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Intracellular localization and membrane topology of 11-cis retinol dehydrogenase in the retinal pigment epithelium suggest a compartmentalized synthesis of 11-cis retinaldehyde

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SUMMARY

11-cis retinol dehydrogenase (EC 1.1.1.105) catalyses the last step in the biosynthetic pathway generating 11-cis retinaldehyde, the common chromophore of all visual pigments in higher animals. The enzyme is abundantly expressed in retinal pigment epithelium of the eye and is a member of the short chain dehydrogenase/reductase superfamily. In this work we demonstrate that a majority of 11-cis retinol dehydrogenase is associated with the smooth ER in retinal pigment epithelial cells and that the enzyme is an integral membrane protein, anchored to membranes by two hydrophobic peptide segments. The catalytic domain of the enzyme is confined to a lumenal compartment and is not present on the cytosolic aspect of membranes. Thus, the subcellular localization and the membrane topology of 11-cis retinol dehydrogenase suggest that generation of 11-cis retinaldehyde is a compartmentalized process.

Key words: Retinol, Retinal, Enzyme, Pigment epithelium, Membrane, Topology, Dehydrogenase

INTRODUCTION

Vitamin A derivatives (retinoids) are essential in a variety of biological processes. Retinoic acid (RA), one of the physiologically active retinoids, has been identified as a ligand for two classes of nuclear, ligand-controlled transcription factors (the retinoic acid receptors and the retinoid X receptors; for a review see Mangelsdorf et al., 1994). 11-cis retinaldehyde (11-cis RAL) is another vitamin A derivative with a well defined physiological function. In vertebrates, this compound acts as the chromophore of all visual pigments in the photoreceptor cells of the eye by forming complexes with the opsins (for a review see Saari, 1994). RA and 11-cis RAL are formed from a common precursor, all-trans retinol (ROH), present in the general circulation bound to a specific plasma protein, the retinol-binding protein (RBP) (for a review see Soprano and Blaner, 1994).

The retinal pigment epithelium (RPE) of the eye has a central role in the visual processes as it is involved in uptake and metabolic processing of retinoids in the visual cycle. This polarized epithelium forms the blood-retina barrier, and all-trans ROH, accumulated from the circulation on the basolateral side, has to transverse the cytoplasm of RPE cells while being metabolized into 11-cis RAL. Generated 11-cis RAL is then secreted on the apical side of the RPE (Carlsson and Bok, 1992) and complexed with the opsins in the photoreceptor cells. As a consequence of light exposure, the isomerization of 11-cis to all-trans RAL initiates the reaction cascade that eventually leads to visual perception (for a review see Wald, 1968). All-trans ROH is subsequently formed in the photoreceptor cells and transported back to the RPE for regeneration of 11-cis RAL (for a review see Saari, 1994). Current data suggest that transport of retinoids between the RPE and the photoreceptor cells is carried out by the interphotoreceptor retinol-binding protein, IRBP (Adler and Martin, 1982; Lai et al., 1982; Liou et al., 1982).

Inside RPE cells, all-trans ROH is esterified with long chain fatty acids (Saari and Bredberg, 1988) and formed retinylesters are believed to be substrates for a hydrolyse-isomerase, which converts all-trans ROH into the 11-cis configuration (for references see Rando et al., 1991). 11-cis ROH is then oxidized to 11-cis RAL by the enzyme 11-cis retinol dehydrogenase (11-cis RDH) (Lion et al., 1975).

The mechanisms involved in cellular uptake of all-trans ROH from RBP to RPE cells have been partially characterized. RBP is recognized by a specific 63 kDa membrane receptor (p63) expressed by RPE cells (Båvik et al., 1991, 1992, 1993). While isolating proteins associated with p63 we discovered a 32 kDa protein from the RPE (Simon et al., 1995). Characterization of this protein revealed that it was a membrane-associated enzyme able to oxidize 11-cis ROH into 11-cis RAL. The abundant expression of this protein in RPE

and the stereo-specific substrate specificity led us to propose that the enzyme is the 11-cis RDH responsible for generation and regeneration of 11-cis RAL. 11-cis RDH is highly conserved during evolution and the human gene has been located to chromosome 12q13-12q14 (Simon et al., 1996).

11-cis RDH belongs to the superfamily of short chain dehydrogenases/reductases (SDR). Members of this highly divergent class of enzymes are either cytosolic or membrane-bound. They have two well conserved and characteristic regions: the active site with an invariable tyrosine residue as part of the motif Y-X-X-X-K, and a cofactor binding site for NAD, NADP, or their reduced forms, consisting of three glycines as part of the motif G-X-X-X-G-X-G (Jörnvall et al., 1995). Substrates for the SDR family of enzymes include steroids, prostaglandins and fatty acids.

Recently, the primary structures of several microsomal SDRs were identified and shown to catalyze the oxidation of all-trans and 9-cis ROH into the corresponding RAL in an NAD/NADP-dependent manner (Chai et al., 1995a,b, 1996, 1997; Mertz et al., 1997; Romert et al., 1998; Su et al., 1998). The oxidation of all-trans and 9-cis ROH are the first and rate-limiting steps in the biosynthesis of the two stereo isomers of RA. Thus, closely related enzymes belonging to the SDR family are involved in processing of retinoids in several tissues.

Despite the identification of these and other retinoid metabolizing enzymes (reviewed in Duester, 1996), the different steps in the metabolic processing of retinoids are poorly understood at the molecular and cell biological levels. RPE cells provide an adequate model system to study cellular uptake and metabolism of retinoids and in this work we have extended the characterization of 11-cis RDH.

MATERIALS AND METHODS

SDS-PAGE, immunoblotting and antibodies

Bovine RPE cells were isolated as previously described (Båvik et al., 1991). Neuroretinas were obtained in the course of isolating the RPE cells by carefully removing the tissue using forceps. Total membrane fractions from neuroretina was obtained essentially as described for the RPE cells. The samples were then separated by SDS-PAGE under reducing conditions. Gels containing radioactive samples were fixed in 10% acetic acid, dried and exposed to Kodak XR film. Protein samples for subsequent immunoblotting analysis were electrotransfered onto nitrocellulose filters. For immunoblotting the filters were probed with polyclonal rabbit antibodies as indicated below. Bound antibodies were visualized using the ECL system (Amersham). 11-cis RDH was detected by incubating filters for 2 hours with 1 μg/ml of affinity-purified Ig to 11-cis RDH (Simon et al., 1995) in PBST (PBS with 0.1% Tween 80) and 5% non-fat dry milk. After extensive washing in PBST, the filters were incubated with horseradish peroxidase-labeled anti-rabbit Ig for 1 hour, washed extensively, incubated with the substrate according to the recommendations of the manufacturer (Amersham, ECL protocols). The filters were then exposed to Kodak XR film. Some blots were stripped for 30 minutes at 50°C with constant shaking under reducing conditions according to the manufacturer's instructions and reprobed with different antibodies several times.

Proteinase K treatment and alkaline extraction of RPE microsomes

RPE cells were prepared as described (Båvik et al., 1991). The last cell pellet was resuspended in 20 volumes of swelling buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM PMSF and 10 units aprotinin/ml). After 10 minutes of swelling, the cells were homogenized by 15-20 up-and-down strokes in a glass Dounce homogenizer. Sucrose concentration was adjusted to 250 mM by addition of 2 M sucrose containing 10 mM Tris-HCl, pH 7.5, and 5 mM benzamidine. Cell debris and nuclei were pelleted at 1,000 ***g*** for 10 minutes. The mitochondrial fraction was pelleted at 7,700 ***g*** for 15 minutes and the resulting supernatant was loaded on a two-step sucrose gradient as follows: 2 ml 2 M sucrose and 4.5 ml 0.35 M sucrose were overlaid by 3.5 ml of the post mitochondrial supernatant in a Beckman ultraclear tube. After an ultracentrifugation for 1.5 hours at 100,000 ***g*** in an SW41 rotor (Beckman), the microsomes were harvested at the 0.35-2 M sucrose interface. This fraction was designated 'total microsomes' and used directly or snap-frozen in liquid nitrogen and stored at –80°C.

The sucrose concentration of total RPE microsomes was diluted to 250 mM and the microsomes were pelletted by centrifugation at 100,000 ***g*** for 60 minutes at 4°C. Fractions of total RPE microsomes were either suspended in PBS or solubilized in PBS containing 1% Triton X-100 and incubated at 4°C in the presence of 10 μg/ml Proteinase K (Boehringer Mannheim). Samples were removed after 0, 5, 15, 25 and 45 minutes of incubation and proteolysis was stopped by the addition of PMSF to a final concentration of 2 mM, subjected to SDS-PAGE and analysed by immunoblotting as described above.

For alkaline extractions total RPE microsomes were incubated in 100 mM sodium carbonate buffer, pH 11.5, for 30 minutes on ice. The membranes were collected by centrifugation at 100,000 ***g*** for 60 minutes. The pellets were resuspended in PBS and equal fractions of the pellets and supernatants were analysed by SDS-PAGE and immunoblotting as described above.

Construction of 11-cis RDH mutants

For construction of 11-cis RDHΔ289-318, a PCR fragment coding for amino acids 26-288 was generated from the bovine 11-cis RDH cDNA as template (Simon et al., 1995) using standard procedures. The primers were 5′-ACGTGGTACCGCCAGCGATGCC (sense) and 5′-ACGTGGATCCTTACTTGGCATCCCA (reverse). The latter included an in-frame stop codon and a *Bam*HI cloning site (nucleotides complementary to 11-cis RDH are underlined). This PCR fragment and the plasmid encoding 11-cis RDH were digested with *Sma*I and *Bam*HI and part of the generated PCR fragment encoding amino acids 156-288 was subcloned into the original 11-cis RDH plasmid, thus creating a stop codon after amino acid 288. For the construction of 11-cis RDH containing a C-terminal hemagglutinin (HA) epitope (Wilson et al., 1984) followed by a glycosylation site (amino acid residues, N-I-T), a PCR fragment was generated as above using the primers 5′-GTAAAACGACGGCCAGT (sense, complementary to the pBluescript SK vector upstream from the 11-cis RDH insert) and the 5′ ACGTGAATTCTTATGCGTAATCT GGAACATCGTATGGGTATGTGGCATTGTAGACTGTCTGGGCA GGC (reverse). The latter primer included the nucleotide sequence for the HA epitope and the glycosylation site, an in-frame stop codon and an *Eco*RI cloning site. The generated PCR fragment and the plasmid encoding 11-cis RDH were digested by *Eco*RI and *Pst*I and the PCR fragment encoding amino acids 224-318, the glycosylation site and the HA epitope was subcloned into the original 11-cis RDH plasmid, creating an enzyme extended with 12 amino acids attached to the C terminus and a stop codon after amino acid 330. In a separate mutant, 11-cis RDH GM71-73, a glycosylation site was introduced (amino acid residues N-I-S) by single strand mutagenesis (Kunkel et al., 1987) at amino acid positions 71-73, in the catalytic domain of the enzyme, using the oligonucleotide 5′-GACCTCCAGCGGAACAT-CACCTCCCGCCTCCAC. The authenticity of the subcloned PCR fragments and of the glycosylation mutant was verified by nucleotide sequencing.

Proteinase K treatment, alkaline extraction and endoglycosidase treatment of in vitro-translated 11-cis RDH

Plasmids encoding wild-type or mutant forms of 11-cis RDH were linearized and in vitro transcribed using T7 RNA polymerase (Promega). In vitro translation were carried out in the presence of ^{35}S-methionine (Amersham) as described earlier (Lévy et al., 1991). Rabbit reticulocyte lysates and canine pancreatic microsomes (Promega) were used and protease inhibitors were excluded from the in vitro translation mixtures. Following the in vitro translation reactions, the microsomes were directly pelleted by centrifugation for 20 minutes at 14,000 ***g*** at 4°C. Pellets and supernatants were subjected to SDS-PAGE using 10% linear slab gels. For Proteinase K treatments the microsomes were pelleted by centrifugation as above, washed once with PBS and resuspended in PBS. Samples of the resuspended microsomes were then incubated with 10 μg/ml Proteinase K for 15 minutes on ice in the absence or in the presence of 1% Triton X-100. Reactions were stopped by the addition of PMSF to a final concentration of 2 mM. The samples were subjected to SDS-PAGE as described above. Alkaline extraction of microsomes containing in vitro-translated 11-cis RDH protein were carried out as described above.

The glycosylation mutant 11-cis RDH GM71-73 was generated by in vitro translation as described above. Washed microsomes were solubilized at room temperature for 15 minutes in 50 mM sodium acetate buffer, pH 5.2, containing 0.3% SDS and 1 mM PMSF and subsequently heated for 2 minutes at 98°C. Debris was removed by centrifugation and 2 μl (2 mU) of endoglycosidase H was added per 20 μl of sample and incubated for 12-14 hours at 34°C. 1 μl (1 mU) of the enzyme was then added and incubated for an additional 3-4 hours before analysis by SDS-PAGE. As a control, samples were incubated under identical conditions with the exception that no endoglycosidase H was added.

Immunohistochemical analysis of RPE cells

Freshly isolated bovine eyes were processed and tissue sections were cut, devaxed and rehydrated essentially as previously described (Båvik et al., 1992). The sections were incubated for 10 minutes in PBS containing 0.3% Triton X-100 and then rinsed repeatedly in PBS. Non-specific binding of Ig to the tissue sections were blocked by incubating the sections in 4% BSA in PBS (blocking solution) for 1 hour at room temperature. Affinity-purified rabbit Ig to 11-cis RDH (Simon et al., 1995), diluted in blocking solution to 10 μg/ml, was added and the sections were incubated overnight at 4°C. Similarly, preimmune IgG (10 μg/ml) was added to control sections. For light microscopy, the sections were stained using a modified avidin-biotin complex technique (Busch et al., 1990). For confocal microscopy analysis, the sections were thoroughly rinsed in several changes of 50 mM Tris-HCl buffer (pH 7.6) and PBS, and were then incubated with Cy-5-labelled goat anti-rabbit antibodies (Jackson Research Laboratories) for 3 hours at room temperature. The sections were then rinsed again several times in Tris-buffer and PBS, and mounted in Vectashield (Vector) to reduce fading.

For laser scanning confocal microscopy analyses, a Molecular Dynamics Multiprobe 2001 instrument, supplied with an Argon/Krypton laser and dual detectors, was used together with ImageSpace 3.10 software™ (Carlsson et al., 1985; Mossberg et al., 1990). Dual scannings were performed using excitation wavelengths of 488 nm to detect autofluorescent lipofucins and of 647 nm for Cy-5-related fluorescence. Fluorescence emission between 580 nm and 620 nm was detected to identify the autofluorescent lipofucins. Fluorescence emission over 660 nm was detected to identify Cy-5-related fluorescence.

Electron microscopy analysis of RPE cells

Electron microscopy

The analyses were performed as previously described (McCaffery and Farquhar, 1995). Freshly obtained bovine eyes were injected at multiple sites with 4% paraformaldehyde containing 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 2.5% sucrose in 100 mM cacodylate buffer, pH 7.4, and allowed to fix for 2-4 hours at room temperature. The retinas were dissected into thin 1-3 mm strips and refixed in 100 mM cacodylate buffer containing 4% formaldehyde, 1.5% glutaraldehyde and 2.5% sucrose, pH 7.4, for 1 hour at room temperature. After washing, the tissue strips were post-fixed in Palade's OsO_4 (1% OsO_4, Kellenberger's buffer, pH 7.4) for 1 hour at 4°C, en bloc-stained in Kellenberger's uranyl acetate overnight at room temperature, dehydrated through graded ethanols and embedded in Epon. Sections were cut on a Reichert OM 2 microtome and observed on a JEOL 1200EX transmission electron microscope.

Immuno-electron microscopy

Freshly obtained bovine eyes were processed as described above and subsequently refixed in the afore-mentioned fixative solution, now containing 0.05% instead of 1.5% glutaraldehyde, and allowed to fix for an additional 1 hour at room temperature. The retina slices were then oriented in liquified, low temperature gelling agarose such that the outer segment/RPE interface was clearly visible; the agarose was then gelled at 4°C. Strips were then infused with 2.3 M sucrose/30% polyvinylpyrolidone (M_r 10,000) for 2 hours, oriented on cryo-pins, and plunge-frozen in liquid nitrogen. Ultrathin cryosections of the outer segment/RPE interface were cut on a Reichert Ultracut S microtome, equipped with an FC-4 cryo stage, and collected onto formvar/carbon coated grids. After a brief wash in wash buffer (PBS containing 10 mM glycine and 2.5% FCS, pH 7.4), the grids were floated on 10% FCS-PBS for 20 minutes. They were rinsed briefly in wash buffer and incubated in the polyclonal rabbit Ig to 11-cis RDH (10 μg/ml in 10% FCS) overnight at 4°C. The grids were then washed for 20 minutes through 10 puddles of wash buffer, and subsequently incubated with goat anti-rabbit secondary IgG absorbed with 5 nm gold particles (diluted 1/50 in 10% FCS, AuroProbe™, Amersham). The grids were then washed for 20 minutes through 10 puddles of wash buffer, washed quickly in water, stained for 10 minutes with 2% neutral uranyl acetate and subsequently adsorption stained for 5 minutes and embedded in a solution containing 0.1% uranyl acetate, 0.2% methylcellulose and 3.2% polyvinyl alcohol and observed as above.

Subcellular fractionation of RPE membranes on sucrose gradients

The subcellular fractionation on discontinuous sucrose gradients was adapted from Saraste et al. (1986). Briefly, a total microsome fraction from isolated RPE cells was generated as described above and mixed with 60% (w/w) sucrose buffer to obtain a final sucrose concentration of 50%. Typically, 3 ml of membranes in 50% sucrose were placed in a Beckman ultraclear tube and overlaid by 6 layers (1.5 ml each) of 45% (ρ=1.204 g/ml), 40% (ρ=1.178 g/ml), 35% (ρ=1.153 g/ml), 30% (ρ=1.129 g/ml), 25% (ρ=1.105 g/ml) and 20% (ρ=1.083 g/ml) sucrose. After an overnight centrifugation at 100,000 ***g*** in a SW41 rotor (Beckman), the membranes fractionated into five visible bands; B1 (the 45-40% sucrose interphase) to B5 (the 25-20% sucrose interphase). The fractions were collected, the sucrose concentration was diluted to 250 mM and the membranes pelleted at 100,000 ***g*** for 1 hour. Each pellet was resuspended in equal volume of PBS and protein concentrations were determined using the Bradford assay. Equal portions from each fraction and equal amounts of total membrane proteins from each fraction (2 μg/lane) were separately analysed by SDS-PAGE and immunoblotting as described above. The rabbit antiserum to megalin was a kind gift from Dr Aaro Miettinen, Helsinki University, the rabbit antisera to p58 and to calnexin were kind gifts from Drs Ulla Lahtinen and Ralf F. Pettersson, Ludwig Institute for Cancer Research, Stockholm, and the rabbit antiserum to ARF6 was a kind gift from Dr Julie Donaldson, NIH.

RESULTS

Hydropathy profile, alkaline extraction and protease protection analysis of 11-cis RDH present in RPE microsomes

11-cis RDH was originally purified from detergent-solubilized RPE membrane fractions, suggesting that it is a membrane-associated enzyme (Simon et al., 1995). Analysis of the hydropathy profile of the amino acid sequence of bovine 11-cis RDH identified three hydrophobic stretches of amino acids which are of sufficient length to span the lipid bilayer, e.g. residues 1-18, 132-154 and 289-310 (Fig. 1A). Immunoblotting analysis of RPE membrane fractions following extraction with 100 mM sodium carbonate buffer, pH 11.5 revealed that the enzyme remained membrane bound, suggesting that it is an integral membrane protein (Fig. 1B).

The presence of an N-terminal signal sequence warranted an analysis of the membrane topology of 11-cis RDH as it could mediate the translocation of parts of the enzyme across the ER membrane. Digestion of RPE microsomes using Proteinase K revealed that the enzyme was protected in intact microsomes for up to 45 minutes of incubation and that it migrated with identical mobility to the intact protein in SDS-PAGE throughout the incubation (Fig. 1C). Proteinase K digestion of microsomes solubilized by the addition of 1% Triton X-100 resulted in a rapid degradation of the protein. These experiments suggest that the major part of the enzyme is confined to a lumenal compartment, e.g. located inside the isolated microsomes.

Protease protection, alkaline extraction and endoglycosidase H-treatments of in vitro-translated wild-type and mutant 11-cis RDHs

As mentioned above, the hydropathy index of the amino acid sequence of 11-cis RDH indicated the presence of three possible membrane anchoring domains, the N terminus and the regions between residues 132-154 and 289-310 (Fig. 1A). The major part of the enzyme was protease-resistant in intact microsomes, suggesting that residues 132-154 are unlikely to span the membrane as that would leave large parts of the enzyme exposed to the cytosolic side of the membranes and thus accessible to proteinase K digestion. In addition, such a topology would separate the cofactor binding motif (G-X-X-X-G-X-G, residues 35-41) and the active site (the invariant Y175) into two different cellular compartments. Furthermore, the corresponding region in soluble SDRs has also a hydrophobic nature without being involved in membrane-association (Persson et al., 1991).

To explore whether residues 289-310 represented a transmembrane segment, we constructed a mutant protein, 11-cis RDH-HA, with a C-terminal extension of 12 amino acid residues consisting of the hemagglutinin (HA) antigenic epitope (Wilson et al., 1984) and a glycosylation site (Fig. 2A). This sequence was selected to allow a detailed future immunochemical characterization of the mutant protein if found to be necessary. However, in vitro translation and SDS-PAGE analysis of 11-cis RDH-HA mutant revealed that it migrated significantly slower than the wild-type protein. Proteinase K treatment of in vitro-produced protein in intact microsomes generated a molecular species which migrated identically to the wild-type protein (Fig. 2B). This showed that the C-terminal extension was exposed on the cytosolic side of the membranes. Control experiments using the wild-type protein confirmed that a major part of the protein was present inside the microsomes and thus protected from the action of Proteinase K. Together these results suggested that residues 289-310 of 11-cis RDH are a transmembrane domain and that amino acid residues 311-318 are located in the cytosol.

As previously pointed out, the N-terminal hydrophobic region (residues 1-18) has features of a typical signal sequence but it lacks a consensus site for signal peptidase cleavage,

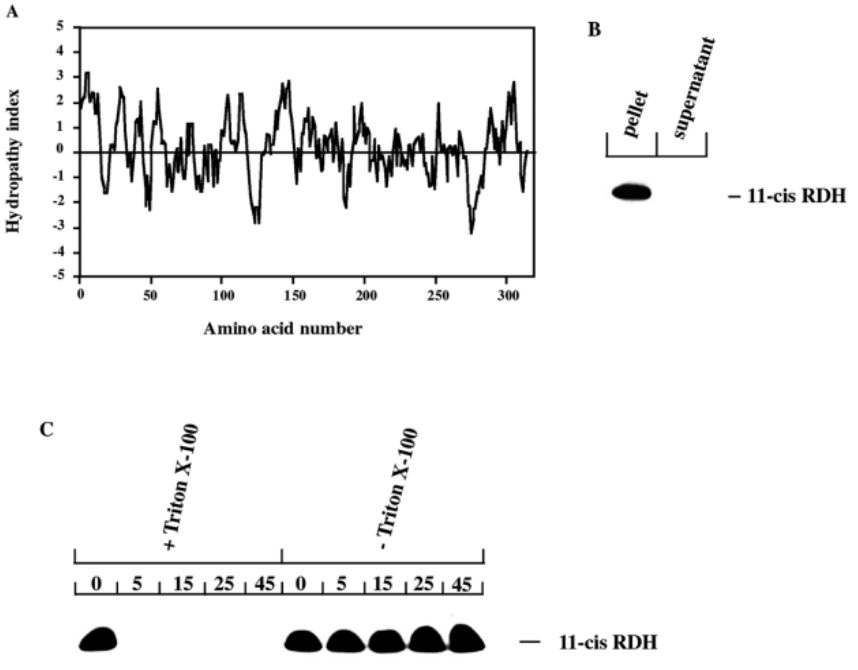

Fig. 1. Hydropathy profile and membrane binding properties of 11-cis RDH. (A) Hydropathy profile of the amino acid sequence of bovine 11-cisRDH using a 9-amino-acid residue window (Kyte and Doolittle, 1982). Long hydrophobic stretches of amino acids, e.g. residues 1-18, 132-154 and 289-310 are potential membrane anchoring domains. (B) RPE microsomes were subjected to alkaline extraction using 100 mM sodium carbonate, pH 11.5. Equal fractions of released proteins present in the supernatant and of membrane bound proteins were analysed by immunoblotting. (C) Protease protection analysis of 11-cis RDH in isolated RPE microsomes. Proteinase K digestion of intact RPE microsomes (–Triton X-100) or Triton X-100-solubilized RPE microsomes (+Triton X-100) were carried out on ice. Samples were withdrawn at the indicated time points and subjected to SDS-PAGE. 11-cis RDH was detected by immunoblotting.

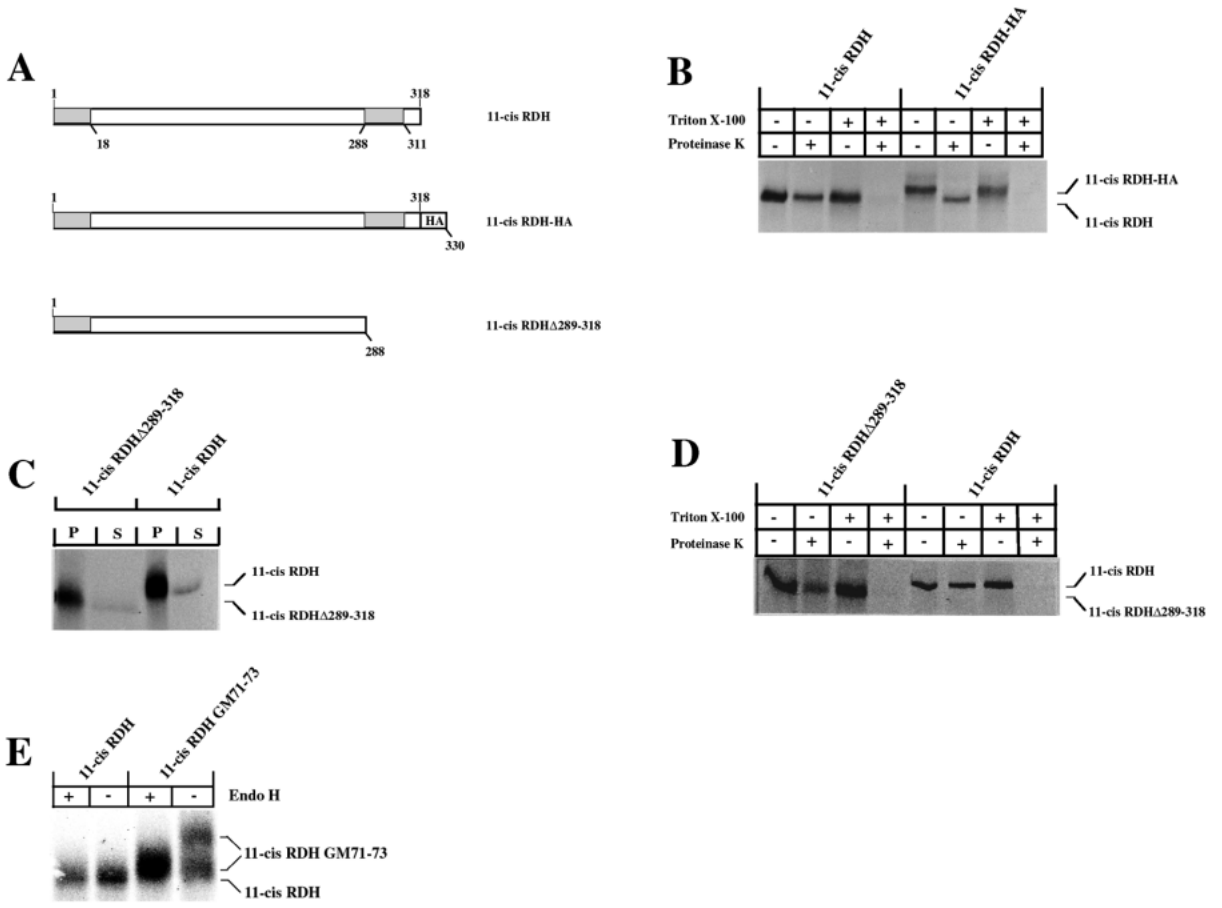

Fig. 2. Analysis of membrane topology, membrane anchoring and N-linked glycosylation of wild-type and mutant 11-cis RDHs. (A) Schematic illustration of wild-type 11-cis RDH and of two mutants of 11-cis RDH. The mutant 11-cis RDH-HA contains a 12-amino-acid residue extension, including the HA epitope at the extreme C terminus, while the second mutant, 11-cis RDHΔ289-318, has a deletion of amino acid residues 289-318. The potential membrane anchoring domains are shaded. The numbering refers to amino acid residues in the different proteins. (B) Protease protection analysis of in vitro-translated 11-cis RDH and 11-cis RDH-HA with a C-terminal HA epitope. The protection of 11-cis RDH upon Proteinase K digestion in intact microsomes and the unchanged mobility in SDS-PAGE of the protected protein demonstrates that in vitro-synthesized 11-cis RDH acquired the same membrane topology as the endogenous protein present in microsomes isolated from bovine RPE. The 11-cis RDH-HA mutant migrated slightly slower than the wild-type protein in SDS-PAGE and the HA epitope was accessible to Proteinase K digestion in intact microsomes, as indicated by the mobility shift. This demonstrates that the HA epitope is exposed to the cytosol and suggests that amino acid residues 311-318 of the wild-type 11-cis RDH are cytosolic and that amino acid residues 289-310 constitute a transmembrane domain. (C) Microsomes containing 11-cis RDHΔ289-318, with a deletion of the C-terminal transmembrane domain, and wild-type 11-cis RDH were subjected to alkaline extraction. Equal fractions of released and membrane-bound proteins were analysed by SDS-PAGE. The results show that both proteins remain membrane-bound, suggesting that the N-terminal hydrophobic signal sequence is uncleaved and acts as an independent membrane anchoring domain. (D) Protease protection analysis of 11-cis RDHΔ289-318. The protection of the truncated protein following Proteinase K digestion shows that it is translocated and present inside the microsomes. Parallel analysis of 11-cis RDH was used as a control. (E) Endoglycosidase H treatment of wild-type 11-cis RDH and of mutant 11-cis RDH GM71-73 containing an introduced N-linked glycosylation site at amino acid position 71-73. The shift in migration following endoglycosidase H treatment shows that the mutant protein becomes glycosylated, suggesting that the catalytic domain of 11-cis RDH has a lumenal orientation.

suggesting that it may not be cleaved cotranslationally. These possibilities were supported by the observations that in vitro translation of mRNA encoding 11-cis RDH results in quantitative membrane association of the generated protein and that no difference in migration in SDS-PAGE could be observed for 11-cis RDH translated in the presence or absence of microsomal membranes (data not shown). Thus, the N-terminal hydrophobic signal sequence is likely to act as a membrane anchor for the enzyme.

To analyse whether the N terminus can act as an independent membrane-anchoring domain and to confirm that it is not cleaved cotranslationally, we generated a mutant 11-cis RDH lacking the transmembrane segment of residues 289-310 and the whole cytosolic tail (Fig. 2A). This protein, 11-cis RDHΔ289-318, remained membrane-associated to the same extent as the wild-type protein when subjected to alkaline extraction using 100 mM sodium carbonate buffer treatment, suggesting that the N-terminal hydrophobic domain is indeed a membrane-anchoring domain (Fig. 2C). To rule out the possibility that deletion of amino acid residues 289-318

compromised the ability of the mutant protein to properly translocate across the ER membrane, Proteinase K treatments were performed. The results showed that 11-cis RDHΔ289-318 was protected in intact microsomes while it was readily degraded in Triton X-100-solubilized microsomes (Fig. 2D).

To independently confirm the lumenal orientation of the catalytic domain of the enzyme, an N-linked glycosylation site (amino acid residues N-I-S) was created by in vitro mutagenesis at positions 71-73 (Simon et al., 1995) to generate the mutant 11-cis RDH GM71-73. SDS-PAGE analysis of in vitro translated mutant and wild-type 11-cis RDHs revealed that the mutant protein migrated as two distinct bands with approximately 50-60% of the protein in the upper band migrating significantly slower than the wild-type protein (Fig. 2E). Treatment with endoglycosidase H, able to digest high mannose glycans added cotranslationally but not terminally modified glycans (for a review see Kornfeld and Kornfeld, 1985), increased the mobility of the upper band of the mutant protein but not of the lower band, or of the wild-type protein. This observation confirms the results of the protease protection experiments, namely the lumenal orientation of the catalytic domain of 11-cis RDH. Furthermore, these data show that wild-type 11-cis RDH is not glycosylated, despite the presence of a putative N-linked glycosylation site at positions 160-163 (amino acid residues N-I-T).

Together these results demonstrate that the catalytic domain of 11-cis RDH is present in a lumenal compartment. Firstly, 11-cis RDH is protected by exogenous proteinase K in intact microsomes but rapidly degraded in detergent-solubilized microsomes. Secondly, introduction of an N-linked glycosylation site in the protein renders it prone to glycosylation, which is known to occur in the lumen of the ER. Furthermore, the data suggest that the protein is anchored to membranes by an N-terminal anchor consisting of an uncleaved signal sequence and a C-terminal transmembrane domain of amino acid residues 289-310. The latter hydrophobic region is followed by a hydrophilic stretch of 8 amino acids, which is exposed to the cytosol.

Immunolocalization of 11-cis RDH in the retina

To define the cellular distribution in the bovine retina and the subcellular localization of 11-cis RDH in RPE, immunohistochemical techniques were employed. Immunoblotting analysis of tissue extracts from bovine RPE and neuroretina demonstrated that the 32 kDa 11-cis RDH was readily detectable in extracts from RPE cells but not in extracts of the neuroretina (data not shown). Staining of tissue sections from bovine eyes confirmed this observation, as intense staining was obtained in the RPE while the neuroretina displayed a weak general staining of the inner segments of the photoreceptor cells and in the optic nerve fibers (data not shown). These observations are in agreement with previously published staining patterns generated by a monoclonal antibody against 11-cis RDH (Driessen et al., 1995; Janssen et al., 1994).

To further analyze the distribution of 11-cis RDH in RPE we employed confocal microscopy and used Cy-5-labelled secondary antibodies to reduce the contribution of the autofluorescent lipofucins to the images. The analysis revealed a distinct cytoplasmic labeling for 11-cis RDH throughout the RPE cells (in red), while the nuclei were negative (Fig. 3A). There was no accumulation of labelling on, or near, the basolateral and apical plasma membranes. Scanning for the autofluorescent lipofucins using other excitation and emission wavelengths, revealed that these pigments were almost exclusively located in the apical part of the RPE cells (in green) and thus distributed differently from 11-cis RDH localization (Fig. 3A). Labelling using preimmune Ig shows only a low general staining and the autofluorescent lipofucins were identified in the apical portion of the RPE cells (Fig. 3B). At higher magnification, it was seen that 11-cis RDH localization was more pronounced in the basolateral half of the cell bodies and concentrated in subregions of the RPE cytoplasm (Fig. 3C).

To localize 11-cis RDH on the ultrastructural level, immunogold electron microscopy (EM) analysis of ultrathin cryosections was carried out. The widespread distribution of gold particles in the RPE cytoplasm confirmed the result from the confocal microscopy analysis. The enzyme was highly represented in the smooth ER (SER) (Fig. 4A) and found to some extent in rough ER (RER) (Fig. 4B). 11-Cis RDH was excluded from mitochondria, pigments, adherence junctions, basement membrane, plasma membranes and phagosomes containing shredded tips of rod outer segments (data not shown). Endosomes appeared to be moderately labelled (Fig. 4C). A routine morphological section of RPE cells indicate the unusual abundance of SER in this cell type (Fig. 4D). Given the extended SER in the RPE, the majority of 11-cis RDH is found in this cellular compartment.

Subcellular fractionation of RPE membranes

To confirm the immunolocalization studies and to get quantitative information on the distribution of 11-cis RDH in different subcellular compartments, total RPE microsome

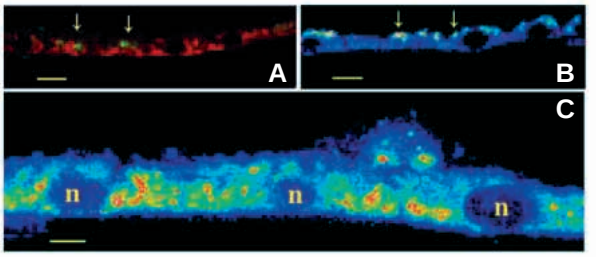

Fig. 3. Confocal microscopy analysis of the localization of 11-cis RDH in RPE. Regular tissue sections from bovine RPE were stained with Ig to 11-cis RDH or with preimmune Ig using Cy-5-labelled secondary antibodies. Cy-5-related fluorescence and the autofluorescent lipofucins were detected by dual scans at different wavelengths. (A) An optical section showing an intense cytoplasmic labelling for 11-cis RDH (in red) and of the autofluorescent lipofucins (in green). Note the apical localization of the lipofucins (arrows) and the widespread cytoplasmic localization of 11-cis RDH. (B) An optical section from a control tissue section stained with preimmune Ig. Note the apical localization of the lipofucins (arrows) and the absence of specific staining in the cell bodies of the RPE cells. (C) An optical section at higher magnification showing the localization of 11-cis RDH in a punctate pattern with a predominant basolateral distribution. Nuclei (n) and plasma membranes appear negative. In B and C the intensities of the signals are color-coded with white-red-yellow-green-blue-magenta in decreasing intensity. Bars, 10 μm (A,B); 5 μm (C).

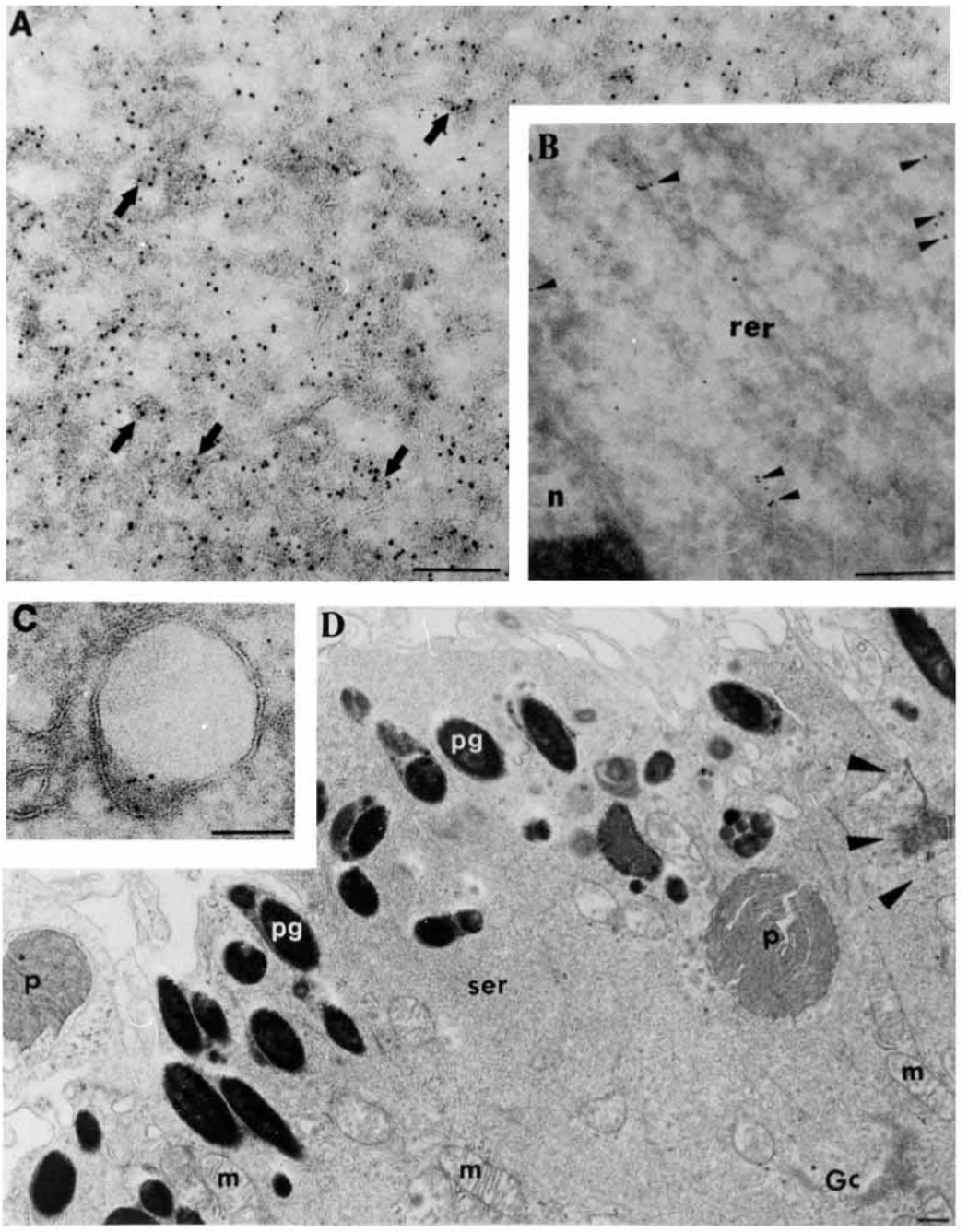

Fig. 4. Localization of 11-cis RDH in RPE by immunogold EM. 11-cis RDH was localized by immunogold EM using ultrathin cryosections from bovine RPE. (A) Immunogold labelling of 11-cis RDH is mainly seen in the SER. Labelled areas with clearly identifiable membrane structures are indicated by the arrows. (B) A representative picture of the RER-containing region in RPE cells shows that labelling is weaker than in the SER. Labelled areas with clearly identifiable membrane structures are indicated by the arrowheads. (C) A representative endosome displaying weak labelling. (D) An overview of the morphology of RPE cells with the pigment (pg) layer in the apical portion of the cells, phagocytosed outer segments of photoreceptor cells (p). Note the extended SER (ser) and the adherence junction (arrowheads). Mitochondria (m) and the Golgi complex (Gc) are indicated. Bars, 100 nm.

fractions were submitted to subcellular fractionation on discontinuous sucrose gradients. The fractions were denoted B1 (heavy) to B5 (light). More than 95% of the total membrane proteins in RPE microsomes were found in the light fractions corresponding to smooth microsomes (B2-B5), while less than 5% of RPE membrane proteins were recovered in fraction B1 (Fig. 5A and see below).

The different fractions were analysed for the presence of 11-cis RDH and various marker proteins by immunoblotting. Analysis of equal portions from each fraction demonstrated that 11-cis RDH was predominantly found in the lighter fractions, B3-B5 (Fig. 5B). In contrast, when analysing equal amounts of protein from each fraction, 11-cis RDH appeared in all fractions with highest specific abundance in fraction B1-B3 (Fig. 5C). Analysis of calnexin, a marker of the RER (Bergeron et al., 1994), revealed that it was enriched in B1. Analysis of p58, a marker for the intermediate compartment and the cis-Golgi compartment, but also present in the RER (Lahtinen et al., 1992, 1996), revealed that it was enriched in fractions B1 and, to some extent, also present in fractions B2 and B3. Megalin/gp330 (Zheng et al., 1994), a marker protein for the endocytic pathway, was enriched in fraction B3 and, to a lesser extent, also present in B4. ARF6 (ADP-ribosylation factor 6), a marker for the plasma membrane and recycling endosomes (Peters et al., 1995), was most abundant in fractions B4 and B5. These data showed that membrane fractions originating from different cellular compartments were separated using this procedure. Fractions B2-B5 represent

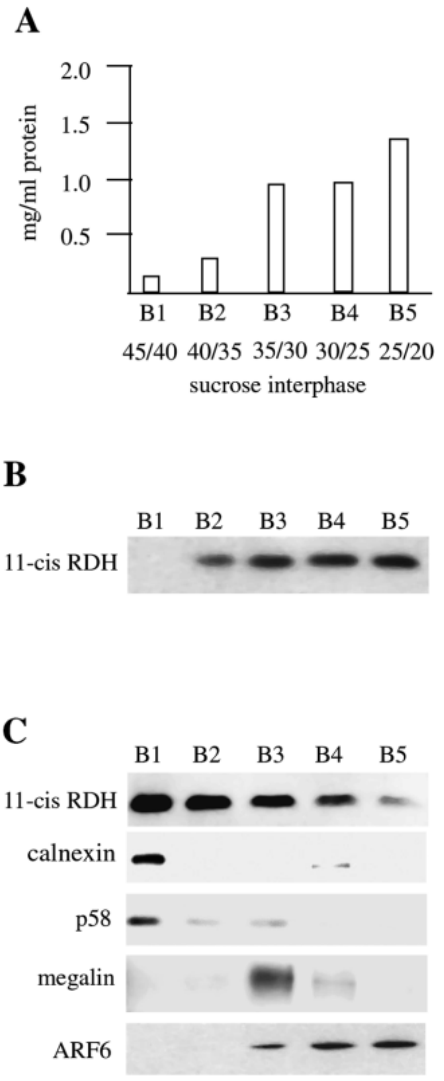

Fig. 5. Subcellular fractionation of total membrane fractions from RPE on discontinuous sucrose gradients. (A) The protein concentrations from fraction B1-B5 are given for a typical experiment (n=2). More than 95% of the total membrane protein in RPE cells is found in the lighter fractions B3-B5, e.g. in the smooth microsomes. (B) Equal portions from each fraction were subjected to SDS-PAGE and analysed by immunoblotting using antibodies to 11-cis RDH. The results of the subcellular fractionation show that 11-cis RDH is predominantly found in B2-B5. (C) Equal amounts of protein (2 μg) from fractions B1-B5 were subjected to SDS-PAGE and analysed by immunoblotting using antibodies to 11-cis RDH and to several marker proteins. Megalin and ARF6, two markers for the endocytic pathway and the plasma membrane, respectively, are present in fractions B3-B5. p58, a marker for the intermediate compartment, the cis-Golgi, and the RER is predominantly found in fraction B1, as is calnexin, a marker for the RER.

smooth microsomes indicated by the lack of calnexin. However, fraction B1 is probably contaminated with smooth microsomes, indicated by the relatively high p58 reactivity. Thus, we conclude that 11-cis RDH is predominantly present in smooth microsomes derived mainly from the SER. These findings are in agreement with the ultrastructural analysis of RPE cells (see Fig. 4D).

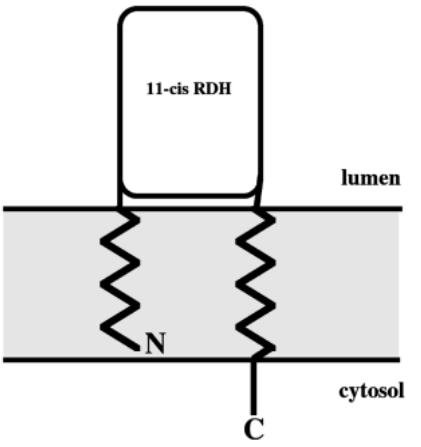

Fig. 6. Schematic illustration of the proposed membrane topology of 11-cis RDH. The N-terminal and the C-terminal hydrophobic membrane-anchoring domains are separated by the centrally located catalytic domain with the active site. The lumenal orientation of the catalytic domain of the enzyme indicates that synthesis of 11-cis RAL from 11-cis ROH occurs in a lumenal compartment in the SER of RPE cells. Only the short hydrophilic extreme C-terminal tail containing a conserved tyrosine residue is exposed to the cytosol.

DISCUSSION

RPE cells synthesize the common chromophore of all visual pigments in higher animals and thus play a unique role in the visual process by generating and regenerating 11-cis RAL. The last step in the biosynthetic pathway generating this compound, the oxidation of 11-cis ROH to 11-cis RAL, is catalyzed by the membrane-bound, stereo-specific enzyme 11-cis RDH. In this work we have extended the characterization of this enzyme.

Protease protection analysis of endogenous 11-cis RDH in RPE microsomes and of in vitro-expressed protein demonstrate that the catalytic domain of the enzyme is located in a lumenal compartment and not present on the cytosolic side of membranes. Consistent with these data, a mutant of 11-cis RDH containing an introduced N-linked glycosylation site in the catalytic domain could be glycosylated. The reason underlying the lack of quantitative glycosylation in the mutant protein, indicated by the fact that it migrates as two different species in SDS-PAGE, is likely to be a suboptimal localization of the introduced glycosylation site. This possibility is strengthen by the observation that the wild-type protein has an endogenous consensus site for N-linked glycosylation, yet the wild-type protein is not glycosylated. Altogether, the two independent types of experiments demonstrate that the catalytic domain of 11-cis RDH has a lumenal orientation (see Fig. 6). This conclusion contrasts with previous observations made with a cell-free experimental system, that a cytosolic binding protein for 11-cis ROH, the cellular retinaldehyde binding protein (CRALBP, Futterman et al., 1977) acts as a substrate carrier for the enzyme (Saari and Bredberg, 1982; Saari et al., 1994). Unless CRALBP uses an unusual mechanism for membrane translocation, the carrier protein and the catalytic domain of the enzyme would localize to different cellular compartments. Based on these data, the suggested role of CRALBP as a substrate carrier for 11-cis RDH appears unlikely.

Following cellular uptake, all-trans ROH must undergo

isomerization to 11-cis ROH before being oxidized to 11-cis RAL by 11-cis RDH. According to the current view, isomerization of all-trans ROH occurs after esterification of ROH, and subsequent hydrolysis of the ester bond serves as the energy source during the isomerization reaction (Rando et al., 1991). At present, the enzymes responsible for esterification and isomerization have not been identified at the molecular level, but the association of these enzymatic activities with microsomal fractions from RPE cells suggests that the enzymes are membrane bound. One of the implications of a lumenal orientation of the catalytic domain of 11-cis RDH is that retinoids, e.g. the substrate 11-cis ROH and the product 11-cis RAL, must be present at some point in lumenal compartments of RPE cells. It will be interesting to determine the membrane topology of the hydrolyse-isomerase enzymes once they become available since this would have a bearing on the need for intracellular transport of retinoids in RPE cells. If isomerization of all-trans ROH occurs in the cytosol it would indicate an extensive shuttling of retinoids across intracellular membranes in RPE cells during biosynthesis of 11-cis RAL.

The immunogold-EM identified 11-cis RDH in several locations but it was mainly present in the tubulo-vesicular network of the SER. Retinoids are generally very hydrophobic compounds and, in the case of 11-cis RAL, also highly reactive through the terminal aldehyde group. This raises the important question of how retinoids are imported to and handled in lumenal compartments of RPE cells. So far, no retinoid binding protein has been shown to be present in the above compartments with the exception of RBP being synthesized and secreted by RPE cells in low amounts (Ong et al., 1994). An appealing possibility is the existence of a so-far-unrecognized, membrane-bound retinoid-binding protein. Such a putative protein may not only act as a substrate carrier for the 11-cis RDH but may also be involved in cellular uptake and transcytosis of retinoids in RPE cells**.**

It is well documented that RPE cells phagocytose the distal tips of the rod outer segments, which are shredded off from the photoreceptor cells in a circadian rhythm (Young and Bok, 1969). The phagosomes contain both opsin and, especially in the dark, 11-cis RAL. A possible role for the enzyme could be the reduction of 11-cis RAL obtained from phagocytosed and degraded outer segments. Members of the SDR family of enzymes are oxido-reductases, i.e. depending on the redox status of available cofactors, they are in general able to catalyze both oxidation and reduction of their substrates (Jörnvall et al., 1995). To our knowledge the ratio of oxidized versus reduced cofactors in lumenal compartments has not been determined, nor has the origin and fate of the necessary pyridine nucleotides been established. Thus, based on the cofactor specificity (NAD versus NADP), it is not possible to decide whether 11-cis RDH acts in vivo as an oxidase, reductase or as both. Nevertheless, it has been shown that NAD and NADP are present at high concentrations in the lumen of the ER (Bublitz and Lawler, 1987). We did not observe any accumulation of 11-cis RDH immunoreactivity in phagosomes but our study does not reveal the dynamics of intracellular transport of 11-cis RDH, and we cannot formally exclude a transient presence of the enzyme in phagosomes. However, these data, together with the general oxidative milieu of lumenal compartments of most cells (Hwang et al., 1992), suggest that the primary role of 11-cis RDH is oxidation of 11-cis ROH rather than reduction of 11-cis RAL. As the cofactors are unlikely to passively diffuse across membranes from the cytosol into the lumenal compartments, an active transport system for pyridine nucleotides is likely to exist. Alternatively, the correct cofactor(s) is generated inside lumenal compartments.

The 11-cis RDH was the first retinol metabolizing enzyme to be identified as a member of the SDR superfamily (Simon et al., 1995, 1996). It is interesting to note that the all-trans RDHs (Chai et al., 1995a,b; 1996) have been shown to utilize the cytosolic cellular retinol binding protein type I (CRBP I) as a substrate carrier in a cell-free experimental system (Boerman and Napoli, 1995). Given the overall close structural similarities between 11-cis RDH and the all-trans RDHs, it is tempting to speculate that the mode of interaction with membranes and the membrane topology of these enzymes are similar. 11β-hydroxysteroid dehydrogenase, a member of the SDR superfamily with 25% amino acid sequence identity to all-trans RDH type I, has been shown to have a lumenal orientation of the catalytic domain (Ozols, 1995). Thus, a close examination of the membrane topology of the all-trans RDHs, and other closely related enzymes, appears warranted.

The mechanisms underlying accumulation, transcytosis and metabolism of retinoids in the RPE remain obscure. The membrane topology of 11-cis RDH indicates that at least part of the metabolic processing of 11-cis ROH occurs in lumenal, non-cytosolic compartments in RPE cells. Interestingly, the enzyme is also able to oxidize 9-cis ROH in vitro (Driessen et al., 1998; Mertz et al., 1997; and our unpublished observations). However, it remains to be elucidated whether the mechanisms used in ROH activation by RPE cells are similar to the pathways used for biosynthesis of RA.

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