RPE65 of Retinal Pigment Epithelium, A Putative Receptor Molecule for Plasma Retinol-Binding Protein, is Expressed in Human Keratinocytes

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Retinoids are important modulators for cell growth and differentiation of normal skin. In plasma, retinol is transported coupled to plasma retinol-binding protein. In this study, we investigated gene and protein expression of RPE65, a putative receptor for plasma retinol-binding protein in human epidermal keratinocytes. We performed real-time PCR analysis to evaluate expression of RPE65 mRNA in proliferating and differentiating keratinocytes. Immunoblotting with anti-RPE65 antibody shows distinct reactivity to a 61-kDa protein. Indirect immunofluorescence on normal human epidermis reveals cell surface labeling of keratinocytes. Laser scan microscopy exhibits colocalization of plasma retinol-binding protein and RPE65 on cultured keratinocytes. Internalization experiments with [^{3}H]retinoic acid–retinol-binding protein complex in the presence and absence of excess of retinol-binding protein indicates receptor-dependent uptake of retinoids. We further show isolation of RPE65 protein by affinity chromatography from lysates of keratinocytes using a retinol-binding protein-matrix gel column. In summary, we demonstrate mRNA and protein expression of RPE65 in epidermal keratinocytes. Colocalization of plasma retinol-binding protein with RPE65 and affinity binding suggest a direct interaction of RPE65 with plasma retinol-binding protein in cultured human keratinocytes that might be involved in retinoid uptake of keratinocytes.

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Vitamin A and its derivatives, the retinoids, play an important role in a wide spectrum of biologic processes such as vision, cell growth, or development (Napoli, 1996, 1999). By regulating gene expression retinoids influence target cells involving specific extra- and intracellular proteins (Blomhoff *et al*, 1990; Roos *et al*, 1998). Cellular retinol-binding proteins (RBP) act as carriers for retinol and affect trafficking, metabolism and storage of retinoids in the cytoplasm (Li and Norris, 1996). In the nucleus, retinoic acid receptors and 9-*cis*-retinoic acid receptors act as ligand-dependent transcription factors mediating gene regulation (Chambon, 1996) and are known to mediate retinoid activity within the cell (Giguere *et al*, 1987; Petkovich *et al*, 1987). Retinoids alter the expression of growth factors, transcription factors, and oncogenes, encoding a number of different regulatory proteins thus modulating epidermal growth and differentiation either directly or indirectly. In the epidermis, retinoic acid suppresses differentiation of basal keratinocytes and promotes cell proliferation (Fisher and Voorhees, 1996) influencing normal squamous differentiation of the skin (Roos *et al*, 1998).

In serum, plasma RBP, a 21-kDa protein of the lipocalin family (Newcomer and Ong, 2000), acts in the transport and delivery of retinol from the liver, where dietary retinoids are stored and secreted as retinol bound to plasma RBP (Blomhoff *et al*, 1990). Retinol–RBP in plasma is also reported to form a complex of approximately 55 kDa with transthyretin to prevent elimination by glomerular filtration in the kidney (Wei *et al*, 1995). Whereas the mode of retinoid action via nuclear retinoid acid receptors and retinoid X receptors (Petkovich *et al*, 1987; Chambon, 1996; Fisher and Voorhees, 1996) regulating gene expression appears to be undisputed, the mechanism of cellular retinol uptake has been reported controversially (Noy, 2000). Some studies have supported the hypothesis of a nonspecific receptor-independent way like diffusion across target cell membranes (Noy and Xu, 1990; Van Bennekum *et al*, 1993). Other investigators have suggested the theory of a specific plasma RBP receptor-mediated uptake involving a receptor that recognizes the protein part of the plasma RBP–retinol complex (Rask and Peterson, 1976; Sivaprasadarao and Findlay, 1988; Bavik *et al*, 1991). A possible receptor for plasma RBP has been studied in tissues like intestine, testis, liver, and placenta (Rask and Peterson, 1976; Shingleton *et al*, 1989; Senoo *et al*, 1993; Sivaprasadarao *et al*, 1994). The mode of uptake of retinol into human keratinocytes is also a matter of controversial discussion (Vahlquist and Torma, 1992; Creek *et al*, 1993). Some authors show data supporting the hypothesis of receptor independent delivery of retinol to human keratinocytes (Hodam *et al*, 1991; Hodam and Creek, 1998), and, in

Abbreviations: C_T, threshold cycle; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RBP, retinol-binding protein; SDS, sodium dodecyl sulfate.

contrast, studies propose that a regulated retinol transfer into human keratinocytes implies involvement of a receptor protein to which the RBP of the RBP–retinol complex is bound (Bavik *et al*, 1995; Smeland *et al*, 1995). A putative receptor for plasma RBP has been assigned to a 61-kDa protein (named RPE65) which was originally identified in retinal pigment epithelium cells (Bavik *et al*, 1992; Hamel *et al*, 1993; Bavik *et al*, 1995; Nicoletti *et al*, 1995). This protein is highly conserved across various species and is predominantly expressed in the retinal pigment epithelium (Bavik *et al*, 1993; Nicoletti *et al*, 1995). The physiologic role of RPE65 is under debate. It has been reported to be membrane associated but not to be an integral membrane protein lacking hydrophobic transmembrane domains and signaling peptides (Bavik *et al*, 1992; Hamel *et al*, 1993; Nicoletti *et al*, 1995; Flower, 2000). The abundant expression in retinal pigment epithelium indicates an important role for RPE65 in retinoid processing and transport in the eye suggesting to be involved in human retinal degenerations (Nicoletti *et al*, 1995). RPE65 gene mutations have been shown to cause inherited retinal dystrophies (Gu *et al*, 1997; Morimura *et al*, 1998; Redmond *et al*, 1998). In this study we demonstrate RPE65 gene and protein expression in human keratinocytes and find evidence for RPE65 to play a role as receptor molecule for RBP in retinoid uptake.

Results

Expression of RPE65 mRNA in human epidermal keratinocytes To demonstrate gene expression of RPE65 originally identified and sequenced in retinal pigment epithelial cells (Nicoletti *et al*, 1995) in cultured human keratinocytes, we performed real-time PCR using specific primers and probes for human RPE65 as given under Materials and Methods. As a control, mRNA of ARPE-19, a cell line expressing RPE65, was used for real-time PCR. TaqMan real-time PCR assay based on measurement of threshold cycle (C_T) values was employed. C_T value is associated with an increasing detection signal as a result of the exponential growth of the PCR product. C_T values of RPE65 and β-actin were measured from ARPE-19 cells and from proliferating and differentiating—induced by confluence as well as by 1 mM Ca^{2+} exposure for 24 h—keratinocytes. C_T and ΔC_T of ARPE-19 cells (mean values from three different runs) were 34.92 ± 0.70 and 18.47 ± 0.24, respectively. To evaluate a possible difference in mRNA expression of keratinocytes at the various conditions we applied a comparative C_T method. Expression of RPE65 mRNA in the differentiating keratinocytes—each—was compared to the proliferating cells. The relative quantity of RPE65 mRNA in proliferating keratinocytes is given 1 (calibrator). The relative quantity ($2^{-\Delta\Delta CT}$) of RPE65 mRNA in differentiating keratinocytes induced by confluence is 0.93, whereas high-calcium-exposed keratinocytes give a value of 0.74. The differences in the relative amount of RPE65 mRNA expression between confluence-induced differentiating and proliferating keratinocytes as well as between calcium-induced differentiating keratinocytes and proliferating keratinocytes were not statistically significant; p values were 0.56 and 0.11, respectively. The mean values with standard deviations of five different donors are shown in Table I.

Protein expression of RPE65 in human epidermal keratinocytes We have raised a peptide-specific rabbit antibody according to previous publications that assigned the peptide NFITKINPETLETIK of the RPE65 protein as antigenic and respective antibodies as useful reagents to identify RPE65 (Redmond and Hamel, 2000; Ma *et al*, 2001). To obtain an affinity-purified antibody to RPE65 the rabbit serum was passed through a column of the respective peptide coupled to a polymer resin. We tested protein expression of RPE65 using the affinity-purified antibody for immunoblotting lysates of cultured human keratinocytes and, for control, lysates of ARPE-19 cells expressing RPE65. The affinity-purified rabbit antibody reacted with a single band of approximately 61 kDa (Fig 1*A*, *lane 1*), whereas the irrelevant rabbit IgG shows no specific reactivity (Fig 1*A*, *lane 3*). Immunoblotting with peptide-specific RPE65 antibodies in the presence of excess free synthetic peptide NFITKINPETLETIK revealed no reactivity (Fig 1*A*, *lane 2*). The peptide-specific RPE65 antibody revealed a single band within protein extracts of retinal pigment epithelial cells (Fig 1*B*, *lane 1*). This reactivity has also been competed by the presence of the respective synthetic peptide (Fig 1*B*, *lane 2*).

Indirect immunofluorescence reveals RPE65 at the cell periphery of epidermal keratinocytes Indirect immuno-

Table I. Comparison of RPE65 mRNA expression among proliferating and differentiating keratinocytes by comparative C_T method

Keratinocytes	RPE65 (mean $C_T^a \pm$ SD)	β-Actin (mean $C_T^a \pm$ SD)	Mean $\Delta C_T^b \pm$ SD	$\Delta\Delta C_T^c \pm$ SD	RPE65 mRNA expression relative to proliferating keratinocytes ($2^{-\Delta\Delta CT}$)
Proliferating	34.54 ± 0.97	17.96 ± 1.43	16.58 ± 1.22	0.0 ± 1.22	1
Differentiating					
Confluence induced	35.54 ± 2.51	18.85 ± 1.68	16.69 ± 1.28	0.11 ± 1.28	0.93 (0.38–2.25)
Induced by high calcium	35.87 ± 2.50	18.93 ± 1.64	17.02 ± 1.19	0.44 ± 1.19	0.74 (0.32–1.68)

[a] C_T, threshold cycle.
[b] $\Delta C_T = C_T$RPE65 $- C_T$ β-actin.
[c] $\Delta\Delta C_T$ = mean ΔC_T (mRNA of confluence induced/calcium induced differentiating keratinocytes) − mean ΔC_T calibrator (proliferating keratinocytes).

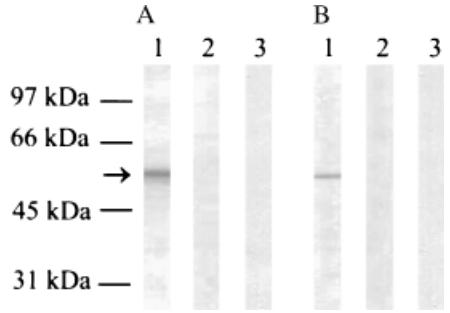

Figure 1
Biochemical detection of RPE65 protein in human epidermal keratinocytes. Lysates of cultured human keratinocytes (*A*) and ARPE-19 cells (*B*) were run on the same gel for SDS-PAGE and transferred to nitrocellulose. Strips of both lysates were cut out, and immunoblotting was performed using the peptide-specific anti-RPE65 antibody. The antibody reacted specifically with an antigen of approximately 61 kDa (*arrow*) within both the keratinocyte extract (*A*, *lane 1*) and the retinal pigment epithelial cell extract (*B*, *lane 1*). The specificity of the antibody was also documented by competitive inhibition of antibody binding with an excess of corresponding synthetic peptide (*A* and *B*, *lane 2*). Immunoblotting with irrelevant rabbit IgG showed no specific reactivity (*A* and *B*, *lane 3*).

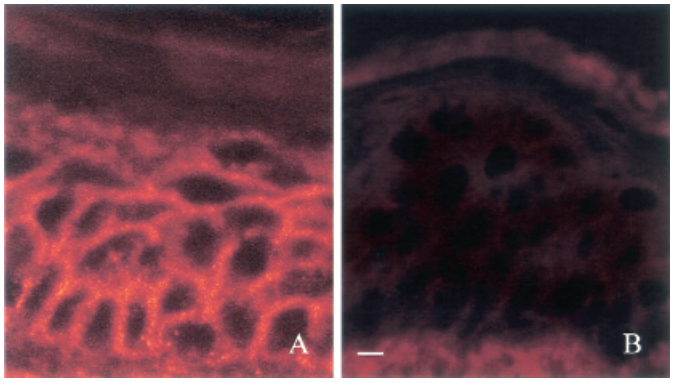

Figure 2
RPE65 expression in normal human epidermis. Indirect immunofluorescence performed on normal skin with peptide-specific anti-RPE65 antibody reveals a distinct cell surface labeling (*A*). Individual keratinocytes predominantly from the basal and suprabasal cell layer exhibit a membrane associated dotted fluorescence pattern (*A*). The upper granular cell layer and the horny cell layer shows no specific labeling (*A*). Immunofluorescence labeling with irrelevant rabbit IgG was negative (*B*). *Bar,* 20 μm.

fluorescence on normal human epidermis performed with affinity-purified anti-RPE65 antibody reveals distinct membrane-associated labeling suggesting RPE65 at the cell surface of keratinocytes. The labeling is predominantly localized to the keratinocytes of the basal and suprabasal cell layers. Individual keratinocytes show also a dotted appearing surface labeling pattern (Fig 2*A*). There appears to be a slight decrease in labeling intensity from the basal to the upper cell layers, leaving the horny cell layers negative (Fig 2*A*).

RPE65 protein and plasma RBP colocalize in human keratinocytes RPE65 expression was also demonstrated on paraformaldehyde-lysine-periodate–fixed, permeabilized cultured human keratinocytes by indirect immunofluorescence. Double labeling was performed to evaluate possible colocalization of plasma RBP and RPE65. We used the affinity-purified rabbit anti-RPE65 antibody and a sheep anti-RBP antibody with respective detector reagents. RBP appears as dotted fluorescence pattern on the cell surface and as vesicular staining in the subcellular cell periphery and throughout the cytoplasm (Fig 3*A*). RPE65 gives a very similar fluorescence signal (Fig 3*B*). To a large degree, RBP is found to be colocalized to RPE65, and colocalization appears to be most prominent in individual vesicles at the cell periphery indicating vesicular uptake of plasma RBP into keratinocytes (Fig 3*C*). Controls using only second step antibodies showed no specific reactivity on cells (not shown).

Uptake experiments suggest RBP-dependent retinoid uptake by human keratinocytes To evaluate uptake of [^{3}H]retinoic acid, proliferating keratinocytes were incubated with culture medium containing [^{3}H]retinoic acid coupled to RBP, as discussed under Materials and Methods, at 37°C and 4°C for different time spans. When the cells were incubated for 1, 3, 5, and 10 min at 37°C with the incubation mixture, measured cell associated radioactivity was between 173 ± 40 and 247 ± 65 cpm per 5×10^2 cells (per μL). Mean values and SD represent triplicate determination of measurement. Cell-associated radioactivity is significantly lower when incubation steps were performed at 4°C ($p = 0.004$) (Fig 4). When an excess of uncoupled RBP

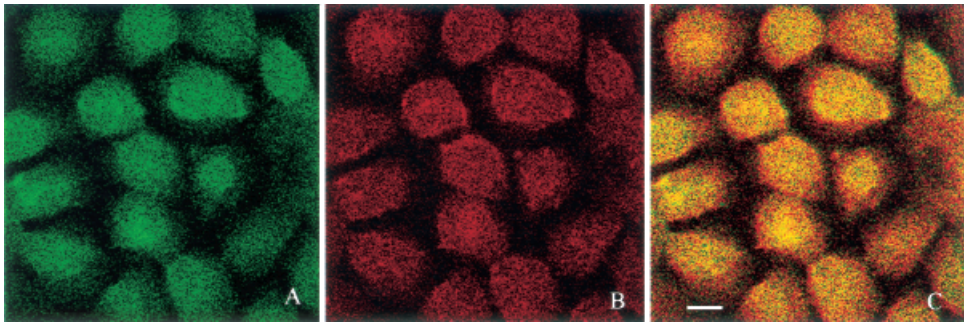

Figure 3
Plasma RBP and RPE65 colocalize in cultured human keratinocytes. Paraformaldehyde-lysine-periodate–fixed, permeabilized cultured keratinocytes were used for indirect immunofluorescence experiments and investigated by laser scanning microscopy. Plasma RBP detected by a sheep anti-RBP antibody and Alexa 488-conjugated donkey anti-sheep second step is found in a cell surface pattern and also in small dots at the subcellular cell periphery indicating a vesicular staining pattern (*green, A*). RPE65 detected by the peptide-specific anti-RPE65 antibody and TRITC-conjugated donkey anti-rabbit second step shows a similar fluorescence labeling (*red, B*). Using the multitrack scan to a great part colocalization of both, RBP and RPE65 could be demonstrated as shown by the orange color in *C*. *Bar*, 20 μm.

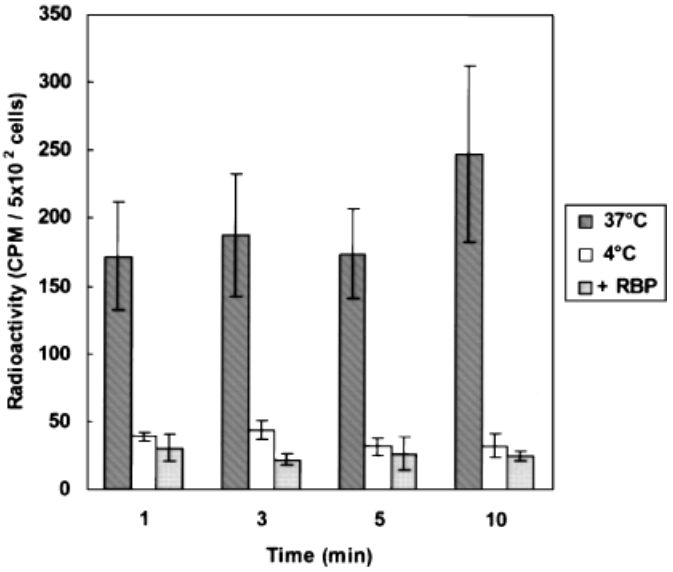

Figure 4
Retinoid uptake in keratinocytes is RBP-dependent. Proliferating cultured human keratinocytes were incubated with culture medium containing [3H]retinoic acid–RBP complex at 37°C for 1, 3, 5, and 10 min. Cell-associated radioactivity was evaluated by liquid scintillation from cells harvested in PBS (300 μL PBS for 1.5×10^5 cells) expressed in cpm per 5×10^2 cells. The values result from three different measurements. There is a slight but statistically not significant increase at 10 min. When incubation steps were performed at 4°C cell-associated radioactivity is drastically reduced compared to incubation at 37°C ($p = 0.004$). Nearly the same effect was reached when incubation was performed in the presence of excess unbound RBP competitively inhibiting the [^{3}H]retinoic acid–RBP complex ($p = 0.003$) compared to incubation at 37°C.

was added to the incubation mixture at 37°C, again, a significant reduction ($p = 0.003$) of [^{3}H]retinoic acid uptake was found (Fig 4) indicating competitive inhibition of [^{3}H]retinoic acid–RBP by pure RBP. These findings strongly suggest receptor associated uptake of RBP-retinoids in human keratinocytes.

Human plasma RBP binds to RPE65 Human plasma RBP (Biotrend) covalently bound to a matrix gel was used as affinity column to specifically bind proteins from NP-40 extracts. To determine possible interactions of plasma RBP as ligand with a putative receptor protein from human keratinocytes, we incubated the RBP-coupled matrix gel with NP-40-extracted proteins of keratinocytes. To separate bound proteins from the RBP-matrix gel, the mixture was boiled in 10% SDS sample buffer and the supernatant was loaded onto a 12% slab gel. After PAGE and electrophoretic transfer to nitrocellulose, strips were cut out and incubated with the affinity-purified anti-RPE65 antibody. The antibody recognizes a distinct, single band of approximately 61 kDa (Fig 5, *lane 1*) indicating RPE65 affinity isolation by the RBP column. Immunoblotting peptide-specific antibody in the presence of excess synthetic peptide (Fig 5, *lane 2*) as well as irrelevant rabbit IgG (Fig 5, *lane 3*) gave no specific reactivity. When RBP-matrix gel alone was exposed to SDS sample buffer, run for SDS-PAGE, and transferred to nitrocellulose, immunoblotting with anti-peptide antibody and irrelevant rabbit IgG showed no reactivity (not shown).

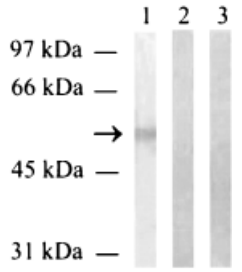

Figure 5
Human plasma RBP coupled to a resin matrix binds to RPE65 of keratinocyte lysates. Ethanol-acetone–precipitated proteins of NP-40 lysates of cultured keratinocytes were dissolved in PBS and exposed to the RBP column. Bound proteins were eluted by SDS-sample buffer and loaded onto a 12% gel for PAGE and transferred to nitrocellulose. Immunoblotting with the peptide-specific anti-RPE65 antibody showed a single specific reacting band of approximately 61 kDa (*lane 1, arrow*). Competitive inhibition of antibody binding with excess of free peptide (*lane 2*) and irrelevant rabbit IgG showed no specific reaction (*lane 3*).

Discussion

In this study we give molecular biologic, immunomorphologic, and biochemical evidence for the presence of RPE65 in cultured human keratinocytes. We used specific primers and probes to show RPE65 gene expression in cultured human keratinocytes by real-time PCR. Originally, RPE65 was identified in retinal pigment epithelium and described to be there expressed abundantly with a calculated molecular mass of 61 kDa (Bavik *et al*, 1992; Nicoletti *et al*, 1995). As control, ARPE-19, a retinal pigment epithelium cell line that expresses RPE65, was applied. Nevertheless, previous observations show a reduced RPE65 mRNA and protein expression in retinal pigment epithelial cells in cell culture (Nicoletti *et al*, 1995), where decreased mRNA suggested regulation of RPE65 protein synthesis occurring at the transcriptional level and reduced protein might reflect decreased promoter activity owing to loss of tissue-specific regulatory factors, inhibition of translation, or degradation of mRNA (Nicoletti *et al*, 1995; Boulanger and Redmond, 2002).

Recent molecular and biochemical investigations have shown that antibodies raised to amino acids 150 to 164 (NFITKINPETLETIK) of RPE65 protein represent specific anti-RPE65 antibodies (Redmond *et al*, 1998; Ma *et al*, 2001). Based on these observations, we generated a polyclonal, affinity-purified peptide-specific antibody. This antibody recognized a single antigen of approximately 61 kDa in lysates of keratinocytes and of ARPE-19 cells. In the presence of an excess of free synthetic peptide (NFITKINPETLETIK), the binding of the antibody could be competed supporting antibody specificity. Indirect immunofluorescence on normal human skin showed distinct surface labeling of the individual keratinocytes predominantly in the basal and suprabasal cell layers. When we performed double immunofluorescence on proliferating cultured keratinocytes, we found to a great extent plasma RBP to be colocalized with the RPE65 protein, suggesting an interaction of RBP with RPE65. The colocalization appeared more concentrated on the cell periphery and largely in a vesicular peripheral pattern. These observations are suggestive of a possible receptor-mediated vesicular uptake of plasma

RBP and go in line with previous studies that have shown internalization of plasma RBP–retinol via receptor-mediated endocytosis in hepatocytes (Malaba *et al*, 1995) and, similarly, in liver stellate cells (Senoo *et al*, 1993). Therefore, one can imagine that the uptake of retinol into cells entails interaction of the carrier protein plasma RBP with the respective receptor molecule (Senoo *et al*, 1993; Sundaram *et al*, 1998). Nevertheless, we find more RBP labeling intracellularly compared to RPE65 that is predominantly seen at the cell surface. This could be explained by vesicular uptake of bound RBP that is enriched in the cell, whereas RPE65 as a possible receptor could be relocated to the cell surface. A very recent study also shows retinol-binding affinity for RPE65 by itself in bovine pigment epithelium membranes (Jahng *et al*, 2003), and studies with cultured keratinocytes have also shown receptor-independent uptake of retinoids (Hodam *et al*, 1991; Hodam and Creek, 1998). Therefore, based on previous studies that show binding affinity of RBP to its ligands like retinol or retinoic acid we performed complexation of [^{3}H]retinoic acid with RBP (Horwitz and Heller, 1973; Zanotti *et al*, 1994) and used it to test uptake of a retinol analog in a form complexed with RBP in cultured human keratinocytes. We performed internalization experiments at 37°C from 1 up to 10 min, a time span where according to standard protocols vesicular uptake is effective. This uptake could be inhibited competitively by an excess of RBP alone and is also significantly reduced at 4°C, when vesicular uptake is arrested. These data demonstrate that receptor-mediated vesicular uptake of retinoid–RBP in complexed form could be involved in the retinoid pathway of epidermal keratinocytes.

To evaluate a possible direct interaction of RBP and the RPE65 protein, we obtained human RBP coupled to a resin matrix and incubated it with NP-40 protein extracts of keratinocytes. NP-40 allows the protein to be extracted in the biologically active form thus enabling RPE65 to bind RBP. The protein eluted from the RBP column was recognized by the anti-RPE65 antibody. This result gives high evidence for direct binding affinity of RBP to RPE65 acting as a putative receptor molecule. Nevertheless, RPE65 has been reported to be membrane associated but not to be an integral membrane protein because it is lacking hydrophobic transmembrane domains and a signal peptide (Bavik *et al*, 1993; Hamel *et al*, 1993; Nicoletti *et al*, 1995). The knowledge on the structure of the protein is limited, and the possible receptor function of RPE65 for plasma RBP has been discussed and is still unclear (Bavik *et al*, 1993; Hamel *et al*, 1993; Nicoletti *et al*, 1995; Redmond *et al*, 1998; Flower, 2000). As abundantly expressed in retinal pigment epithelial cells, RPE65 was shown to be involved in the all-*trans*- to 11-*cis*-isomerization reaction that regenerates the 11-*cis*-retinal chromophore of rhodopsin in the visual cycle of the retina (Redmond *et al*, 1998). Recent studies have shown high sequence homology between bovine RPE65 and mouse α-carotene 15,15′-dioxygenases (Redmond *et al*, 2001; von Lintig and Wyss, 2001), an essential enzyme in metabolism of carotene to vitamin A. The functional role of RPE65 in the enzymatic processing is not yet clear whether this homology is due to a conserved carotenoid/retinoid binding motif or related biochemical function (von Lintig and Wyss, 2001). Studies consider RPE65, primarily also described as p63, a likely receptor molecule (Bavik *et al*, 1991, 1993) for plasma RBP and therefore of importance for retinol uptake.

Differentiation of epidermal keratinocytes could be influenced by a monoclonal anti-p63 (means RPE65) antibody when RBP–retinol complex was administered in cell culture (Bavik *et al*, 1995) demonstrating that retinol might block differentiation of cultured human keratinocytes when administered as a complex with RBP to the culture medium. When we compared mRNA from proliferating to differentiating keratinocytes, we found a slight, but not statistically significant, decrease of RPE65 mRNA expression. Retinoids are important for proliferation of keratinocytes in cell culture. When epidermal keratinocytes are cultured in the absence of vitamin A, the cells display features of terminal differentiation (Fuchs and Green, 1981) including stratification, cell adhesiveness, and expression of keratin 1 and 10 (Fuchs, 1990). Colocalization of RPE65 and RBP, which might also be produced by the keratinocytes (Torma *et al*, 1994), and *in vitro* binding of RBP to RPE65 suggest direct interaction of these molecules and a likely receptor function of RPE65 in epidermal keratinocytes.

Many signal-transducing receptors are plasma membrane proteins that bind specific extracellular molecules such as growth factors, hormones, or neurotransmitters transmitting signals eliciting specific responses. Posttranslationally modified proteins with hydrophobic anchors that include fatty acids, isoprenoids and glycosyl-phosphatidyl inositol by which membrane-associated proteins might be bound to the plasma membrane have been identified (Magee *et al*, 1989). For example, glycosyl-phosphatidyl inositol acts as membrane anchor for the Fc receptor molecule FcγRIIIB, which may exert receptor function (Ravetch and Perussia, 1989). FcγRIIIB has no triggering capability by itself but appears to transduce signals with additive receptor proteins like FcγRIIA (Heijnen and van de Winkel, 1997). It could well be that RPE65 exerts peculiar receptor function eliciting ligand binding and cell signaling in an appropriate fashion influenced by additional plasmalemmal proteins or cell-specific regulation factors influencing transcription and protein function. Studies supporting that possibility demonstrated RPE65 in some transformed kidney cells and not in primary kidney cultures indicating that RPE65 gene transcription might for example be dependent on different neoplastic transformation (Ma *et al*, 1999). More recently, human RPE65 expression using the baculovirus system revealed the protein in two forms, one of which is a cytosolic form, partly found in microsomes and a membrane associated form exhibiting significant posttranslational modifications (Ma *et al*, 2001).

Summarizing our data, we demonstrate RPE65 gene and protein expression in cultured human keratinocytes and find evidence for receptor-mediated endocytosis involved in retinoid–RBP uptake. Double immunofluorescence on keratinocytes shows colocalization of RBP and RPE65 and affinity binding of RPE65 to plasma RPB suggests interaction of these two molecules in cultured human keratinocytes supporting the hypothesis of a possible receptor function of RPE65 protein.

Materials and Methods

Cell culture, antibodies, and reagents Normal human keratinocytes were isolated from skin samples of adults, obtained from healthy volunteers after cutaneous surgery. Donors gave informed consent. The protocol was approved by the Institutional Review Board. Keratinocytes were grown to confluence in keratinocyte growth medium (Clonetics, San Diego, CA) including bovine pituitary extract as described previously (Boyce and Ham, 1983). Briefly, epidermis was separated by incubation in 50 caseolytic units per mL dispase (Collaborative, Bedford, MA) for 2 h at 37°C. After incubation in trypsin/ethylenediaminetetraacetic acid (Clonetics) for 30 min at 37°C, a single-cell suspension of keratinocytes was released by pipetting and cultured to near confluence using keratinocyte growth medium (Clonetics). In addition, keratinocytes were grown to confluence until spike formation resulting in differentiation and used after 24 h. Alternatively, cells grown to near confluence were incubated for 24 h with 1 mM Ca^{2+} to induce differentiation. ARPE-19, a human retinal pigment epithelium cell line expressing RPE65, was purchased from American Type Culture Collection (ATCC, Manassas, VA) and grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (ATCC) supplemented with 10% fetal bovine serum for 4 d. Cells were grown in 100 × 20-mm petri dishes using appropriate growth medium to obtain extracts for immunoblotting, in eight-well chamber slides (Becton Dickinson, Franklin Lakes, NJ) for immunofluorescence experiments and in T-150 flasks (Techno Plastic Products, Trasadingen, Switzerland) for mRNA isolation.

For colocalization by indirect immunofluorescence, a sheep antibody against plasma RBP was purchased from Chemicon (Temecula, CA). As second step antibodies, Alexa 488-conjugated donkey anti-sheep IgG from Molecular Probes (Eugene, OR) and TRITC-conjugated donkey anti-rabbit IgG from Chemicon were used. For immunoblotting alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega, Madison, WI) was used as second antibody. For control studies, irrelevant rabbit IgG was obtained from Sigma Chemical Co. (St. Louis, MO). Human RBP was purchased from Biotrend (Cologne, Germany).

Generation of affinity-purified peptide-specific anti-RPE65 antibody A synthetic peptide comprising a 15-amino-acid sequence (amino acids 150–164), NFITKINPETLETIK, of RPE65 protein (Nicoletti *et al*, 1995) described earlier as antigenic target for antibody generation (Redmond and Hamel, 2000) synthesized on branched oligolysines as carrier was produced by piChem R & D (Graz, Austria) and used as antigen to raise an anti-peptide rabbit antibody (Posnett *et al*, 1988; Butz *et al*, 1994). The rabbit was immunized by Charles River (Kisslegg, Germany) according to standard protocols. For affinity purification of the antibody, the peptide NFITKINPETLETIK was synthesized on a beaded polystyrene polyoxyethylene graft copolymer resin with a coupling intensity of 0.17 mmol per g by piChem R & D (Butz *et al*, 1994). A quantity of 330 mg of that gel matrix was loaded onto a 10-mL disposable column (Pierce, Rockford, IL), swollen in 70% ethanol, and conditioned with 10 mM Tris-HCl, pH 7.4. A quantity of 200 μL of polyclonal rabbit serum diluted in 1800 μL ImmunoPure Gentle binding buffer (ImmunoPure Gentle Ag/Ab Buffers, Pierce, Rockford, IL) was incubated overnight at room temperature under gentle shaking, washed with 20 column vol 10 mM Tris-HCl, pH 7.4, followed by elution with 4 mL ImmunoPure Gentle elution buffer (ImmunoPure Gentle Ag/Ab buffers, Pierce). Eluted affinity-purified anti-RPE65 antibodies were pooled, dialyzed against phosphate-buffered saline (PBS), pH 7.4, and concentrated (to 100 μg per 100 μL) with Centricon-30 microconcentrators (Amicon, Beverly, MA). In addition, free peptide NFITKINPETLETIK was purchased from piChem R & D and used for competitive inhibition of antibody binding.

Qualitative and quantitative real-time PCR (TaqMan) analysis Real-time PCR experiments were performed as described previously (Cauza *et al*, 2002). Human keratinocytes obtained from five different donors were cultured and cell pellets of proliferating and differentiating—induced by confluence as well as by 1 mM Ca^{2+}—keratinocytes were used for RNA isolation. For control, mRNA of ARPE-19 cells was used. Total RNA was extracted using Qiagen RNeasy Midi kit (Qiagen, Valencia, CA) according to the manufacturer's protocol (*RNeasy Midi/Maxi Handbook,* second edition, November 2001, Qiagen). Approximately 600 ng of total RNA diluted in sterile distilled water was reverse transcribed using TaqMan reverse transcription reagents kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction (TaqMan universal master mix protocol, Applied Biosystems). The reaction was performed in the presence of 10 μL of 10 × TaqMan reverse transcription buffer, 22 μL of 25 mM magnesium chloride (5.5 mM), 20 μL deoxy-NTPs mixture (500 μM of each dNTP), 5 μL of random hexamer (2.5 μM), 2 μL of RNase inhibitor (0.4 U per μL), 2.5 μL of MultiScribe reverse transcriptase (1.25 U per μL), and 38.5 μL of RNA (about 600 ng in sterile distilled water). The final solution (100 μL) was incubated for 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C. Five microliters of resulting cDNA solution was used as PCR template in the presence of 12.5 μL of TaqMan universal master mix (Applied Biosystems), 1 μL (5 pmol per μL) of gene-specific TaqMan probe, 3.7 μL (6 pmol per μL) of gene-specific forward and 2 μL (11 pmol per μL) of reverse primer, and 0.8 μL of sterile distilled water. Each cDNA was also amplified in the presence of 12.5 μL TaqMan universal master mix, 1.25 μL of β-actin (Applied Biosystems) as endogenous control, and 6.25 μL of water. Specific primers and probes for RPE65 were obtained from Applied Biosystems. The used sequences were designed using Primer Express 1.0 software (supplied by Applied Biosystems) with known gene-specific DNA and appropriate mRNA sequence of RPE65 gene (Nicoletti *et al*, 1995) from Entrez Nucleotide's search tool on the World Wide Web (National Center of Biotechnology Information (NCBI), Bethesda, MD). The following primers were used: CGCCGCTCACAGCTCAT (forward) and ATCGAAGGAGACTGCCGGT (reverse). The sequence for specific fluorogenic probe was ACAGGCAGGATCCCCCTCTGGC for RPE65. This probe was labeled with FAM as reporter, whereas the probe for endogenous control was VIC-associated. The sequence of PCR product was tested for specificity using NCBI BLAST search database (standard nucleotide–nucleotide BLAST) to exclude possible cross-reactivity and amplification of another target gene.

The samples underwent following stages: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; and stage 3, 95°C for 15 s followed by 60°C for 1 min. Stage 3 was repeated 45 times. Gene-specific products were measured by means of an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA) continuously for 45 cycles.

The relative quantity of specific RPE65 mRNA in the epidermal keratinocytes was evaluated according to the manufacturer's protocol using comparative threshold cycle (C_T) method (user bulletin for ABI Prism 7700 sequence detection system), where the relative quantity of RPE65 mRNA sample, normalized to α-actin as endogenous control and relative to a calibrator, is given by $2^{-\Delta\Delta CT}$. $\Delta\Delta C_T$ results from mean ΔC_T (mRNA from respective sample) minus mean ΔC_T calibrator. The mean ΔC_T value is the average ΔC_T value of five different donors. C_T of β-actin subtracted from gene-specific C_T gives ΔC_T. Calibrator is a basis sample and defined as 1 × sample. Before using the comparative C_T method for quantitation, validation experiments to demonstrate that efficiencies of target and reference (endogenous control) are approximately equal, were performed according to the instructions in the user bulletin for the ABI Prism 7700 detection system. Variables are described by means ± SD.

Immunoblotting Immunoblotting was performed according to methods described previously (Foedinger *et al*, 1998). Human keratinocytes and ARPE-19 cells were used to obtain cell extracts. Samples were washed with PBS and homogenized in 2% sodium dodecyl sulfate (SDS, Bio-Rad, Hercules, CA) in 0.0625 M Tris-buffered saline, pH 6.8, supplemented with 2 mM phenymethyl-

sulfonyl fluoride (Sigma Chemical Co.), boiled, and centrifuged. Extracted proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% polyacrylamide gel and transferred onto nitrocellulose membranes. Strips of nitrocellulose were exposed to affinity-purified peptide specific rabbit anti-RPE65 antibody diluted 1:100 for both extracts, overnight. Additionally, nitrocellulose strips of keratinocyte and ARPE-19 cell extracts were exposed to the antibody in the presence of an excess of free synthetic peptide. For control, irrelevant rabbit IgG (Sigma Chemical Co.) was used for immunoblotting. Strips were washed and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega, Madison WI). Bound antibody was visualized by nitroblue tetrazolium/bromochloroindolyl phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) enzyme reaction.

Indirect immunofluorescence Four-micrometer cryosections of snap-frozen paraformaldehyde-lysine-periodate-fixed normal human skin were prepared, and indirect immunofluorescence was performed. Sections were incubated with affinity purified anti-RPE65 antibody, diluted 1:25, washed, and exposed to respective second-step reagent. After being washed, sections were coverslipped, and examined using an Olympus BH 2-RFCA fluorescence microscope (Olympus, Albertville, MO). For control, normal rabbit IgG was used as first antibody.

Normal human keratinocytes were grown to near confluence in eight-well chamber slides, fixed with paraformaldehyde-lysine-periodate (McLean and Nakane, 1974) for 20 min, and permeabilized with 0.1% Triton X-100 for 10 min. This fixative preserves antigenic property of proteins as well as cell morphology and is routinely used for ultrastructural studies in our laboratory (Hinterhuber *et al*, 2002). For colocalization studies, we incubated the keratinocytes with RBP (0.1 μg/100 mL) in culture medium for 1 min at 37°C, washed, fixed, and exposed them to sheep anti-RBP antibody diluted 1:50 and affinity-purified rabbit anti-RPE65 antibody diluted 1:25. Cells were washed in PBS and incubated with a mixture of Alexa 488-conjugated donkey anti-sheep and TRITC-conjugated donkey anti-rabbit IgG, diluted 1:100 for 1 h.

Cells were washed, embedded in Fluoprep (BioMérieux, Marcy l'Etoile, France), and examined by confocal laser scan microscope LSM 510 using multitracking mode (Zeiss, Oberkochen, Germany) to separate the signal of each fluorescence and to prevent cross-talk.

Complexation of RBP and [^{3}H]retinoic acid and uptake experiments To examine uptake of conjugated RBP–retinoid, a complex of [^{3}H]retinoic acid and RBP was performed according to previous publications (Horwitz and Heller, 1973; Hodam et al, 1991; Zanotti et al, 1994). [11,12-^{3}H]Retinoic acid (1 mCi/mL) was purchased from American Radiolabeled Chemicals (St. Louis, MO) and used upon delivery under light protection. One-hundred microliters of RBP (Biotrend) resolved in 100 μL of PBS and 50 μL of [^{3}H]retinoic acid (1 μCi/μL) were added to 850 μL of PBS and incubated for 1 h at room temperature under gentle shaking and light protection. Thereafter, this solution was sent over a Sephadex LH20 (Amersham Bioscience, Piscataway, NJ) column to separate unbound [^{3}H]retinoic acid from the final preparation. The counts per minute per microliter of the complex solution were measured before (12007.3 $\pm$ 364 cpm/μL) and after (5596 $\pm$ 445 cpm/μL) passing the Sephadex LH20 column. Proliferating human keratinocytes grown in 35-mm petri dishes were used. After being washed in PBS, cells were exposed to culture medium, containing 80 μL of the final [^{3}H]retinoic acid–RBP complex preparation per mL of culture medium, at 37, 4, and, at 37°C in the presence of an excess of unconjugated RBP (75 μg/mL culture medium), for different time spans: 1, 3, 5, and 10 min. The culture medium temperature was brought to 37°C in a water bath before the respective labeled RBP complex was added. 4°C exposure was performed on ice. After incubation, cells were washed, harvested in PBS (1.5×10^5 cells per 300 μL of PBS), and counted for radioactivity using Wallac 1410 liquid scintillation counter (Pharmacia, Wallac Oy, Turku, Finland).

Statistics Statistical analysis was performed using the paired Student's t test and p values of less than 0.05 were considered to indicate statistical significance. Variables are given as means $\pm$ SD.

Affinity purification of RPE65 from keratinocyte lysates Human RBP (Biotrend) was covalently bound to a beaded polystyrene polyoxyethylene graft copolymer resin with a coupling intensity of 0.22 mmol per g by piChem R & D. A quantity of 100 mg of that RBP-coupled matrix gel was incubated in PBS for 20 min, followed by washing. Cultured human keratinocytes of one petri dish were lyzed in 1 mL of 1% Nonidet P-40 (NP-40) in Tris-HCl, pH 7.4, with proteinase inhibitors and centrifuged at 3100 g. Proteins were precipitated with ethanol:acetone 1:1 overnight at -20°C, pelleted, and air-dried. Then, the protein sample was dissolved in 1 mL of PBS with 2 mM phenymethylsulfonyl fluoride and transferred to the RBP-coupled matrix gel for 2 h under gentle shaking. Afterwards, the matrix gel was washed with PBS, for 3×5 min, and centrifuged. Thirty microliters of a 10% SDS sample buffer in 0.0625 M Tris-buffered saline, pH 6.8, supplemented with 2% dithiothreitol (Sigma Chemical Co.) was added to the pellet, boiled, and centrifuged. The supernatant was loaded onto a 12% polyacrylamide gel separated by SDS-PAGE and transferred electrophoretically onto nitrocellulose. Strips of nitrocellulose were cut, exposed to affinity-purified anti-RPE65 antibody with or without the presence of excess free peptide, and further processed for immunoblotting as described above. Control immunoblots were performed with irrelevant rabbit IgG. For additional control, only RBP-matrix gel without NP-40 lysate was exposed to SDS sample buffer, boiled, loaded, and processed for immunoblotting.

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