FEBS Letters 584 (2010) 753–758

FEBS Letters

journal homepage: www.FEBSLetters.org

Panky, a novel photoreceptor-specific ankyrin repeat protein, is a transcriptional cofactor that suppresses CRX-regulated photoreceptor genes

Rikako Sanuki [a,b], Yoshihiro Omori [a], Chieko Koike [a,c], Shigeru Sato [a], Takahisa Furukawa [a,*]

[a] *Department of Developmental Biology, Osaka Bioscience Institute, 6-2-4 Furuedai, Suita, Osaka 565-0874, Japan*
[b] *Department of Molecular Development, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Sho-goin, Sakyo-ku, Kyoto 606-8507, Japan*
[c] *PRESTO, JST, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan*

ARTICLE INFO

Article history:
Received 4 October 2009
Revised 16 December 2009
Accepted 16 December 2009
Available online 22 December 2009

Edited by Ned Mantei

Keywords:
Retina
Repressor
Photoreceptor cell
Ankyrin repeat
Transcriptional cofactor

ABSTRACT

Neuronal gene transcription is regulated by both transcriptional activators and repressors. While the roles of transactivators in retinal photoreceptor development have been well characterized, the roles of repressors have been poorly understood. We isolated *Panky/Ankrd33*, a gene encoding an ankyrin repeat-containing protein. *Panky-A* was specifically expressed in retinal photoreceptors and the pineal gland, and its expression was directly up-regulated by the CRX transcription factor. Subcellular localization of PANKY-A was observed in the nucleus and cytoplasm. Additionally, transactivation analysis suggested that PANKY-A is a transcriptional cofactor that suppresses CRX-activated photoreceptor genes. Furthermore, we found by an electrophoretic mobility shift assay that PANKY inhibited the DNA-binding activity of CRX.

1. Introduction

The neural retina is an exquisitely sensitive light detector. The vertebrate retina is formed of six types of neurons and one type of glial cell in three cell layers: the outer nuclear layer (ONL), which contains rod and cone photoreceptors; the inner nuclear layer (INL), the location of bipolar, horizontal and amacrine interneurons, and Müller glia; and the ganglion cell layer (GCL), which harbors ganglion cells. Each retinal cell is generated from common precursors that retain the capacity to trans-differentiate into each retinal cell type both in vivo and in vitro [1,2]. It is known that this neural differentiation is controlled by the functions of both transcriptional activators and repressors [3].

We previously reported that *orthodenticle homolog 2* (*Otx2*) plays a critical role in the cell fate determination of photoreceptor cells. In the *Otx2* CKO retina, differentiating photoreceptor precursor cells are converted to amacrin-like neurons [4,5]. In terminal differentiation of photoreceptor cells, CRX, another *Otx* family transcription factor, is essential for the formation of outer segments, synaptic terminals, and phototransduction pathways [1,6]. CRX functions together with several other transcriptional cofactors [2,7–12], including NRL and NR2E3/PNR. NRL is expressed in rods in the developing retina, and transactivates the *Rhodopsin* promoter in cooperation with CRX [11,13]. The deletion of *neural retina leucine zipper* (*Nrl*) in the genome converts the developing rods to the S-opsin expressing cones, indicating the importance of NRL in rod cell fate determination [14,15]. The rod photoreceptor development also requires another transcription factor, NR2E3 which interacts with CRX [11]. NRL and NR2E3 function not only to activate rod genes, but also to repress cone genes in developing rods.

To further identify genes that may regulate photoreceptor development and functions, we performed a microarray analysis of the wild-type and *Otx2* CKO retinas [5]. We identified the *Panky* (*photoreceptor ankyrin repeat protein*) gene, which was significantly down-regulated in the *Otx2* CKO retina, in this analysis. *Panky-A*, a splicing variant form of *Panky* dominantly expressed in the retina, encodes a protein containing five repeats of the ankyrin motif. The ankyrin repeat motif consists of repeated α-helices of 30–33 amino acid residues linked by loops. The ankyrin repeat is one of the most common motifs and has also been found in various transcription factors and transcriptional cofactors [16]. In this study, we carried out histological and biochemical analyses of *Panky-A*. We found that PANKY-A is localized in the nucleus and cytosol in mammalian cells, and *Panky* expression is regulated by CRX. We also found that PANKY-A functions as a repressor for transcription of CRX-regulated photoreceptor genes.

Abbreviations: Crx, cone–rod homeobox; Otx2, orthodenticle homolog 2; Nrl, neural retina leucine zipper; Nr2e3, nuclear receptor subfamily 2 group E member 3

* Corresponding author. Fax: +81 6 6872 3933.
E-mail address: furukawa@obi.or.jp (T. Furukawa).

0014-5793/$36.00
doi:10.1016/j.febslet.2009.12.030

2. Materials and methods

2.1. Identification of mouse Panky cDNA and phylogenetic analysis

Based on the mouse spliced EST data in the UCSC Genome Browser (http://genome.ucsc.edu), a forward primer (5′-TTGGTATCAGCTACCCCACAGA-3′) and a reverse primer (5′-GACGACCTACACAGAATACAGGTT-3′) were used for PCR amplification of a *Panky* cDNA fragment containing an entire open reading frame. The PCR reaction was carried out with KOD-Plus DNA polymerase (TOYOBO, Japan). The mouse PANKY amino acid sequence was aligned with human, bovine, chick, and zebrafish putative PANKY sequences (NP_872414, XP_001788577, XP_419009, and XP_685746, respectively) by CLASTAL-W, and the guide tree was calculated using the neighbor-joining method (Kimura's two-parameter distance method) by DNA Data Bank of Japan (DDBJ; http://clustalw.ddbj.nig.ac.jp/top-j.html).

Full methods and materials are presented in Supplementary data.

3. Results and discussion

3.1. Expression patterns of Panky

We previously screened retinal genes, which are down-stream targets of the OTX2 transcription factor, using microarray (our unpublished data). In this screen, we identified a photoreceptor-enriched gene encoding an ankyrin repeat protein with an unknown function. We referred to this gene as *Panky* (*photoreceptor ankyrin repeat protein-A*) (Genbank #FJ895380). To confirm that *Panky* expression was regulated by *Otx2*, we performed quantitative RT-PCR (Q-PCR) analysis and compared *Panky* expression in the *Otx2* CKO and control retinas. Expression of *Panky* was markedly reduced in the *Otx2* CKO retina compared to the control (Supplementary Fig. S2A). To obtain a cDNA containing a full open reading frame (ORF) of the *Panky* gene, we performed RT-PCR using cDNA synthesized from adult mouse retinal RNA, and obtained a 1.5-kb cDNA predicting an ORF of 385 amino acids in length with a 5′ in-frame stop codon (Supplementary Fig. S1). We identified two splice variants of *Panky,* and designated them *Panky-A* (a longer form) and *-B* (a shorter form). PANKY-A and -B contain five and four ankyrin repeats, respectively, in the N-terminal portion (Fig. 1A and B). The PANKY amino acid sequence is conserved from human, bovine, and chick, through to zebrafish, which display 71%, 70%, 37% and 37% identity with the mouse PANKY-A protein, respectively (Supplementary Fig. S2B). The ankyrin repeat motifs of human, bovine, chick and zebrafish PANKY display 81%, 82%, 58% and 58% identity with those of mouse PAKNKY-A protein (Supplementary Fig. S2C). Human and bovine PANKY also contains five repeats of the ankyrin motif as mouse PANKY-A dose (Supplementary Fig. S2C)**.** Human and bovine PANKY also contains five repeats of the ankyrin motif as mouse PANKY-A dose (Supplementary Fig. S2C). The chromosomal localizations of mouse and human *PANKY* genes were determined by searching the mouse and human genome databases (NCBI). Mouse *Panky* is mapped to chromosome 15F2. Human *PANKY* maps to chromosome 12q13.13. These regions do not contain a candidate mutant or disease locus so far mapped. In order to examine the tissue specificity of *Panky* expression, we performed Northern blot analysis of the *Panky* transcripts in various adult tissues. We detected a strong 1.7-kb band specifically in the retina (Fig. 1D). To examine the tissue distribution of the *Panky* variants, we examined the expression of *Panky* transcripts in the pineal gland, retina, brain and liver by RT-PCR using primers which can both amplify and distinguish the two isoforms, *Panky-A* and *-B* (Fig. 1C). We detected *Panky* expression in the retina and pineal gland where *cone–rod homeobox* (*Crx*) is also known to be expressed (Fig. 1C) [6,17]. We observed that *Panky-A* expression was predominant and *Panky-B* expression was almost undetectable in the retina. A small amount of *Panky-B* was detected in the pineal gland (Fig. 1C). We then investigated the expression patterns of *Panky* in the developing retina by section in situ hybridization (Fig. 1E–J). The *Panky* signal was first detected in the presumptive photoreceptor layer at P1 and it increased at P5 (Fig. 1G and H). A strong *Panky* signal was detected in the outer nuclear layer, which is a photoreceptor cell layer, at P9 (Fig. 1J), and then the signal diminished slightly but was significantly maintained in the adult retina (Fig. 1J). Our results indicate that *Panky* is predominantly expressed in developing and mature photoreceptors and the pineal gland. This expression pattern correlates well with that of *Crx*, *Rhodopsin* and the other photoreceptor genes at P6–P9 [18]. Around P6, photoreceptors begin to undergo terminal differentiation, forming the outer segment [19]. We therefore hypothesized that *Panky* plays a significant role in late development of photoreceptors and maintenance of mature photoreceptors. These results indicate that *Panky* is expressed at least in rods, however, it is difficult to clearly examine whether or not *Panky* is expressed in cones, because the resolution of in situ hybridization is not high enough. Previously Hsiau et al. reported the expression profiles of the *Nrl* KO and *nuclear receptor subfamily 2 group E member 3* (*Nr2e3*) KO retinas [20]. It is known that expression of many cone-specific genes is up-regulated in these KO mice, and in contrast, the expression of many rod-specific genes is down-regulated. We examined *Panky* expression in the *Nrl* KO and *Nr2e3* KO retinas, and found that Panky expression was not significantly affected between the KO and control retinas (1.4-fold in *Nrl* KO, 1.4-fold in *Nr2e3* KO). These data suggest that *Panky* is expressed in both cones and rods.

In order to further characterize the *Panky-A* protein, we raised an anti-mouse PANKY antibody. We expressed *PANKY-A* by transiently introducing a *Panky-A* expression plasmid into 661W cells, a cone photoreceptor-like cell line. We detected a PANKY-A signal of 42 kDa on cell lysates using the anti-PANKY antibody by Western blot analysis (Fig. 1K). We detected a very faint PANKY-A band in the retinal extract (data not shown). However, we failed to detect a significant signal on mouse retinal sections using the anti-PANKY antibody (data not shown). These results might be due to the low sensitivity of the anti-PANKY antibody. In order to determine the subcellular localization of the PANKY-A protein in mammalian cells, we then observed 661W cells transfected with the *Panky-A* expression plasmid by immunostaining using the anti-PANKY antibody. We co-transfected the *Panky-A* expression plasmid together with a plasmid to express either EGFP or EGFP containing an endoplasmic reticulum localization signal (ER-EGFP) into 661W cells, and immunostained transfected cells with the anti-PANKY antibody. Both EGFP and PANKY-A signals were detected in the cytoplasmic region and the nucleus (Fig. 1L–Q and U), however no PANKY-A signal was observed in the endoplasmic reticulum marked with ER-EGFP (Fig. 1R–T). To exclude the possibility that the PANKY-A signal observed in the nucleus was due to the cytosolic signal overlaying the nucleus, we examined nuclear localization of PANKY-A protein by z-stacked image analysis on the 661W cells co-transfected with both *Panky-A* and EGFP expression plasmids. We confirmed that PANKY-A signals were localized in the nucleus (Fig. 1U). To investigate the localization of PANKY-A protein in cultured retinal cells, we transfected the *Panky-A* expression vector into 661W cells and detected the PANKY protein by Western blotting. We detected PANKY-A bands in both the cytosolic and nuclear fractions, however, we detected a stronger signal in the nuclear fraction (Fig. 1V). These data showed that the PANKY-A protein was localized in both the cytosol and nucleus in cultured retinal cells.