Retinol-induced secretion of human retinol-binding protein in yeast

Sjur Reppe[a], Sigbjørn Smeland[b], Jan Ø. Moskaug[b], Rune Blomhoff[b,*]

[a] *Institute for Medical Biochemistry, University of Oslo, P.O. Box 1112, Blindern, 0317 Oslo, Norway*
[b] *Institute for Nutrition Research, University of Oslo, P.O. Box 1046, Blindern, 0316 Oslo, Norway*

Received 13 March 1998

Abstract Retinol-binding protein (RBP) functions as a transporter for retinol (vitamin A) in plasma in higher eukaryotes. We have successfully expressed human RBP in *Saccharomyces cerevisiae*, and its secretion was found to be induced by retinol also in this lower eukaryote. Reduced induction of secretion by retinol in a temperature-sensitive *sec18-1* mutant that is blocked in secretion at the restricted temperature suggests that as in mammalian cells, RBP can be released from the endoplasmic reticulum upon addition of retinol. Thus, the molecular mechanism involved in retinol-dependent secretion of RBP appears to be conserved in yeast, and this points to yeast as a putative model system for studying retinol-regulated secretion of RBP. RBP purified from yeast was found to be indistinguishable from RBP purified from human plasma in several functional assays.

Key words: Retinol-binding protein; Secretion; Yeast

1. Introduction

In plasma of mammals, retinol is transported bound to a specific retinol-binding protein (RBP). The majority of RBP is produced in the liver, but mRNA for the protein has also been found in several extrahepatic tissues [1]. Retinol-induced secretion of RBP has been demonstrated in human and rat liver cells, and in human HeLa cells (which normally do not produce RBP) transfected with an expression plasmid for rat RBP [2]. Several authors have presented data demonstrating that retinol deficiency reduces RBP secretion in vivo, and that this does not affect the mRNA level in living rats [3–5]. Common for all the systems studied is that when retinol-depleted cells or animals are given retinol, secretion of RBP is rapidly restored. In the absence of retinol, RBP accumulates in the endoplasmic reticulum (ER) [6–8] probably due to specific retention mechanisms. These mechanisms are, however, not known.

Several proteins have been found to be retained in the ER due to signals within the proteins. Examples of such signals include relatives of the C-terminal sequences KKXX and KXKXX. Membrane proteins carrying these sequences are cycled between the ER and Golgi compartments [9]. Proteins can also be retained in the ER due to retrieval signals like the C-terminal sequence KDEL (HDEL in yeast) [10]. Also misfolded or partly folded proteins can be retained by specific retention signals. Hence a protein's folding or oligomerization may determine if the protein is retained or not. Formation of disulfide bonds appears to be important for correct folding, and in yeast, proteins containing disulfide bonds have been shown to be retained intracellularly in the presence of 20 mM of the reducing agent dithiothreitol (DTT), while secretory proteins without disulfide bonds were not affected [11]. RBP contains three disulfide bonds, but in contrast to secretory proteins in yeast, its secretion from HepG2 cells is stimulated at low levels of reducing agent [6]. Several intracellular intermediate forms of RBP have been identified, termed pre-RBP, compact I, compact II, and mature RBP [7,8]. The conversion process from compact II to mature RBP was found to take several hours. Thus, the rapid retinol-induced secretion of RBP observed when retinol-depleted cells are supplemented with retinol most likely involves rapid conversion of compact II to a mature form of RBP which is rapidly secreted.

Secretion of both protein and low molecular signal substances have been shown to be regulated at the level of fusion of secretory vesicles with the plasma membrane (reviewed in [12]). However, ligand-regulated secretion, as in the case of RBP, appears to be a more unique process, perhaps paralleled only by the secretion of apolipoprotein B-100 (apoB) which is secreted as a part of very low density lipoproteins (VLDL). However, in the case of apoB secretion, triglycerides may not act as true ligands with specificity for unique binding sites on the protein. Secretion of apoB has been shown to be increased in the presence of triglycerides and oleate which is part of the VLDL particles. The apoB protein is rapidly degraded within the ER in the absence of triglycerides [13–15]. This is in contrast to the situation for RBP which accumulates in the ER [3,16–18] in the absence of its ligand. We have taken advantage of yeast as a eukaryotic expression system to express human RBP and shown that ligand-induced secretion of this protein can be reproduced in yeast, and that the properties of the secreted RBP are indistinguishable from those of RBP purified from human plasma. This makes it possible to utilize the numerous secretion mutants and powerful genetic properties of yeast to further investigate the molecular mechanisms involved in retinol-induced secretion of RBP.

2. Materials and methods

2.1. Materials

N-(4-Hydroxyphenyl)retinamide (4-HPR) was a generous gift from Dr. Richard Moon at Specialized Cancer Center, University of Illinois at Chicago, IL, USA. Other retinoids, cycloheximide, phytol and β-mercaptoethanol were purchased from Sigma (St. Louis, MO, USA). Except for cycloheximide, which was dissolved in water, these chemicals were dissolved in ethanol prior to use. Zymolyase 100T was purchased from Seikagaku (Tokyo, Japan). Substrate for β-glucanase (Azo-barley glucan) was purchased from Megazyme (Sydney, Austral-

*Corresponding author. Fax: (47) 22 85 13 96.
E-mail: rune.blomhoff@basalmed.uio.no

Abbreviations: hRBP, human retinol-binding protein; TTR, transthyretin; 4-HPR, *N*-(4-hydroxyphenyl)retinamide; ER, endoplasmic reticulum; HepG2, hepatoma G2; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; PVDF, polyvinyl difluoride; TCA, trichloroacetic acid; RA, all-*trans* retinoic acid; RAL, all-*trans* retinal; ROH, all-*trans* retinol; 2-MEtOH, β-mercaptoethanol; EtOH, ethanol

PII S0014-5793(98)00428-1

ia). DNA modification enzymes were purchased from Promega (Madison, WI, USA). DNA primers were purchased from MWG-Biotech (Ebersberg, Germany). Antibodies were purchased from DAKO (Copenhagen, Denmark). Attophos substrate were purchased from JBL (San Luis Obispo, CA, USA).

2.2. Plasmid constructs

To express RBP in yeast, a construction was made based on the plasmid pαUXPTH-2 [19]. The *Bam*HI-*Ecl*136II fragment was excised from this construct, and replaced with a *Bam*HI-digested PCR fragment from the same plasmid made by primers I and II, leading to the construct pPαUX39PTH. From this construct an *Ecl*136II-*Sal*I fragment was excised, and the *Sal*I site of the vector was filled in to create blunt ends. Into this vector a PCR fragment encoding the mature form of hRBP (made by primers III and IV) was inserted, giving the construct pPαUXhRBP.

2.3. Primers

Primers I, II, III and IV used in the present study were:

I TGT GGA TCC TCT AGA GAA ATC;
II CTC TTT TAT CCA AAG ATA CCC;
III AAA GAG ACT GCA GAG TGA GCA GC;
IV CAG CTA TGA CCA TGA TTA CGA A.

2.4. Strains, transformation and culture conditions

Saccharomyces cerevisiae strains FL200 (α *leu2 ura3*), RSY271 (α, *sec18-1, ura3-52, his4-619*) and H758 (α *sec6-4 his4-260 leu2-3,112 trp1* (*Hin*dIII) *ura3-52*) were transformed with the pPαUXhRBP plasmid and cultured in 0.67% YNB without amino acids, 2% glucose, 1% casamino acids and supplemented with amino acids (50–75 μg/ml). Histidine was added to 0.4% to enhance the buffer capacity at pH 6.0. Strain M994 (*a, leu2, thr4*) was transformed with PCNN-MH(A16-M) and was cultured in 0.67% YNB without amino acids, 4% sucrose, supplemented with amino acids (50–75 μg/ml). All strains were transformed by a lithium salt-based method [20]. Plasmid PCNN-MH(A16-M) and yeast strain M994 were generous gifts from Ole Olsen and Morten Meldgaard at the Carlsberg Laboratory, Copenhagen, Denmark. Strain RSY271 was a gift from Randy Schekman (University of California, Berkeley, CA, USA).

2.5. Preparation of spheroplasts

Spheroplasts were prepared by incubating yeast cells at 37°C in a solution of 1 M sorbitol/0.15 M phosphate buffer pH 7.4 with 1 mg/ml Zymolyase 100T, and 1.4 mM β-mercaptoethanol.

2.6. Preparation of intracellular and periplasma fractions

After preparation of spheroplasts the cells were spun down and the supernatant was taken as the periplasma fraction. The cells were washed once in 1 M sorbitol/0.15 M phosphate buffer pH 7.4, before resuspension and lysis in 0.1% SDS. This sample was taken as the intracellular fraction.

2.7. Purification of RBP from yeast growth medium

Yeast cells were grown at 25°C to late log phase in the presence of 10 μM retinol and concentrated to 1×10^9 cells/ml. The cells were then incubated for several hours in the presence of 10 μM retinol. To remove the cells they where pelleted twice at 10 000 rpm in a SS34 rotor (Sorvall) for 5 min. RBP was concentrated using Ultrafree with a cutoff of 5 kDa. Subsequently RBP was purified from the concentrated sample by gel filtration on a Superdex 75 10/30 column (Pharmacia).

2.8. Purification of RBP from human plasma

RBP was purified from plasma essentially as described [21]. The procedure involves three steps: chromatography on DEAE Sepharose Fast Flow, followed by gel filtration on a HiLoad 26/60 Superdex 200 prep grade column and finally affinity chromatography on a NHS activated HiTrap column with coupled TTR (Pharmacia) according to instructions from the manufacturer.

2.9. Labeling of RBP

RBP was iodinated at Tyr residues of RBP using Iodo-Beads according to instructions from the manufacturer.

2.10. Receptor-binding assay

The assay was performed by measuring bound ^{125}I-RBP to placenta vesicles using an oil-centrifugation method as described [22]. Three parallel incubations were done in presence and absence of 2 μM unlabeled RBP purified from plasma. Human placenta vesicles were prepared as described [23].

2.11. Assay for secreted RBP and β-glucanase

Yeast cells were grown to late log phase (5×10^7–1×10^8 cells/ml) in shake flask cultures at 30°C. Cells were washed once in growth medium at 30°C before resuspension in growth medium at 1×10^9 cells/ml containing 0.1 mg/ml cycloheximide. Aliquots of 10 ml were then transferred to 50 ml tubes, and placed in a shaker at 30°C. Retinoids or other chemicals were then added, and aliquots were taken at indicated time points for immediate separation of cells from growth medium by centrifugation. RBP was quantified by immunoblotting as described [24] except for blotting onto PVDF membranes instead of on nitrocellulose. The relative intensity of the bands from immunoblotting was detected by densitometry or fluorography. β-Glucanase was assayed as described [25].

2.12. Electrophoresis

Electrophoresis was performed under reducing conditions in the presence of 0.1% SDS on commercially available 14% Tris-glycine gels (Novex).

2.13. Immunoblotting

Immunoblotting was done as described [24]. When Attophos was used as a substrate, alkaline phosphatase conjugated to pig anti-rabbit immunoglobulins was used as secondary antibody. Attophos was used according to instructions from the manufacturer.

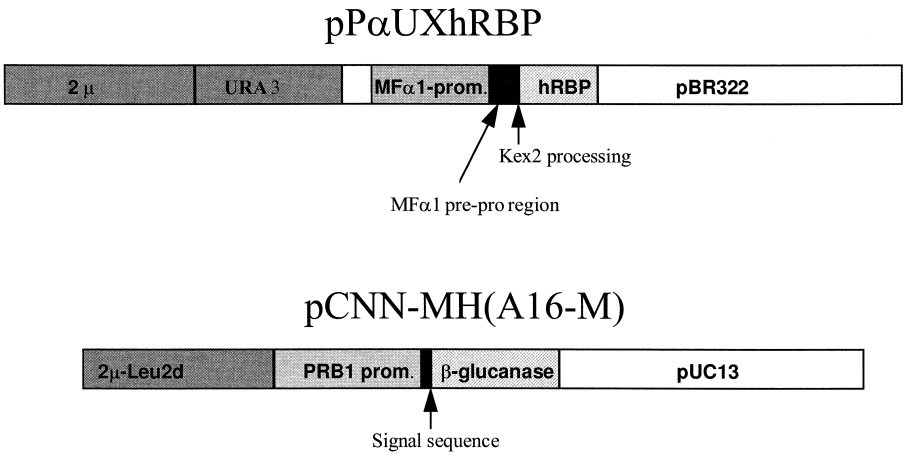

Fig. 1. Schematic representation of expression plasmids. The plasmids pPαUXhRBP and PCNN-MH(A16-M) direct expression and secretion of hRBP and (1-3,1-4)-β-glucanase, respectively, in yeast.

2.14. Silver staining
Staining with silver was done as described [26].

3. Results

To examine whether retinol-regulated secretion as it is observed in mammalian cells is conserved in *S. cerevisiae*, yeast cells were transformed with a plasmid that directs expression of RBP under the control of the constitutive MFα1 promoter (Fig. 1). The MFα1 pro-region contains the basic sequence Lys-Arg that is recognized by the *KEX2*-encoded endoprotease yscF residing in the Golgi region [27]. Cleavage of the fusion protein by the *KEX2*-encoded enzyme would thus give RBP with a correct N-terminus.

3.1. Effect of incubation time on secretion of RBP in yeast

Yeast cells containing pPαUXhRBP were incubated in the presence and the absence of retinol and samples from the growth medium were collected after various time points and analyzed with Western blotting with anti-hRBP antibodies as described in Section 2. The results are shown in Fig. 2A. It is clear that much more RBP was secreted from the transformed yeast in the presence of retinol compared to the control. Interestingly, retinol was most effective in inducing secretion of RBP the first 15 min after addition of retinol. The effect of retinol on secretion is independent of transcription and translation since these experiments were carried out in the presence of cycloheximide. As can be seen in Fig. 2, secretion also occurs in the absence of retinol. This is in line with results obtained with mammalian cells, where moderate levels were found to be secreted also in the absence of retinol [28,29]. To rule out the possibility that retinol has a more general stimulatory effect on secretion of proteins in yeast, we tested the effect of retinol on secretion of (1-3,1-4)-β-glucanase. Yeast cells were transformed with a plasmid containing the gene for β-glucanase under control of the PRB1 promoter [27,30,31]. The cells were then incubated with retinol or phytol for increasing time periods. The results (Fig. 2B) show that retinol has no effect on the secretion of β-glucanase. Also secretion of (1-3,1-4)-β-glucanase in the presence of ethanol (solvent for phytol and retinol) was comparable to that in the presence of phytol and retinol (data not shown), demonstrating that retinol does not have a general stimulatory effect on protein secretion in yeast.

3.2. The effect of other retinoids and β-mercaptoethanol on secretion of RBP

To determine whether the secretion of RBP to the growth medium can be induced by some other retinoids as has previously been observed in rat and human cells, we incubated transformed yeast cells in the presence of retinol, retinal or retinoic acid or the synthetic retinoid 4-HPR, which have all been found to bind to RBP [32,33]. Also the effect of β-mercaptoethanol was tested. Retinoic acid has been shown to stabilize folding intermediates of RBP in the same way as retinol [7,8] and to promote secretion of RBP in retinol deficient primary hepatocytes [28]. Retinal has been found to stimulate secretion of RBP in HeLa cells in vitro [2], and β-mercaptoethanol stimulates secretion of RBP in HepG2 cells [6]. Growth media from the cells incubated with the different substances were collected and RBP secreted was measured by quantitative Western analysis. The results in

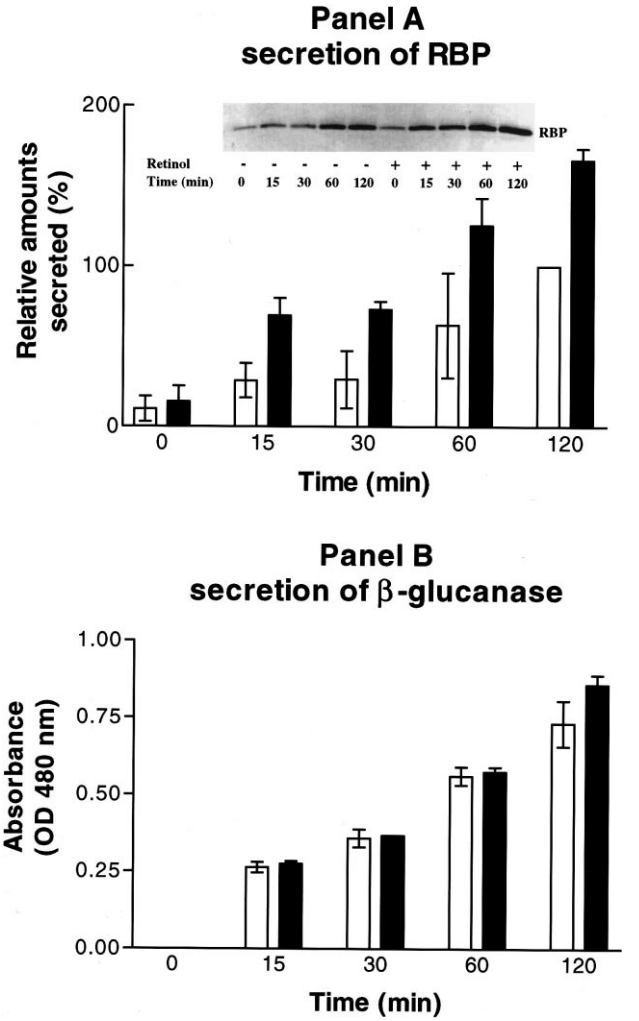

Fig. 2. Time course comparing secretion of hRBP in the presence and absence of retinol. Transformed yeast cells were incubated with 10 μM retinol or ethanol as described in Section 2. Samples from cultures expressing hRBP were subjected to immunoblotting at various time points. The same amount of growth medium was added to each lane. A: In the upper part of the panel a typical Western blot of hRBP are presented. Yeast cell cultures where incubated in the presence and absence of retinol as indicated. Samples for Western analysis were taken after indicated times of incubation. The relative amounts of hRBP were quantitated at indicated time points by Western analysis and densitometry. Error bars represent standard error of the mean from three different experiments. Values obtained in the absence of retinol at 120 min was chosen as 100%, other values are presented as percentages of this value. White bars represent control while black bars represent presence of retinol. B: The cultures expressing β-glucanase were analyzed by a method based on degradation of a dyed Azo-barley glucan substrate to fragments which are soluble in the presence of precipitant solution. The absorbance at 490 nm is thus proportional to β-glucanase activity which was measured at indicated time points in presence of 10 μM phytol or 10 μM retinol. Error bars represent standard error of the mean from three replicates in one typical experiment.

Fig. 3 show that retinol was most potent in stimulating secretion of RBP to the growth medium. 4-HPR seems to be nearly as efficient as retinol in stimulation of secretion, which is in agreement with results obtained with HepG2 cells [34] and liver cells in vivo [35], where retinol and 4-HPR had comparable effects on stimulation of secretion. Retinal and retinoic acid also stimulates the secretion of RBP although not as

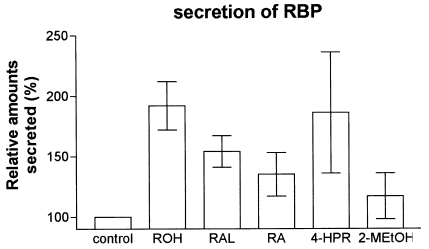

Fig. 3. Effects of retinoids and other substances on secretion of hRBP. Transformed yeast cells were incubated in 10 μM of the indicated substances (except ethanol) as described in Section 2. Samples were collected for analysis by immunoblotting after 30 min of incubation. The relative amounts of hRBP after incubation with various substances were quantified with densitometric analysis on immunoblots. The results shown are mean of four or five individual experiments where values obtained with ethanol were set at 100%. Error bars represent standard error of the mean from different experiments.

efficiently as retinol and 4-HPR. The effect of β-mercaptoethanol on RBP secretion in yeast is not pronounced.

3.3. Intracellular retention of RBP in yeast

RBP expressed in yeast is clearly secreted to a larger extent in the presence of retinol as compared to in the absence of retinol. As expression of RBP is not altered as a function of retinol, RBP has to be retained in a cell associated compartment, either intracellularly or in the periplasma in the absence of retinol. To determine where RBP is retained, a periplasmic fraction, an intracellular fraction and the growth medium were prepared as described and the fractions were characterized with respect to the presence of RBP. The Western blot analysis of these fractions shows (Fig. 4) that there are several RBP-related forms found in the intracellular as well as the periplasmic fraction. RBP secreted to the medium migrates as hRBP purified from plasma and is most likely identical to hRBP. The bands that migrate faster than the standard hRBP purified from plasma are probably degradation products, while those of higher molecular weight could be due to incomplete processing of the pro-region by the *KEX2*-encoded protease. The majority of RBP and RBP-related proteins are retained intracellularly, while some RBP is also retained in the periplasmic space. Quantitative analysis on a FluorImager (Vistra) indicates that only 5% of the band comigrating with purified plasma RBP is released to the growth medium.

3.4. Secretion of RBP from temperature-sensitive sec mutants

A relatively large fraction of the expressed RBP is retained in intracellular and the periplasmic space. The observed increase in secretion upon addition of retinol could be explained by release from both compartments. To clarify whether secretion of RBP was induced from the periplasmic space or from an intracellular compartment, secretion of RBP was studied in a yeast strain with a temperature-sensitive secretion phenotype (*sec18-1*). The secretion from the *sec18-1* mutant strain RSY271 is blocked at the non-permissive temperature (37°C) whereas secretion is apparently normal at the permissive temperature (25°C) [36]. The secretion of RBP from this mutant was compared to that of FL200 (which is wild-type with respect to secretion) at 25°C and 37°C.

If retinol-dependent release of RBP in yeast is due to release of RBP from the ER, secretion in the *sec* mutant should be strongly reduced at 37°C as compared to 25°C, and retinol-induced secretion should take place at 25°C but not at 37°C. First the cells were grown for 24 h at 25°C, where both the FL200 and the RSY271 display wild-type phenotype. Then the cells were split and incubated for 2 h at the permissive or non-permissive temperature, and RBP in the growth medium was analyzed by Western blotting (Fig. 5B). The results show that the pattern of secretion from the *sec18-1* mutant strain RSY271 is consistent with retinol-induced secretion from the ER and not the periplasm. In the presence of retinol the immunoreactive bands containing RBP was significantly reduced when incubated at 37°C compared with incubation at 25°C.

More important, a clear difference between the presence and the absence of retinol was found at 25°C whereas no difference was found at 37°C (this applies also when all immunoreactive bands are grouped (Fig. 5A)). This would not have been the case if retinol induced release of RBP from the periplasma. This experiment also demonstrated that high molecular weight forms of RBP (marked * and ** in Fig. 5B) were secreted to a lesser extent at 25°C than at 37°C in FL200 as well as in the RSY271 strain. The bands marked * and ** are probably due to incomplete cleavage between the MFα1 pro-region and RBP by the *KEX2*-encoded protease. *O*-Glycosylation is a less likely explanation for this band, as this would require glycosylation of most of the 11 Ser and 9 Thr residues of RBP.

Release of RBP to the growth medium was also tested in another *sec18-1* strain as well as in *sec6* and *sec1* mutants at permissive and non-permissive temperatures. Secretion from the *sec6* mutant resembled that of RSY271, while the results with the two other strains were inconclusive as more RBP was released at 37°C as compared to the RSY271 strain (data not shown).

3.5. RBP secreted from yeast has similar binding properties as RBP from human serum

When RBP is expressed in yeast from the pPαUXhRBP

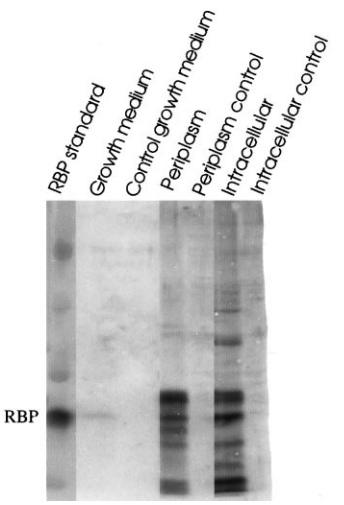

Fig. 4. Distribution of RBP between extracellular and intracellular compartments. Growth medium, the periplasmic fraction and the intracellular fraction from 3×10^7 cells cultured in the presence of retinol and cycloheximide were subjected to SDS-PAGE and immunoblotting. As control, fractions were prepared from the same number of cells not transfected with pPαUXhRBP.

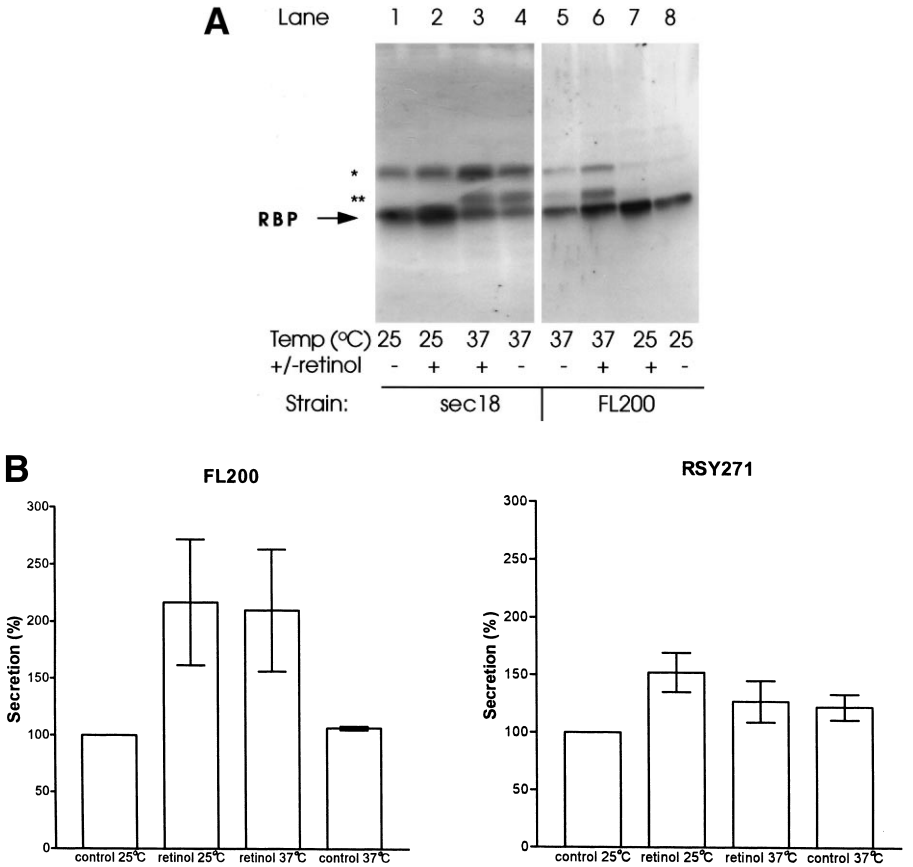

Fig. 5. Secretion of RBP from sec mutants. A: Cultures of strain FL200 and the *sec18-1* mutant RSY271 were grown at 25°C to late log phase. The cultures were then split, and washed and concentrated to 10^9 cells/ml before incubation at 25°C or 37°C in the presence or absence of 10 μM retinol for 30 min. The same amount of medium from each incubation was then subjected to SDS-PAGE and immunoblotting. B: The immunoblot in A was analyzed by densitometry and the relative amounts of RBP secreted under different conditions were plotted. The amount of RBP secreted from cells incubated at 25°C with no retinol was set at 100%. Error bars represent standard error of the mean from three different experiments.

construct, the protein undergoes several processing steps on its way through the secretory pathway due to its fusion to the MFα1 pro-region. These processing events take place in different compartments. As these processing events in *S. cerevisiae* may result in different forms of RBP, it is important to verify that the secreted protein has the same properties as the protein secreted from mammalian cells. Therefore, we subjected RBP secreted to the yeast growth medium to characterization with respect to the biological function of RBP, i.e. retinol binding, binding to TTR and binding to RBP receptors in placenta vesicles. With respect to these biological functions, RBP purified from the growth medium of transfected yeast cells had similar receptor-binding affinity as RBP purified from plasma (Fig. 6C). The K_d for the interaction between RBP and its receptor was found to be 34.5 nM, which is similar to the value (i.e. 79 nM) observed by Sivaprasadarao and Findlay [37]. The secreted RBP did also interact with retinol and TTR as shown (Fig. 6A,B). Although these data do not directly determine the binding affinity of RBP to retinol and TTR, they clearly demonstrate that the protein interacts with retinol and TTR.

4. Discussion

In this work we describe how retinol-binding protein is expressed and secreted in *S. cerevisiae* transfected with a plasmid encoding human RBP. Secretion of many proteins by mammalian cells appears to be a regulated process. For example, in mouse mammary cells casein secretion is to some extent regulated by the intracellular Ca^{2+} concentration. In the absence of stimulation, casein appears to be accumulated in secretory vesicles [38]. In hepatic cells apolipoprotein B-100 is secreted upon incubation of cells with triglycerides and this protein is degraded within the ER when secretion is not stimulated [18]. Retinol-induced secretion of RBP from the liver in vivo and from hepatocytes and transfected non-hepatic cells in vitro have been described in several studies [16,29,39,40]. When secretion is not induced by retinol, RBP is retained and accumulates in the ER [3,6]. This type of ligand-induced secretion seems so far to be unique for RBP and the mechanisms involved are poorly understood.

Our results clearly demonstrate that RBP secretion from the yeast cells can be induced with RBP's physiologically functional ligand, retinol. One implication of these results is that the retention mechanisms involved in keeping RBP in the ER in the absence of its ligand is conserved even in organisms where retinol appears to play no role for the viability or function of the organism. Moreover, RBP secreted from yeast cells is indistinguishable from human plasma RBP

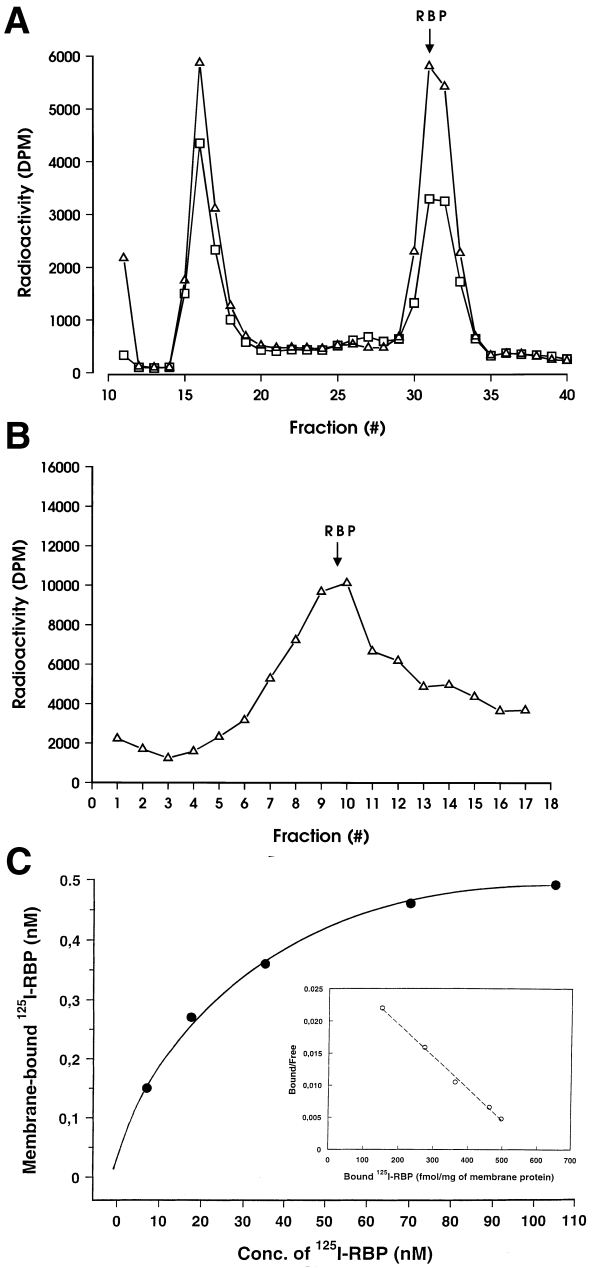

Fig. 6. Binding properties of RBP secreted from yeast cells. A: Binding of retinol to RBP. [^{3}H]Retinol-labeled RBP (0.5 ml) was loaded onto a Superose 12 column before (triangles) and after (squares) incubation with cold retinol. The samples were eluted with phosphate buffered saline (PBS) pH 7.4 at 0.5 ml/min. Fractions of 0.5 ml were collected and 10 μl of each fraction were counted. The arrow marked RBP denotes the fraction in which plasma-purified RBP was eluted in a parallel experiment. B: Binding of RBP to TTR. [^{3}H]Retinol-labeled RBP in PBS was loaded onto a TTR column (TTR coupled to a 1 ml NHS-activated HiTrap column (Pharmacia)). The column was washed with 5 ml PBS and eluted with water adjusted to pH 10 with NH_3. The radioactivity in 10 μl of each fraction was counted. The arrow marked RBP indicates the fraction in which plasma purified RBP was eluted in a parallel experiment. C: Receptor binding. Placenta vesicles (1 mg/ml) were incubated with various concentrations of ^{125}I-RBP at 22°C for 15 min, and bound RBP was plotted against concentration of RBP during binding. The binding data were then subjected to a Scatchard plot (insert). The calculated K_d and B_{max} were 34.5 nM and 590 fmol of RBP bound/mg of membrane protein, respectively. The data represent mean values of three experiments.

in terms of retinol binding, TTR binding and binding to cell surface receptors on mammalian cells.

Our interpretation of the data for the RSY271 strain in Fig. 5 is as follows: RBP is made as a pre-pro precursor. As the precursor is transported into the ER the signal sequence is cleaved off. The resulting pro-RBP is then transported to the Golgi compartment before processing of the pro-region by the *KEX2*-encoded protease, which resides in trans-Golgi. At 37°C however, secretion is blocked between ER and Golgi, and whatever comes out of the cell at the non-permissive temperature is released from a post-ER compartment. The same set of different RBP forms are seen in RSY271 as in periplasma and the intracellular compartment of the FL200 strain at both permissive and non-permissive temperatures (Fig. 5B), indicating that the RBP forms released to the medium at the non-permissive temperature could come from the Golgi or the periplasmic space.

Mechanisms involved in folding, ER retention and secretion of proteins has been studied in great detail in *S. cerevisiae* and our results showing that retinol-induced secretion of RBP can be reproduced in this organism opens up the possibility of studying this process in yeast strains where one or several components required for folding, retention and secretion have been mutated.

RBP could possibly be retained in the ER by its association to other proteins that are retained by the well characterized mechanisms based on the KDEL (HDEL) or KKXX motifs as both these mechanisms are conserved between yeast and mammalian cells [10]. We have recently found that the RNLL sequence in the C-terminal of RBP, although similar to a KDEL motif, is not responsible for its intracellular retention [29]. RBP has under certain conditions been found associated with calnexin [41], which is considered to be a part of an apparatus responsible for control of protein conformation in the ER [42,43] and thus calnexin could be responsible for RBP's retention in the absence of its ligand. This association was, however, detected only when RBP was exposed to reducing agents, which may induce alterations in RBP's conformation and may therefore not be involved under the normal conditions. A member of the calnexin/calreticulin family of genes has recently been described by Parlati et al. They have named the gene *CNE1* and made strains with a disruption in this gene [44]. By transfecting these mutant strains with our expression vector it is possible to study the involvement of this member of the calnexin family in retention of RBP.

Formation of disulfides during folding of newly synthesized and translocated RBP has been implicated in retention and retinol-induced secretion. Kaji and Lodish have found that RBP goes through several intermediate conformations during folding and that retinol stabilizes certain disulfide configurations in RBP [7,8]. This is in line with the observation that β-mercaptoethanol modifies secretion of RBP from HepG2 cells [6]. The reason why β-mercaptoethanol has little effect on secretion of RBP in yeast could be that secretory proteins containing disulfide bonds have been shown to be retained intracellularly in yeast in the presence of reducing agents [11]. Formation and rearrangements of disulfides in mammalian cells and yeast are mediated by protein disulfide isomerase (PDI). Indeed, PDI has been shown to enhance the rate of unfolding of RBP by DTT in vitro [8]. As yeast strains with mutations in PDI have already been described [45], this enzyme's role in retention and secretion of RBP can be studied by

transfection of these mutant strains with our expression vector and our assay for retinol induced secretion.

Additionally, yeast may be used for expression and purification of RBP for functional studies. Small amounts (approx. 200 μg/l) of RBP can be obtained in its functional form when it is expressed in *Escherichia coli* [46], whereas we can obtain 10 times more RBP in the concentrated yeast growth medium. This RBP can be either holo- or apo-RBP and it can be labeled to high specific activity with [^{35}S]methionine. This is important for studies of RBP-receptor interactions as iodination of RBP has been shown to modify its behavior in cellular binding, uptake and degradation [47]. In summary, this work describes and characterizes some of the tools required for elucidating the mechanisms responsible for ligand-induced secretion of RBP.

Acknowledgements: We thank Tordis B. Øyen for helpful discussions, and Sirkka Keränen and Randy Schekman for the yeast strains. The work was supported by the Norwegian Cancer Society and the Research Council of Norway.

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