Human pancreas protein 2 (PAN2) has a retinal reductase activity and is ubiquitously expressed in human tissues

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Abstract Human gene for pancreas protein 2 (PAN2) is a novel member of the short-chain dehydrogenase/reductase gene superfamily. The properties of PAN2 protein have not yet been characterized. We present the first evidence that human PAN2 is a ubiquitously expressed microsomal enzyme that recognizes retinoids but not steroids as substrates with the apparent K_m values between 0.08 μM and 0.4 μM. PAN2 is ~4-fold more efficient in the reductive than in the oxidative direction. The apparent K_m values for $NADP^+$ and NADPH are 0.65 μM and 0.32 μM versus 1200 μM and 1060 μM for NAD^+ and NADH, respectively. Kinetic constants and expression pattern of PAN2 suggest that it is likely to function as a reductase in vivo and might contribute to the reduction of retinaldehyde to retinol in most human tissues.

Key words: Retinaldehyde; Reductase; Microsomal; Human

1. Introduction

Short-chain dehydrogenases/reductases (SDRs) are a rapidly growing family of proteins, which includes steroid, retinoid, and prostaglandin dehydrogenases [1–3]. Similar to many other SDR proteins, pancreas protein 2 (PAN2) (GenBank accession number AF237952) was initially identified as a member of the SDR superfamily based on the presence of signature SDR motifs in its primary structure: the cofactor binding domain GxxxGxG at Gly-50 and the putative active site consensus sequence YxxxK at Tyr-217. To our knowledge, the substrate and cofactor specificity of PAN2 has not yet been reported and its physiological function is not known. PAN2 shares ~40% sequence identity with the recently characterized all-*trans*-retinal reductase type 1 (RalR1), which is highly expressed in prostate [4,5] (Fig. 1). RalR1 prefers all-*trans* isomers over *cis* isomers of retinoids and is 50-fold more efficient in the reductive direction, suggesting that it might contribute to the conversion of all-*trans*-retinaldehyde to all-*trans*-retinol in prostate epithelium [4]. Retinaldehyde can be formed in the prostate epithelium by a symmetrical cleavage of β-carotene catalyzed by the recently cloned β-carotene 15,15′-monooxygenase [6–11]. Besides prostate, β-carotene monooxygenase is expressed in many other tissues [6–11], suggesting that various types of cells can supplement their local retinoid stores by converting β-carotene into retinol via retinaldehyde as an intermediate product. While RalR1 could reduce retinal to retinol in prostate, different enzymes could contribute to the reduction of retinal in other tissues. In the present study, we investigated the tissue distribution of PAN2 and determined whether it is active toward retinoids.

2. Materials and methods

2.1. Northern blot analysis

The IMAGE consortium clone #4053893 that contained the full-length cDNA for PAN2 was obtained from Research Genetics (Huntsville, AL, USA). The cDNA probe for Northern blot analysis was prepared by digestion of the cDNA clone with *Eco*RI and *Xho*I restriction endonucleases, followed by gel purification of the ~1.1-kb pair DNA fragment. Human 12-lane multiple tissue mRNA blots (Clontech, Palo Alto, CA, USA) were hybridized with ^{32}P-labeled cDNA probe in ExpressHyb hybridization solution (Clontech), according to the manufacturer's instructions. Briefly, the blots were prehybridized in ExpressHyb solution for 30 min at 68°C and transferred to a fresh solution containing 2×10^6 cpm/ml denatured radiolabeled cDNA. The hybridization was carried out at 68°C for 1 h. The blots were rinsed in 2×SSC (2×SSC = 0.3 M NaCl, 0.03 M trisodium citrate), 0.05% sodium dodecyl sulfate (SDS) several times at room temperature and washed in 0.1×SSC, 0.1% SDS for 10 min at 50°C. The mRNA bands were visualized by exposure to X-ray film at 70°C with two intensifying screens for 3 days.

2.2. Expression in Sf9 cells

To express PAN2 cDNA in insect cells, the cDNA clone #4053893 was digested with *Eco*RI and *Pst*I restriction endonucleases. The 1327-bp fragment was gel purified and ligated into the corresponding sites of pVL1393 baculovirus transfer vector. The construct was verified by DNA sequencing. Cotransfection of Sf9 cells with the transfer vector and linearized BaculoGold DNA was performed according to the manufacturer's protocol (Pharmingen, San Diego, CA, USA). The recombinant virus was amplified and used to produce PAN2 protein essentially as described for human retinol dehydrogenase 4 (RoDH-4) [12]. After 3 days of incubation at 27°C, the infected cells were collected by centrifugation and homogenized using French press. The unbroken cells, cellular debris, and nuclei were removed by centrifugation at 1000×*g* for 10 min. Mitochondria were pelleted by centrifugation at 10 000×*g* for 30 min, and microsomal fraction was isolated by centrifugation at 105 000×*g* for 1 h through a 0.6 M sucrose cushion. Microsomes were resuspended in 0.1 M potassium phosphate, pH 7.4, 0.1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol, 20% glycerol, aliquoted and stored frozen at −70°C. Protein concentration was determined by Lowry et al. [13] using bovine serum albumin as a standard.

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Abbreviations: PAN2, pancreas protein 2; SDR, short-chain dehydrogenase/reductase; RalR1, retinal reductase 1; HPLC, high-pressure liquid chromatography; 2×SSC, 0.3 M NaCl, 0.03 M trisodium citrate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; retSDR1, retinal SDR1; prRDH, photoreceptor retinol dehydrogenase

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hPAN2     MAVATAAAVL AALGGALWLA ARRFVGPRVQ RLRRGGDPGL MHGKTVLITG   50
hRalR1    MVELMFPLLL LLLPFLLYMA APQIR--KML SSGVCTSTVQ LPGKVVVVTG   48
          *         *   *   *  * *                              ** *  **

hPAN2     ANSGLGRATA AELLRLGARV IMGCRDRARA EEAAGQLRRE LRQAAECGPE  100
hRalR1    ANTGIGKETA KELAQRGARV YLACRDVEKG ELVAKEIQ-- ----------   86
          ** * *  **  **   ****    ***     *  *

hPAN2     PGVSGVGELI VRELDLASLR SVRAFCQEML QEEPRLDVLI NNAGIFQCPY  150
hRalR1    -TTTGNQQVL VRKLDLSDTK SIRAFAKGFL AEEKHLHVLI NNAGVMMCPY  135
             *       ** ***     * ***    *  **  * *** ****   ***

hPAN2     MKTEDGFEMQ FGVNHLGHFL LTNLLLGLLK SSAPSRIVVV SSKLYKYGDI  200
hRalR1    SKTADGFEMH IGVNHLGHFL LTHLLLEKLK ESAPSRIVNV SSLAHHLGRI  185
           ** *****   ********* ** ***  **  ******* * **     * *

hPAN2     NFDDLNSEQS YNKSFCYSRS KLANILFTRE LARRLEGTNV TVNVLHPGIV  250
hRalR1    HFHNLQGEKF YNAGLAYCHS KLANILFTQE LARRLKGSGV TTYSVHPGTV  235
           *  *  *   **    *  * ******** * ***** *  * *    *** *

hPAN2     RTNLGRHIHI PLLVKPLFNL VSWAFFKTPV EGAQTSIYLA SSPEVEGVSG  300
hRalR1    QSELVRHSSF MRWMWWLFS- ---FFIKTPQ QGAQTSLHCA LTEGLEILSG  281
             * **          **       * ***   *****   *       *  **

hPAN2     RYFGDCKEEE LLPKAMDESV ARKLWDISEV MVGLLK- 336
hRalR1    NHFSDCHVAW VSVQARNETI ARRLWDVSCD LLGLPID 318
            * **          *  *   ** *** *     **
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Fig. 1. Alignment of protein sequences of human PAN2 (hPAN2) and human RalR1 (hRalR1). Identical residues are indicated by a star symbol. The cofactor binding motif and the active site consensus sequence are underlined. Numbers on the right indicate the positions of the amino acid residues.

2.3. Analysis of enzymatic activity

Catalytic activity of PAN2 was assayed in 90 mM potassium phosphate, pH 7.4, and 40 mM KCl at 37°C (reaction buffer) in siliconized glass tubes [4]. The oxidative and reductive activity of PAN2 toward retinoid substrates was analyzed using all-*trans* and *cis* isomers of retinoids (Sigma-Aldrich). The stock solutions of retinoid substrates were prepared in ethanol and their concentrations were determined based on the corresponding extinction coefficients at the appropriate wavelengths [14]. Ethanol-dissolved retinoids were solubilized in the reaction buffer by a 10-min sonication in the presence of equimolar delipidated bovine serum albumin. The concentration of ethanol in the reaction mixture did not exceed 1%. The 500-μl reactions were started by the addition of cofactor and carried out for 10–30 min at 37°C as described before [4], except retinaldehyde produced in the oxidative reactions was converted into retinal oximes by the addition of 20 μl of 2 M hydroxylamine (pH 6.3) [15]. The amount of protein in the reaction mixture varied from 0.02 to 250 μg. Reaction products were extracted twice using 2 ml of hexane [15]. Hexane was evaporated under nitrogen flow and retinoids were dissolved in 200 μl of high-pressure liquid chromatography (HPLC) mobile phase (hexane:acetone, 90:10). Samples were analyzed at 350 nm using 2487 Dual Absorbance Detector integrated into Waters Alliance HPLC System. The stationary phase was Waters Spherisorb S3W column (4.6 mm × 100 mm). The flow rate was 1 ml/min. Under these conditions, all-*trans*-retinaldehyde and all-*trans*-retinol eluted at 2.09 and 4.095 min, respectively. The peak detection limits were ~1.0 pmol for all-*trans*-retinal and ~2.5 pmol for all-*trans*-retinol. The elution times for 9-*cis*-retinol and 9-*cis*-retinaldehyde were 3.96 and 1.94 min, respectively. Retinoids were quantitated by comparing their peak areas to a calibration curve constructed from peak areas of a series of standards.

PAN2 activity toward steroid substrates was assayed using ^{3}H-labeled 5α-androstan-3α-ol-17-one (androsterone), 5-androsten-3β-ol-17-one (dehydroepiandrosterone), 4-pregnen-3,20-dione (progesterone), 4-pregnen-11β,21-diol-3,20-dione (corticosterone), 5α-pregnan-3α-ol-20-one (allopregnanolone) and ^{14}C-labeled dihydrotestosterone (5α-androstan-17β-ol-3-one) (NEN Life Science Products, Boston, MA, USA). Aqueous solutions of steroids were prepared as described previously [12]. Reactions were carried out for 1 h at 37°C in 150 μl of reaction buffer in the presence of 1 mM NADPH and 5 μM steroid compound. The amount of the microsomal protein in the reaction mixture was 5 μg. Reaction products were extracted and analyzed by thin-layer chromatography (TLC) [12]. To visualize tritiated steroids, TLC plates were exposed to tritium phosphorimager screens. ^{14}C-labeled steroids were detected by exposure to an X-ray film.

2.4. Determination of kinetic constants

Steady-state kinetic analysis was performed in the reaction buffer under linear conditions with time and protein. In the reductive direction, the reaction rate was linear with up to 1.28 μg of microsomes per 500 μl volume for 15 min. In the oxidative direction, the rate was linear with up to at least 0.5 μg of microsomes per 500 μl volume for 10 min. Kinetic constants were determined using 0.02 and 0.1 μg of protein in the reductive and oxidative direction, respectively. The amount of product did not exceed 10% of the initial substrate amount. The background value without cofactor was determined for each concentration of substrate and was subtracted from each data point. The experimental values were at least 4-fold higher than the background and were expressed as means ± S.D.

The apparent K_m values for the oxidation and reduction of retinoids were determined at fixed $NADP^+$ (1.0 mM) or NADPH (1.0 mM) concentrations. Each K_m determination was repeated at least three times using six to 10 concentrations of all-*trans*-retinal (0.05–5.0 μM) and all-*trans*-retinol (0.1–3.2 μM). The values of initial velocities (nmol/min of product formed per mg of protein) were obtained by non-linear regression analysis. The apparent K_m values for cofactors were determined at a fixed saturating concentration of all-*trans*-retinal or all-*trans*-retinol with seven concentrations of each cofactor: NADPH (0.05–6.4 μM), NADH (0.1–6.4 mM), $NADP^+$ (0.064 μM–1.0 mM) and NAD^+ (0.1–6.4 mM).

3. Results

3.1. Tissue distribution of PAN2 mRNA

Tissue distribution of PAN2 was analyzed by Northern blotting using PAN2 cDNA as a probe. The cDNA was hybridized to the poly(A)$^+$ mRNA samples from 23 different human tissues (brain was represented twice). A single major band of approximately 1.5 kb was detected in each mRNA sample (Fig. 2). The intensity of the band was especially strong in the heart and skeletal muscle, followed by adrenal, thyroid, kidney, brain, liver, trachea, stomach, prostate, spinal cord, placenta, etc. Thus, PAN2 mRNA was present at significant levels in a wide variety of human tissues.

3.2. Characterization of PAN2 protein

To determine whether PAN2 mRNA encoded a functional enzyme, we expressed the corresponding cDNA in insect Sf9 cells using the BaculoGold baculovirus system. The recombinant protein was detected in the microsomal fraction of Sf9 cells (Fig. 3). SDS–polyacrylamide gel electrophoresis (PAGE) analysis revealed a clearly visible protein band of ~37 kDa, which was not present in the control microsomes from the cells infected with wild-type virus. The size of this protein agreed with the predicted subunit molecular mass of PAN2 protein (36 864 Da).

To test whether the recombinant protein was active toward retinoids, we assayed the retinal reductase activity of Sf9 cells infected with the recombinant virus versus control cells using 5 μM all-*trans*-retinaldehyde as substrate and 1 mM NADPH as cofactor. The assays showed that only the cells infected with PAN2 virus exhibited a reductive activity toward retinaldehyde. Furthermore, distribution of the retinal reductase ac-

Table 1
Kinetic constants of PAN2 for retinoid substrates and cofactors

Substrate/cofactor	K_m (μM)	V_{max} (nmol/min × mg microsomes)
All-*trans*-retinal	0.08 ± 0.02	27 ± 1
All-*trans*-retinol	0.4 ± 0.07	31 ± 2
NADPH	0.32 ± 0.04	–
$NADP^+$	0.65 ± 0.11	–
NADH	1060 ± 70	–
NAD^+	1200 ± 120	–

Kinetic constants were measured as described in Sections 2 and 3.

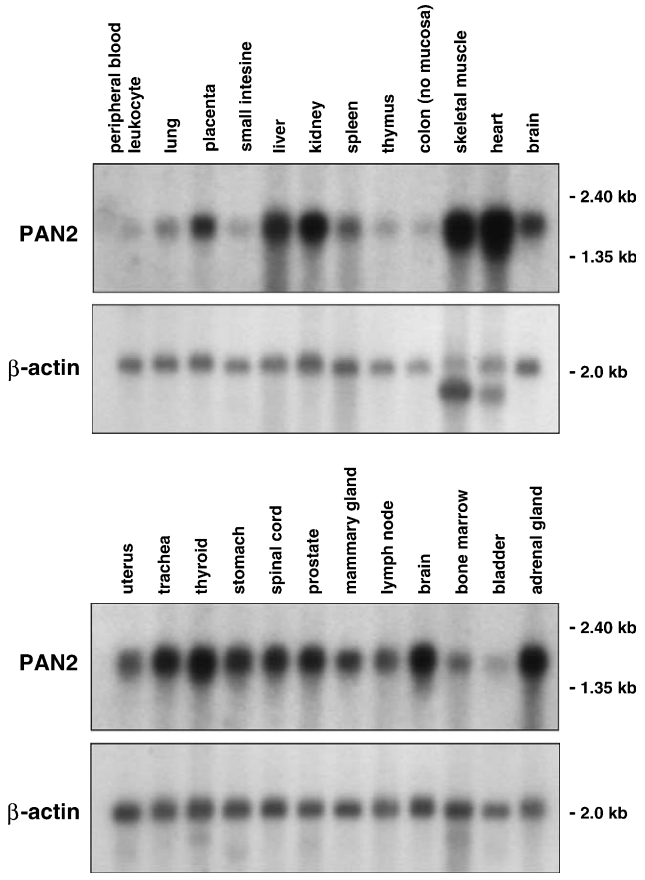

Fig. 2. Northern blot analysis of PAN2 expression in human tissues. Human multiple tissue Northern blots (Clontech) containing a minimum of 1 μg of poly(A)$^+$ RNA per lane were hybridized with human radiolabeled PAN2 cDNA at high stringency conditions. The blots were exposed to X-ray film for 3 days, then stripped of residual radioactivity, reprobed with a β-actin cDNA, and exposed for 3 h (lower panel). Positions of size standards are indicated on the right.

tivity among the subcellular fractions of Sf9 cells agreed well with the distribution of PAN2 protein. About 90% of the total activity was recovered in the microsomal fraction. The control microsomes did not have significant retinal reductase activity.

3.3. Analysis of PAN2 substrate and cofactor specificity

To establish whether PAN2 preferred all-*trans* or *cis* isomers of retinoids, we compared PAN2 activity toward all-*trans*-retinal and 9-*cis*-retinal in the presence of 1 mM NADPH as cofactor. At 0.02 μg of microsomes per reaction and two different concentrations of substrate (0.1 and 1 μM), PAN2 activity toward all-*trans*-retinal was consistently ~2.5-fold higher than toward 9-*cis*-retinal, suggesting that all-*trans*-retinal was the preferred substrate. PAN2 activity in the oxidative direction was characterized using all-*trans*-retinol in the presence of 1 mM NADP$^+$. The apparent K_m value of PAN2 for all-*trans*-retinol was ~5-fold higher than the apparent K_m value for all-*trans*-retinal (Table 1). The V_{max} values in the oxidative and reductive directions were approximately similar. The resulting V_{max}/K_m value of PAN2 for oxidation of all-*trans*-retinol, 78 (nmol/min × mg)/μM was 4-fold lower than that for the reduction of retinal, 338 (nmol/min × mg)/μM, suggesting that PAN2 was less efficient in the oxidative direction.

The cofactor specificity of PAN2 was determined using 5 μM all-*trans*-retinol as substrate in the oxidative direction and 5 μM all-*trans*-retinal in the reductive direction. The apparent K_m values for NADP$^+$ and NADPH were less than 1 μM (Table 1). In contrast, the apparent K_m values for NAD$^+$ and NADH were in the millimolar range (Table 1). Thus, PAN2 exhibited strong preference for the phosphorylated nucleotide cofactors, NADP$^+$ and NADPH.

Since a number of retinol-active SDRs recognize steroid compounds as substrates [16], we assayed PAN2 activity toward 5 μM androsterone, dehydroepiandrosterone, progesterone, corticosterone, allopregnanolone, and dihydrotestosterone. The reactions were incubated for 1 h at 37°C in the presence of 33.3 μg/ml of microsomal protein. No additional spots besides those corresponding to the substrates were detected in the reactions with androsterone, dehydroepiandrosterone, progesterone, corticosterone, or allopregnanolone. A weak spot corresponding to 5α-androstan-3α,17β-diol was detected in the reaction with dihydrotestosterone (reduction of 3-ketone group) in the presence but not in the absence of cofactor. The estimated rate of this reaction was 0.036 nmol/min × mg microsomes, ~750-fold lower than the rate of all-*trans*-retinal reduction catalyzed by the same PAN2 microsomes. Thus, PAN2 did not exhibit significant activity toward steroid compounds.

4. Discussion

Human PAN2 (AF237952) was originally identified at the gene level by Brereton et al. (unpublished). The authors indicated that PAN2 is a novel member of the SDR superfamily similar to 11β-hydroxysteroid dehydrogenase type I. Whether PAN2 is active toward 11β-hydroxysteroids was not reported. We were interested whether PAN2 could recognize retinoids as substrates because it shares about 40% amino acid sequence identity with the novel prostate retinal reductase RalR1 recently characterized in our laboratory [4]. Interestingly, despite significant protein similarity, the genomic structure of PAN2 is quite different from that of RalR1. PAN2 gene ap-

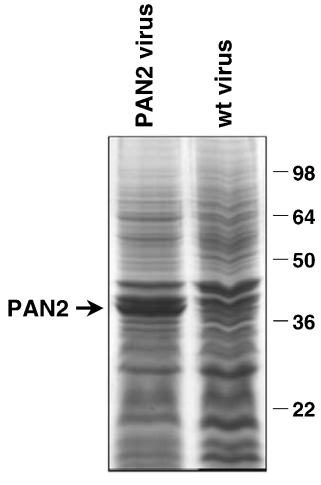

Fig. 3. SDS–PAGE analysis of PAN2 expression in Sf9 cells. 20 μg of microsomal protein isolated from Sf9 cells infected with either recombinant virus carrying PAN2 cDNA (PAN2 virus) or wild-type virus (wt virus) were separated by SDS–PAGE and the gel was stained by Coomassie R-250. The positions of SeeBlue Plus2 prestained protein standards (Invitrogen) are indicated on the right.

pears to consist of only two exons and is located on chromosome 2, whereas RalR1 gene consists of seven exons and is present on chromosome 14. Furthermore, the protein products of the two genes have different lengths: PAN2 is a 336 amino acid polypeptide, whereas RalR1 is only 318 amino acids long. A mouse ortholog of the human PAN2 has also been identified (accession number AF303831). Mouse PAN2 shares 84% identity with the human protein and is two residues shorter (334 amino acids).

To determine whether PAN2 is active toward retinoids, we expressed the corresponding cDNA in insect cells. Analysis of the subcellular localization of PAN2 showed that, like RalR1, PAN2 is a membrane-bound protein. Both SDS–PAGE and activity assays localized PAN2 protein to the microsomal fraction of Sf9 cells. The primary structure of PAN2 polypeptide contains an N-terminal hydrophobic domain (residues 2–21) that is long enough to serve as a transmembrane segment. This segment could serve as an anchor for PAN2 attachment to the membranes.

Kinetic analysis revealed that, first of all, PAN2 is active toward retinoids. Secondly, similar to RalR1, PAN2 is more efficient in the reductive direction than in the oxidative and prefers all-*trans*-retinaldehyde over 9-*cis*-retinaldehyde as substrate. In fact, PAN2 appears to be a better all-*trans*-retinaldehyde reductase than RalR1 with the apparent K_m value for all-*trans*-retinal about 6-fold lower and the V_{max} value about 1.5-fold higher than the corresponding values of RalR1 at approximately similar level of protein expression in Sf9 cells.

The cofactor preference of PAN2 is consistent with its potential role as a retinal reductase. Similar to RalR1, the apparent K_m values of PAN2 for $NADP^+$ and NADPH are three orders of magnitude lower than those for NAD^+ and NADH. Since in the cytosol of liver and presumably other cells $NADP^+$ exists mainly in the reduced form [17], the cofactor preference of PAN2 suggests that it is likely to function predominantly in the reductive direction in vivo.

PAN2 is the fourth member of the SDR superfamily shown to reduce all-*trans*-retinaldehydes to all-*trans*-retinol in the presence of NADPH. Besides RalR1, two other less related to PAN2 enzymes, retinal SDR1 (retSDR1) [18] (gene located on chromosome 1) and photoreceptor retinol dehydrogenase (prRDH) [19] (gene located on chromosome 19) catalyze retinal reduction. Kinetic constants of retSDR1 and prRDH have not been reported, however, it appears that the main difference between PAN2 and all previously characterized retinal reductases lies in their tissue distribution pattern. RalR1 is highly expressed in human prostate [5], prRDH is restricted to photoreceptor outer segments [19], and retSDR1 mRNA is present in placenta, lung, liver, kidney, pancreas, and retina but not in brain [18]. In contrast, PAN2 is surprisingly widespread with its mRNA present at relatively high levels in all 23 different human tissues examined thus far. Interestingly, neither of the currently known $NADP^+$-dependent SDR retinal reductases is active toward steroid substrates. Specificity for retinoids distinguishes these enzymes from the NAD^+-dependent SDR retinol dehydrogenases, which are also highly active as 3α- or 17β-hydroxysteroid dehydrogenases [16].

The expression pattern of PAN2 is consistent with its potential role in retinoid metabolism. All-*trans*-retinal, which is recognized by PAN2 as substrate, is the immediate precursor for a biologically active all-*trans*-retinoic acid that functions as an activating ligand for a family of nuclear retinoic acid receptors (RARs) [20]. All-*trans*-retinoic acid is produced from all-*trans*-retinol in two oxidative steps: first, retinol is oxidized to retinal by retinol dehydrogenases, and then, retinal is oxidized to retinoic acid by retinal dehydrogenases [21]. The enzymatic machinery for retinoic acid biosynthesis is present in many cell types and tissues [21]. The substrate, all-*trans*-retinol, normally stored in the liver in the form of retinyl esters, is delivered to peripheral tissues by the plasma retinol binding protein (RBP) [22]. In addition, a number of cell types appear to be able to supplement their retinoid stores directly from the dietary β-carotene by converting β-carotene into retinol via retinaldehyde as an intermediate product [23–25]. In many tissues including intestinal tract, liver, kidney, brain, stomach, testis, prostate, ovary, colon, and skeletal muscle [7–11], the expression pattern of β-carotene 15,15′-monooxygenase, the enzyme that cleaves β-carotene into two molecules of retinal, overlaps with the expression of PAN2, the enzyme that reduces retinal to retinol. In combination, the two enzymes provide the enzymatic pathway for the conversion of dietary β-carotene into all-*trans*-retinol.

The intracellular levels of retinoic acid in tissues are tightly controlled. Aberrations in retinoid signaling are early events in carcinogenesis, and vitamin A deficiency has been associated with a higher incidence of cancer [26]. The molecular mechanisms that control the levels of retinoic acid in tissues during embryogenesis and in adulthood are poorly understood. The reduction of retinal back to retinol by retinal reductases to prevent excess of retinoic acid in tissues could serve as a potential regulatory mechanism. In fact, a recent study by Cerignoli et al. [27] presented the first evidence that one of the retinal reductases, retSDR1, is retinoic acid inducible and is frequently deleted in human neuroblastoma cell lines. These observations provide further rationale for the identification and characterization of all enzymes that could be involved in retinoic acid homeostasis.

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