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Glycodelin and β-lactoglobulin, lipocalins with a high structural similarity, differ in ligand binding properties

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Abstract Human glycodelin, a lipocalin with a high amino acid similarity to β-lactoglobulins, appears as various glycoforms with different biological activities in endometrium (glycodelin-A) and seminal plasma (glycodelin-S). We found that the structures of these glycodelins and β-lactoglobulin are similar. Despite this structural similarity, unlike β-lactoglobulin, glycodelin-A binds neither retinoic acid nor retinol. It was impossible to detect any endogenous retinoids or steroids in any of the two purified glycodelins. Both their glycoforms share similar thermodynamic parameters of reversible denaturation suggesting that native folding of glycodelin-A and glycodelin-S is not influenced by the differences in glycosylation or by ligand binding.

Key words: β-Lactoglobulin; Glycodelin; Glycosylation; Lipocalin; Protein structure

1. Introduction

The lipocalin superfamily is a group of small extracellular proteins [1,2], the members of which exhibit structural and functional diversity but share three conserved sequence motifs. Their overall folding patterns are similar [1]. The gene structures and localisations of their genes are also highly-conserved [2,3]. Many, if not all, members of this family bind small hydrophobic molecules. They also bind to specific cell surface receptors [1].

Glycodelin, previously known as placental protein 14 or progesterone-associated endometrial protein, can be classified in the lipocalin superfamily on the basis of its protein sequence [4]. Purified glycodelin-A (GdA) from amniotic fluid displays contraceptive [5] and immunosuppressive properties [6,7]. In the human male, seminal plasma contains an immunoreactive glycodelin called glycodelin-S (GdS). It cannot be distinguished from GdA by currently available immunological methods [9]. However, GdS is a differentially glycosylated form of glycodelin [8–10]. While their primary structures are identical, unlike GdA, GdS has no contraceptive activity [10,11]. This suggests that glycosylation determines the biological activities of these two glycodelins. In addition to the differences in glycosylation, alternatively-spliced glycodelin transcripts have been reported [11–13]. Interestingly, transfection of glycodelin cDNA into MCF-7 breast cancer cells induces epithelial differentiation, decreases the proliferation rate and induces apoptosis [14]. Retinoic acid treatment can induce similar effects [15].

Glycodelin exhibits a significant amino acid sequence similarity with β-lactoglobulins, which strongly bind retinoic acid and retinoids [4,16–18]. Hence, it was of interest to study whether glycodelins would also bind these biologically active substances and whether the differences in their biological activities are related to the structure or endogenously bound ligand(s).

2. Materials and methods

2.1. Tertiary structure

The tertiary structure of glycodelin was deduced using the automated Swiss-Model service, which extrapolates a model for a target sequence from the known three-dimensional (3D) structure of related family members (template) [19–21]. We employed the Swiss-Model with the ProMod protein modelling tool [21] for generating the primary model and the 3D profile of the model [22] was used for quality controls. The crystal structure of bovine β-lactoglobulin [23] (entry 1beb in the Brookhaven Protein Data Bank) was used as a template.

For searching proteins with a similar 3D structure, the proposed 3D coordinates were compared to those from the Brookhaven Protein Data Bank using the Dali (version 2.0) service [24]. Comparison between the β-lactoglobulin ligand binding site and the corresponding region in glycodelin was performed using a Swiss-PDB Viewer.

2.2. Proteins

Glycodelin-A from amniotic fluid and the differently-glycosylated GdS from seminal plasma were purified as described before [9,25]. For the detection of endogenously bound ligands, we omitted the addition of Tris-buffer after elution from an anti-glycodelin Sepharose column. For the elution, we used 0.1% trifluoroacetic acid containing 1 mM $CaCl_2$. The subsequent steps of dialysis of GdA and GdS and the HiQ purification of GdS were also omitted in the experiments in which endogenously bound ligands were studied. β-lactoglobulin B was isolated from the milk of a homozygous cow according to Maillart and Ribadeau-Dumas [26]. Homogeneity of the protein preparation was assessed by high performance gel permeation chromatography and SDS gel electrophoresis. The obtained preparations of β-lactoglobulin were over 98% pure.

2.3. Detection of endogenous retinoids or steroids

For the detection of endogenously bound ligands, we treated 32 nmol purified GdA (1 nmol equals to 28 μg) or 17 nmol purified GdS three times with 5 ml chloroform [27] to extract endogenous retinoids, steroids or other ligands. We also treated 12 nmol purified GdA and 17 nmol purified GdS with ethanol plus *n*-hexane [28]. The chloroform and hexane phases were evaporated under nitrogen and dissolved in chloroform or hexane, respectively. The extracts were analysed by thin layer chromatography (TLC) using SIL G-25 UV_{254} plates (Macherey-Nagel, Düren, Germany) and benzene/acetone/methanol (8/1/2, v/v/v) as a solvent. Hydrophobic ligands from the hexane extraction were also analysed using hexane/diethyl ether/acetic acid (80/20/2, v/v/v) as a solvent. All-*trans*-retinol, all-*trans*-retinoic acid, progesterone and prostaglandin E_2 (all from Sigma) were used as molecular size and extraction controls.

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2.4. Exogenous binding

All-*trans*-retinol and all-*trans*-retinoic acid binding to GdA and β-lactoglobulin were tested by fluorescence measurements according to Dufour and Haertlé [29]. Fluorescence spectra were recorded with the Aminco SLM 4800C spectrofluorometer at 20°C in the ratio mode. The concentrations of retinol and retinoic acid in ethanol stock solutions were 686 and 705 μM, respectively. Aliquots (1–1.5 μl) of the ligand stock solutions were added to 1 ml protein solution containing 50 mM phosphate buffer, pH 7.0. Two sets of measurements were carried out with 10.2 and 5.1 μM GdA solutions. The relative intensity of fluorescence caused by the protein was measured at 330 nm (excitation 282 nm) as a function of the ligand concentration. The titration curves were corrected for the blank values provided by injections of the corresponding stock solutions of the ligands into a solution of *N*-acetyl-L-tryptophanamide, adjusted to the concentration giving the same absorbance at 280 nm as did the glycodelin solution.

2.5. Circular dichroism spectroscopy

Circular dichroism spectra were measured at 25°C with the Jobin Yvon CD6 dichrograph. The spectra were an average of 10 accumulated scans with subtraction of the baseline recorded for buffer solution (10 mM potassium phosphate, 1 mM EDTA at pH 6.6 and 20 mM glycine at pH 2.1). The protein concentration was 0.4–0.5 mg/ml. Cylindrical cells of 0.02 cm path length were used.

2.6. Differential scanning calorimetry

Calorimetric measurements were carried out using a high sensitivity differential scanning microcalorimeter (VP-DSC, MicroCal, Northampton, MA, USA) with a temperature range of 6–110°C at a heating rate of 60°C/h and excess pressure of 32 psi. The protein solution was dialysed overnight at 4°C against 10 mM potassium phosphate containing 1 mM EDTA at pH 6.6 or against 40 mM glycine at pH 2.1. After dialysis, the protein concentration was determined spectrophotometrically assuming an extinction value of $E^{1\ mg/ml}_{280nm} = 1.0$ in 1 cm cell [30]. Calorimetric measurements were carried out using a 0.2 mg/ml protein concentration. The molar heat capacity of GdA and GdS was calculated assuming the molecular mass of the monomer (28 kDa). Data processing was carried out with Origin 4.1 software (MicroCal).

3. Results

The sequence alignment of glycodelin with bovine β-lactoglobulin is shown in Fig. 1. The modelled 3D structure of the glycodelin monomer (Fig. 2) was found to be similar to the crystal structure of bovine β-lactoglobulin [23]. These two structures differed only by ≤0.5 Å root mean square deviation (rmsd) in the α-carbon positions. Interestingly, all three potential glycosylation sites of the glycodelins are localised on the same side of the modelled molecule. The 3D profile (not shown) displays two regions with scores falling around 0.1 (average 0.44), which may reflect unfavourably exposed nonpolar surfaces. At a physiological pH, the circular dichroism spectrum of GdS is close to that of bovine β-lactoglobulin (Fig. 3), suggesting similarity in their secondary structures.

In order to address whether GdA binds retinol or retinoic acid, we used the fluorescence quenching method, which is widely used for lipocalin ligand binding studies (Fig. 4). In

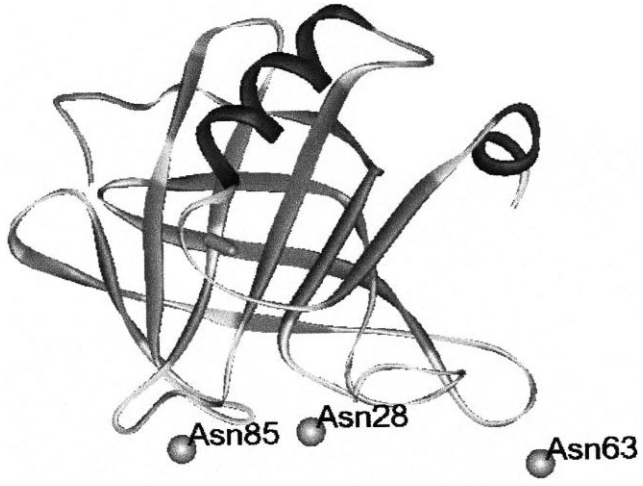

Fig. 2. The Swiss-Model-deduced tertiary structure of the glycodelin monomer. The S-S bridge is shown as a cylinder and the side chain nitrogen atoms of asparagines of the potential glycosylation sites are shown as balls. The model is generated by the automated Swiss-Model service and visualised using a WebLab Viewer (Molecular Simulations, San Diego, CA, USA).

the case of GdA, one would expect that the effect of bound ligand on the quenching of tryptophan fluorescence should be similar to that observed in β-lactoglobulin, since both tryptophans in β-lactoglobulin are conserved in the glycodelin sequence (Fig. 1). However, we found that with an increasing retinol concentration, the fluorescence intensity of GdA varies only slightly and almost linearly. The corresponding curve for retinoic acid shows more deviation, probably due to turbidity in the glycodelin/retinoic acid mixtures when the ligand concentration was high. The titration curves of glycodelin differed significantly from that of bovine β-lactoglobulin (Fig. 4). The latter curve was typical for the binding isotherms with saturation at the protein/ligand ratio > 1. The results suggest that there is no specific binding of any tested retinoid ligand to GdA under the conditions used. This means that, if any interaction between glycodelin and retinol or retinoic acid exists, it must be very weak, with binding constants below 10^5–10^6 l/mol.

No endogenously bound ligands could be extracted from GdA or GdS as detected by TLC suggesting that, if any hydrophobic ligands remain attached to the glycodelins during purification, the amount must be very low. Compared to the molar concentrations of glycodelins used in this study, the methods we used could readily detect smaller or equimolar quantities of retinoic acid, retinol, progesterone and prostaglandin E_2 from aqueous solutions in the control experiments (not shown).

We also measured by differential scanning calorimetry the

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     5          15         25         35         45         55
Gd   QTKQDLELPK LAGTWHSMAM ATNNISLMAT LKAPLRVHIT SLLPTPEDNL EIVLHRWENN
BLG  QTMKGLDIQK VAGTWYSLAM AASDISLLDA QSAPLRVYVE ELKPTPEGDL EILLQKWENG
     65         75         85         95         105        115
Gd   SCVEKKVLGE KTENPKKFKI NYTVANEATL LDTDYDNFLF LCLQDTTTPI QSMMCQYLAR
BLG  ECAQKKIIAE KTKIPAVFKI DALNENKVLV LDTDYKKYLL FCMENSAEPE QSLVCQCLVR
     125        135        145        155
Gd   VLVEDDEIMQ GFIRAFRPLP RHLWYLLDLK QMEEP
BLG  TPEVDDEALE KFDKALKALP MHIRLSFNPT QLEEQ
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Fig. 1. Sequence alignment of glycodelin (Gd) with bovine β-lactoglobulin (BLG) (amino acids 5–159 after the signal sequence from both proteins) [4,23]. Identical residues are shaded (43% identity in the shown region).

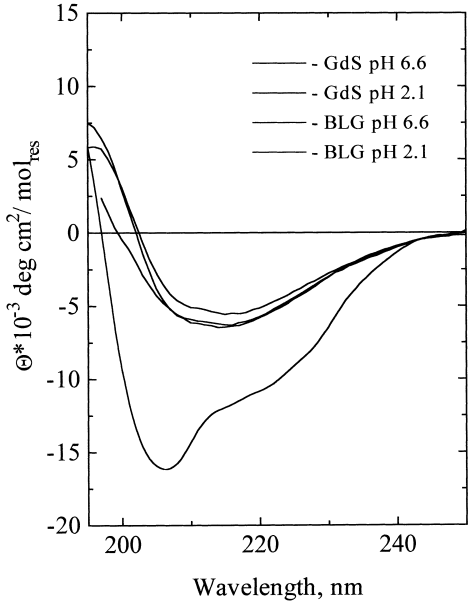

Fig. 3. Far-UV circular dichroism spectra of GdS and bovine β-lactoglobulin (BLG).

stability of the native conformations of GdA, GdS and bovine β-lactoglobulin (Fig. 5). At pH 6.6, a single symmetrical peak of the heat capacity was observed for both glycodelin glycoforms, typical of small globular proteins. Under these conditions, the thermal denaturation of glycodelins was reversible: the degree of reversibility for GdA and GdS was 81% and 93%, respectively, estimated from the percentage of reproducible peak area during re-heating of the protein sample. The following values of thermodynamic denaturation parameters were obtained for GdA and GdS, respectively: denaturation temperature $T_d = 65.65°C$ and 66.23°C, denaturation enthalpy per monomer $\Delta H_{cal} = 296.0$ kJ/mol and 312.2 kJ/mol, denaturation heat capacity increment $\Delta C_p = 7.22$ kJ/mol/K and 10.10 kJ/mol/K. The difference between GdA and GdS does not exceed the usual level of accuracy in high sensitivity differential scanning calorimetry. We conclude that the two glycodelin glycoforms do not differ from each other by their thermodynamic stability at a physiological pH.

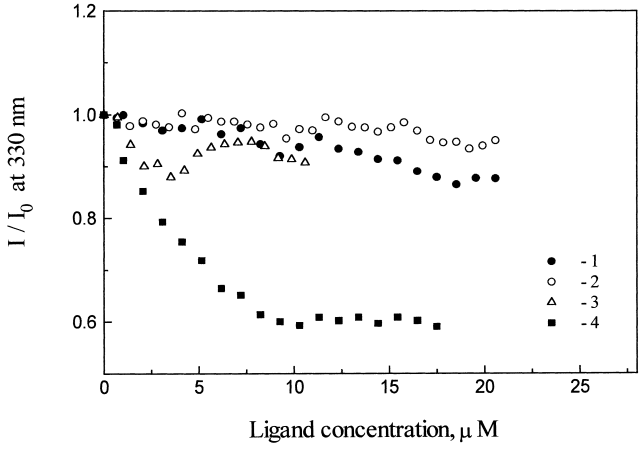

Fig. 4. Corrected fluorescence titration curves of (1–3) GdA and (4) β-lactoglobulin with (1, 2, 4) retinol and (3) retinoic acid. Protein concentration: (1) 10.2 μM, (2, 3) 5.1 μM and (4) 12.5 μM. 50 mM phosphate buffer, pH 7.0.

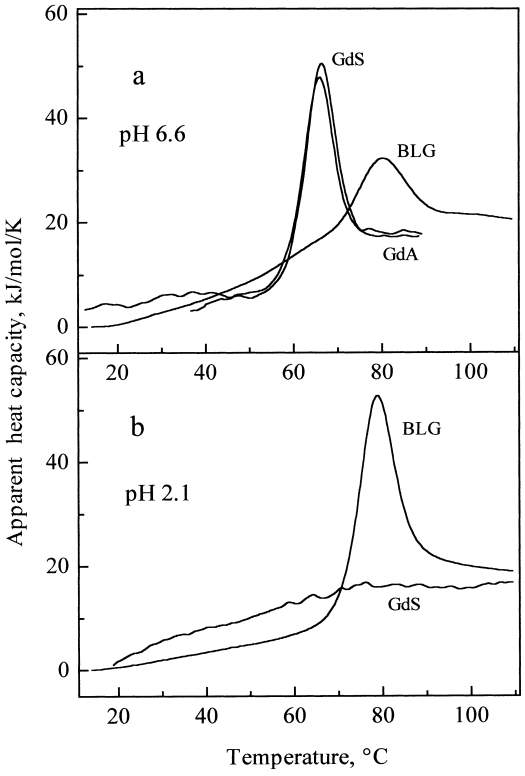

Fig. 5. Heat capacity curves of GdA, GdS and bovine β-lactoglobulin (BLG) (a) at pH 6.6, (b) pH 2.1. Heating rate = 60°C/h; glycodelin concentration 0.20 mg/ml; β-lactoglobulin concentration 0.41 mg/ml.

Fig. 5a shows that, at a physiological pH, the transition temperature and unfolding profile of glycodelin differs substantially from that of bovine β-lactoglobulin. Compared to β-lactoglobulin, the maximum of the unfolding peaks for glycodelins is observed at temperatures of about 15°C lower and the unfolding transition is much narrower. The difference in stability of the tertiary structures of GdS and β-lactoglobulin is especially pronounced at acidic pH. At pH 2.1, β-lactoglobulin shows a symmetrical denaturation peak at about 80°C, whereas GdS reveals no cooperative transitions on the thermogram (Fig. 5b).

4. Discussion

The Swiss-Model-deduced tertiary structure of glycodelin was found to be similar to that of β-lactoglobulin. This remains in agreement with previous work of Sansom et al. [37] who have classified PP14 protein (glycodelin) in what they called the α2UG subfamily in which glycodelin is the closest known 'relative' of BLG in the lipocalin phylogenetic tree drawn by these authors.

Interestingly, in the Swiss-Model-deduced 3D structure, the 3D profile of glycodelin shows two regions which may contain unfavourably exposed non-polar surfaces. These regions are apparently located on the same side of the glycodelin molecule. Like some other lipocalins, glycodelin is a homodimeric protein in its native state [1]. Therefore, it is possible that regions with a low 3D score take part in the dimerisation process. This would be expected because in β-lactoglobulin, the corresponding regions form the dimer interface [23].

At a physiological pH, the circular dichroism spectrum of GdS was found to be similar to that of β-lactoglobulin (Fig. 3). This observation is in agreement with a recent report by Pala and co-workers [31] who found that GdA contains significant amounts of the β-sheet, which is almost identical to that of β-lactoglobulin and other lipocalins [1,37].

The apparent similarity of the tertiary and secondary structures of glycodelin and β-lactoglobulin led us to investigate how far their functional similarity might reach. The capacity of glycodelin to bind retinol and retinoic acid was of great interest because β-lactoglobulin (and also some other lipocalins) can bind these so physiologically important retinoids. First, retinol and retinoic acid were added to purified GdA, β-lactoglobulin or buffer alone and the bound ligands were separated by gel filtration from the unbound, then extracted and detected by TLC. However, the added retinoids precipitate in aqueous solutions, hampering separation of unbound retinoids from protein. Therefore, the fluorescence quenching method was used demonstrating that, unlike bovine β-lactoglobulin, GdA does not bind retinol or retinoic acid. The purified GdA was not found to contain any detectable amount of endogenously bound retinoids, steroids or other hydrophobic ligands that would have been detected by the TLC method used. Protein stability measurement by differential scanning calorimetry showed that, at a physiological pH, the thermal stability of the two glycodelins differs significantly from that of β-lactoglobulin (Fig. 5a). Because β-lactoglobulin is irreversibly denatured when the homodimer is dissociated, comparison of the unfolding of the two proteins by quantitative thermodynamic parameters was impossible. Although denaturation of the glycodelin homodimer is reversible, glycodelins appear less stable than β-lactoglobulin against thermal denaturation. Glycodelins reveal more cooperativity of their tertiary structure than β-lactoglobulin does. This means that, despite considerable similarity in the overall folding patterns of glycodelin and β-lactoglobulin, the stabilising contacts in the folded conformations of these proteins must be different. This is also supported by differences in the denaturation behaviour between GdS and β-lactoglobulin, observed at a very low pH (Fig. 5b). Unlike GdS, β-lactoglobulin retains cooperative properties of its tertiary structure down to pH 1.0 and unfolds into a single cooperative folding unit [32]. Compared to its behaviour at neutral pH (Fig. 3), the GdS thermogram lacks any cooperative transitions at a low pH and its secondary structure is also substantially perturbed. Thus, the observed difference in stability and cooperativity of these two proteins [33] reflects a deeper singularity in their native folding patterns than those predicted from their structural similarity. Perhaps this is one of the reasons why GdA does not bind retinol or retinoic acid, while β-lactoglobulin does. Data of Beste et al. [39], who managed to engineer considerable changes in the bilin binding protein binding specificity through randomised mutagenesis of the loop fragments, indicate that binding specificities of various lipocalins are defined by the composition of these fragments of their 3D structure.

From two existing hypotheses on the localisation of the β-lactoglobulin retinol binding site, preponderance of the central cavity was proposed by Bronlow et al. [23] and in case of palmitate binding by Wu et al. [38]. When the modelled glycodelin structure is compared with β-lactoglobulin, there is only 0.25 Å rmsd between α-carbons of the palmitate binding site of β-lactoglobulin described by Wu et al. [38] as compared to corresponding α-carbons of glycodelin. Of these, Val-41, Leu-46, Ile-56, Lys-69, Ile-84 and Leu-103 were conserved. Lys-60, Ile-71, Val-94, Phe-105, Met-107 of BLG were Arg-60, Val-71, Leu-94, Leu-105, Leu-107 in glycodelin, respectively.

An important difference between the structures of β-lactoglobulin and glycodelins is in their glycosylation. Unlike β-lactoglobulin, both GdA and GdS are glycosylated [8,10,34]. Some of the glycans are very unusual for any secreted human protein so far reported and some of these glycans were reported to play a role in the cell adhesion processes [8,10]. Interestingly, in the Swiss-Model-deduced tertiary structure presented here, these glycans are located in a way which would allow them to form a clustered saccharide patch, i.e. sugars from more than one glycosylation site could form a cluster [35]. GdS is identical to GdA in its polypeptide sequence with the same glycosylation sites occupied by completely different glycans. Therefore, these two glycodelins provide an excellent model to study the effect of differential glycosylation on the conformational stability of a given glycoprotein. As derived from high sensitivity differential scanning calorimetry data, the thermodynamic parameters of reversible unfolding of GdA and GdS are very similar. Thus, the unusual glycosylation patterns of GdA and GdS do not affect their folding. Despite the similar folding conformations of the two glycodelin glycoforms, their functions are very different. Unlike GdS, GdA was shown to inhibit human sperm-zona pellucida binding [5,10]. Because GdA and GdS do not appear to contain any hydrophobic ligands and their primary and tertiary polypeptide structures are similar, it is likely that the contraceptive mechanism involves competitive interaction between the GdA-type oligosaccharide moieties and the surface carbohydrates of the oocyte. This type of functional behaviour makes glycodelin different from most other lipocalins, including β-lactoglobulin and retinol binding protein. The latter reveals a 3D binding site, which is conformation-dependent [36].

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References

[1] Flower, D.R. (1996) Biochem. J. 318, 1–14.
[2] Toh, H., Kubodera, H., Nakajima, N., Sekiya, T., Eguchi, N., Tanaka, T., Urade, Y. and Hayaishi, O. (1996) Protein Eng. 9, 1067–1082.
[3] Holzfeind, P. and Redl, B. (1994) Gene 139, 177–183.
[4] Julkunen, M., Seppälä, M. and Jänne, O. (1988) Proc. Natl. Acad. Sci. USA 85, 8845–8849.
[5] Oehninger, S., Coddington, C.C., Hodgen, G.D. and Seppälä, M. (1995) Fertil. Steril. 63, 377–382.
[6] Bolton, A.E., Pockley, A.G., Clough, K.J., Mowles, E.A., Stoker, R.J., Westwood, O.M.R. and Chapman, M.G. (1987) Lancet 1, 593–595.
[7] Okamoto, N., Uchida, A., Takakura, K., Kariya, Y., Kanzaki, H., Riittinen, L., Koistinen, R., Seppälä, M. and Mori, T. (1991) Am. J. Reprod. Immunol. 26, 137–142.
[8] Dell, A., Morris, H.R., Panico, M., Oehninger, S., Patankar, M., Koistinen, R., Koistinen, H., Seppälä, M. and Clark, G.F. (1995) J. Biol. Chem. 270, 24116–24126.
[9] Koistinen, H., Koistinen, R., Dell, A., Morris, H.R., Easton,

R.L., Patankar, M.S., Oehninger, S., Clark, G.F. and Seppälä, M. (1996) Mol. Hum. Reprod. 2, 759–765.
[10] Morris, H.R., Dell, A., Easton, R., Panico, M., Koistinen, H., Koistinen, R., Oehninger, S., Patankar, M.S., Seppälä, M. and Clark, G.F. (1996) J. Biol. Chem. 271, 32159–32167.
[11] Koistinen, H., Koistinen, R., Kämäräinen, M., Salo, J. and Seppälä, M. (1997) Lab. Invest. 76, 683–690.
[12] Garde, J., Bell, S.C. and Eperon, I.C. (1991) Proc. Natl. Acad. Sci. USA 88, 2456–2460.
[13] Morrow, D.M., Xiong, N., Getty, R.R., Ratajczak, M.Z., Morgan, D., Seppälä, M., Riittinen, L., Gewirtz, A.M. and Tykocinski, M.L. (1994) Am. J. Pathol. 145, 1485–1495.
[14] Kämäräinen, M., Seppälä, M., Virtanen, I. and Andersson, L.C. (1997) Lab. Invest. 77, 565–573.
[15] Toma, S., Isnardi, L., Raffo, P., Dastoli, G., De Francisci, E., Riccardi, L., Palumbo, R. and Bollag, W. (1997) Int. J. Cancer 70, 619–627.
[16] Huhtala, M.L., Seppälä, M., Närvänen, A., Palomäki, P., Julkunen, M. and Bohn, H. (1987) Endocrinology 120, 2620–2622.
[17] Dodin, G., Andrieux, M. and al Kabbani, H. (1990) Eur. J. Biochem. 193, 697–700.
[18] Dufour, E., Marden, M.C. and Haertlé, T. (1991) FEBS Lett. 277, 223–226.
[19] Peitsch, M.C. (1995) Bio/Technol. 13, 658–660.
[20] Peitsch, M.C. (1996) Biochem. Soc. Trans. 24, 274–279.
[21] Peitsch, M.C. and Jongeneel, C.V. (1993) Int. Immunol. 5, 233–238.
[22] Lüthy, R., Bowie, J.U. and Eisenberg, D. (1992) Nature 356, 83–85.
[23] Brownlow, S., Cabral, J.H.M., Cooper, R., Flower, D.R., Yewdall, S.J., Polikarpov, I., North, A.C.T. and Sawyer, L. (1997) Structure 5, 481–495.
[24] Holm, L. and Sander, C. (1993) J. Mol. Biol. 233, 123–138.
[25] Riittinen, L., Närvänen, O., Virtanen, I. and Seppälä, M. (1991) J. Immunol. Methods 136, 85–90.
[26] Maillart, P. and Ribadeau-Dumas, B. (1988) J. Food Sci. 33, 743–752.
[27] Glasgow, B.J., Abduragimov, A.R., Farahbakhsh, Z.T., Faull, K.F. and Hubell, W.L. (1995) Curr. Eye Res. 14, 363–372.
[28] Lin, Z.-S., Fong, S.-L. and Bridges, C.D.B. (1990) Methods Enzymol. 189, 325–330.
[29] Dufour, E. and Haertlé, T. (1991) Biochem. Biophys. Acta 1079, 316–320.
[30] Riittinen, L., Julkunen, M., Seppälä, M., Koistinen, R. and Huhtala, M.-L. (1989) Clin. Chim. Acta 184, 19–30.
[31] Pala, A., Padula, F., Barteri, M., Benagiano, M., Gaudiano, M.C., Moro, M. and Benagiano, G. (1997) J. Chromatogr. B 704, 25–34.
[32] Burova, T.V., Choiset, Y., Tran, V. and Haertlé, T. (1998) Protein Eng. 11, 1065–1073.
[33] Murphy, K.P. and Freire, E. (1992) Adv. Protein Chem. 43, 313–361.
[34] Bohn, H., Kraus, W. and Winckler, W. (1982) Placenta 4, 67–81.
[35] Varki, A. (1994) Proc. Natl. Acad. Sci. USA 91, 7390–7397.
[36] Bychkova, V.E., Berni, R., Rossi, G.L., Kutyshenko, V.P. and Ptitsyn, O.B. (1992) Biochemistry 31, 7566–7571.
[37] Sansom, C.E., North, A.C.T. and Sawyer, L. (1994) Biochim. Biophys. Acta 1208, 247–255.
[38] Su-Ying, W., Perez, M.D., Puyol, P. and Sawyer, L. (1999) J. Biol. Chem. 274, 170–174.
[39] Beste, G., Schmidt, F.S., Stibora, T. and Skerra, A. (1999) Proc. Natl. Acad. Sci. USA 96, 1898–1903.