes using Pax6 antibody from Pax6 mutant cortex tissue. As predicted, we did not amplify any DNA above background in the PCR reaction (data not shown). Our *in vivo* results indicate that Pax6 binds the putative promoter region of *CRALBP*.

3.4. Pax6 activates CRALBP promoter *in vitro*

To evaluate whether binding of Pax6 on *CRALBP* promoter induces expression of CRALBP, a luciferase-reporter assay was performed. CHO cells were transiently cotransfected with Pax6 cDNA plasmid and with a plasmid that contains previously identified CRALBP promoter coupled with luciferase gene . The CRALBP promoter plasmid also contains the 5' upstream sequence of exon 1 of CRALBP gene that were positive in our ChIP experiments (see above). Co-transfected cells were harvested 24hrs after transfection and luciferase activity was measured. The reporter activity of CRALBP promoter plasmid was significantly increased with increasing concentrations of Pax6 cDNA plasmid, whereas the control G5-luciferase reporter activity showed only a basal level of expression (Fig: 4). These *in vitro* experiments indicate that Pax6 can positively regulate the activity of *CRALBP* promoter *in vitro*. In order to find out whether any aspects of Pax6 mutant phenotype where recapitulated in CRALBP knock-out animals we analyzed CRALBP mutant brains. We failed to detect any changes in the developing brain of CRALBP mice. Both the overall morphology and cortical layering were not changed. We did not detect any changes in the neuronal migration, proliferation or radial glia morphology during embryonic development (data not shown).

4. Discussion

Here, we identified CRALBP as a direct down-stream target of Pax6 transcription factor.

Pax6 is expressed in the neocortical ventricular zone (VZ), pallial-subpallial (PSPB)

boundary and the thalamus (Fig: 1b). CRALBP was found to be expressed in all these

regions (Fig: 1c). The borders of CRALBP protein expression coincided with Pax6

expression borders in various regions of the neocortex and thalamus (Fig: 2a-l).

Interestingly, CRALBP protein expression was also detected throughout the cortical plate

(Fig: 2e), while Pax6 protein expression was limited to the VZ (Fig: 2d). There are two

possible ways to explain why CRALBP protein is detected all over the cortex while the

mRNA is seen only in the VZ. One possibility is that young neurons derived from

cortical progenitors in the VZ continue expressing CRALBP after its expression was

initiated by Pax6 in the progenitors. Even though these neurons migrate away from the

VZ towards the cortical plate, we continue to observe CRALBP protein possibly due to

high stability and low turnover rate of the protein. Second possibility is that CRALBP

protein, being produced by Pax6 positive radial glia progenitors, is transported along

radial glial processes towards the pial surface. This however does not explain why

CRALBP staining cannot delineate individual radial glial processes. Pax6 is also

expressed in the differentiating field of the amygdala and in the hypothalamus

surrounding the third ventricle (Fig: 1b; indicated by double arrow heads). However, we

did not find expression of CRALBP in these regions (Fig: 1c). Since the expression of a

gene might depend on binding of many transcription co-factors, such co-factors required

for CRALBP expression could be lacking in these regions.

Abolishment of CRALBP expression in the spinal cord of Pax6 mutants (Fig: 1h)

indicates that CRALBP is a target of Pax6 not only in the neocortex but also in other

regions of the central nervous system. This also implies that CRALBP might play a role in Pax6 mediated development of the spinal cord.

Using chromatin immunoprecipitation assay, we showed that Pax6 binds to two sites upstream of exon1 of CRALBP gene, at 2kb and 2.9kb (Fig: 3c). Additionally, we could confirm the regulatory activity of Pax6 on CRALBP promoter *in vitro* in luciferase reporter assay (Fig: 4).

Pax6 is considered a master regulatory gene of eye development . We found expression of Pax6 in the neuroretina (NR), (Fig: 2m) and that of CRALBP in the retinal pigment epithelium (RPE) that surrounds the neuroretina (Fig: 2n). During eye morphogenesis, Pax6 is expressed in the optic vesicle (OV) and the surface ectoderm (SE) that surrounds the OV. SE thickens to form the lens placoid (LP), whereas the distal OV invaginates to form the optic cup (OC) with the inner layer developing into the NR . The optic vesicle contains bipotential progenitors which can give rise to both RPE and NR cell types. Separation of progenitors into NR and RPE domains is mediated by external cues . We suggest that expression of Pax6 in the bipotential progenitor cells of the OV promotes CRALBP expression. After receiving signals from external cues, the bipotential progenitors differentiate into NR and RPE cells. The RPE (expressing CRALBP) then migrate and occupy the periphery surrounding the NR (Fig: 2n). After the optic cup is formed, Pax6 is downregulated in the optic stalk and the RPE, but retained in the neuroretina . This could possibly explain the expression of CRALBP and Pax6 in distinct non- overlapping regions of the eye (Fig: 2o).

We did not observe any defects in either layer-specification or migration of neurons in CRALBP mutants. However, it is probable that other retinal binding proteins (RBP's)

expressed in the brain compensate for the loss of CRALBP. Further experiments need to be carried out to uncover the role of CRALBP in embryonic development.

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Figure legends

Fig 1: *In situ* hybridization with Pax6 and CRALBP probes in Wt and Pax6 mutant.

Comparison of Pax6 and CRALBP expression in the cortex (a-d) and spinal cord (e-h) at E14.5. Dark-field images (b-d; f-h) and bright-field images (a and e). Pax6 and CRALBP are expressed in the cortical hem, VZ of neocortex and spinal cord, PSPB, and around the third ventricle as shown by single arrowheads (b-c; f-g). Pax6, and not CRALBP, is also expressed in some postmitotic areas as shown by double arrow heads (b). In Pax6 mutants, expression of CRALBP is completely abolished in the cortex and spinal cord (d and h). The anatomical structures are mentioned in the bright field images (VZ; ventricular zone, PSPB; pallial-subpallial boundary, hypo T; hypothalamus, C hem; cortical hem and amygdala).

Fig 2: Comparison of Pax6 and CRALBP expression in the brain and eye by Immunohistochemistry. Pax6 and CRALBP expression in the cortex (a-f), thalamus (g-l) and eye (m-o) at E14.5. Pax6 expression (a, d, g, j, m), CRALBP expression (b, e, h, k, n) and overlay of both proteins (c, f, i, l, o). Pax6 and CRALBP are excluded from the cortical hem (a, b and c). Both proteins share similar borders of expression at the cortical hem (a, b and c), pallial- subpallial boundary (PSPB; d, e and f) and regions around the third ventricle in the epithalamus (g, h and i) and hypothalamus (j, k and l) as indicated by arrows. In the eye, Pax6 is expressed in the neuroretina (m) and CRALBP is expressed in the retinal pigment epithelium that surrounds the neuroretina (n). In the eye, they do not share overlapping domains of expression (o).

Fig 3: Identification of putative Pax6 binding sites using Chromatin Immunoprecipitation (ChIP) assay. Six putative Pax6 binding sites on CRALBP promoter, their distance from exon1 of CRALBP gene and PCR primers to amplify Pax6 binding sites are indicated (a). The 2.3kb segment used for the luciferase reporter assay is also indicated (a). A known Pax6 binding site on its own promoter was used as a positive control for the PCR (b). PCR amplification for the putative Pax6 binding sites on CRALBP promoter are shown (c). DNA-protein complexes precipitated with Pax6 Ab (+) and without Ab (-) are indicated (b and c). ChIP assay revealed two Pax6 binding sites on CRALBP promoter (c; 1+ and 2+). PCR amplification of ChIP input DNA with primers for putative Pax6 binding sites is also shown (d).

Fig 4: Co-transfection of CHO cells with Pax6 and CRALBP plasmids. Reporter activity of luciferase cDNA under the CRALBP promoter was measured in CHO cells by transfecting them along with Pax6 cDNA plasmid. Increasing concentrations of Pax6 cDNA (50ng, 100ng, 150ng), increases CRALBP-luciferase reporter activity (grey bars). The G5-luciferase control plasmids do not show significant increase in activity with increasing concentrations of Pax6 cDNA plasmid (striped bars). Background expression of Pax6 and CRALBP plasmids are also shown (dotted bars). The genomic position of 2.3kb DNA fragment used for luciferase assay is indicated on Fig 3a.