Acta Cryst. (1998). D**54**, 1408–1411

Crystallization of ovine placental lactogen in a 1:2 complex with the extracellular domain of the rat prolactin receptor

Hans W. Christinger,[a] Patricia A. Elkins,[a] Yael Sandowski,[b] Edna Sakal,[b] Arieh Gertler,[b] Anthony A. Kossiakoff[a] and Abraham M. de Vos[a]* *at* [a]*Department of Protein Engineering, Genentech Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080, USA, and* [b]*Institute of Biochemistry, Food Science and Nutrition, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100, Israel. E-mail: devos@gene.com*

(*Received 12 January 1998; accepted 20 April 1998*)

Abstract

Growth hormone and prolactin control somato-lactogenic biology. While high-resolution crystal structures have been determined for receptor complexes of human growth hormone, no such information exists for prolactin. A stable 1:2 complex was formed between ovine placental lactogen, a close prolactin homologue, and two copies of the extracellular portion of the rat prolactin receptor. Using synchrotron radiation, native data have been collected to 2.3 Å. Crystals contain one complex per asymmetric unit. The crystal structure of this complex will shed light on the structural reasons for cross-reactivity and specificity among the endocrine hormones, placental lactogen, prolactin and growth hormone.

1. Introduction

Physiological activity in the endocrine family of hormones is regulated through activation of two receptors, the growth-hormone and prolactin receptors (Kelly *et al.*, 1991). The trigger for this activation process is ligand-induced homodimerization of the receptor, which results in trans-phosphorylation of the JAK2 kinase molecules associated with the receptor cytoplasmic domains. Proper regulation of signaling by the growth-hormone and prolactin receptors involves a delicate balance between specificity and cross-reactivity: whereas the growth-hormone receptor (GHR) can only be activated by growth hormone, prolactin-receptor (PRLR) signaling can be induced by binding of both prolactin and growth hormone.

A comprehensive mutagenesis analysis of human growth hormone (hGH) has identified the residues responsible for tight binding and cross-reactivity (for a review, see Wells & de Vos, 1996). Crystal structures are known for a number of receptors of this family and are listed in Table 1. A comparison between the crystal structures of the 1:1 complexes of hGH bound to the extracellular domain (ECD) of the human GHR (hGHR) and of the human PRLR (hPRLR) revealed significant differences in the orientation of the two receptors with respect to the hormone (Somers *et al.*, 1994; Kossiakoff *et al.*, 1994), resulting in distinct sets of interactions between the cross-reactivity determinants and the receptors. This raised the question as to the manner in which prolactin interacts with the PRLR, but unfortunately it proved impossible to produce high-quality crystals of a complex between the hPRLR ECD and human prolactin.

The crystal structure of the 1:2 complex between hGH and the hGHR ECD provided direct structural evidence for ligand-induced receptor dimerization (de Vos *et al.*, 1992). Mutagenesis studies established that hGH binds first to one hGHR, forming an inactive intermediate complex, and subsequently to a second hGHR, forming an active signaling complex (Fuh *et al.*, 1992). In the active complex, both receptor molecules interact with the ligand, but also form a substantial interface with each other, suggesting that the structural requirements for receptor activation include direct receptor–receptor interactions. A modeling experiment based on the structure of the 1:1 complex between hGH and the hPRLR ECD led to the conclusion that addition of a second hPRLR molecule to form the dimeric signaling state would result in a distinct but also substantial receptor–receptor interface (Kossiakoff *et al.*, 1994). Thus, from the crystal structure of the 1:2 complex between hGH and the hGHR ECD and the modeling experiment on the complex between hGH and the hPRLR ECD, it appeared that receptor–receptor interaction might be a requirement for formation of an active signaling complex. Surprisingly, in the recent structure of an active complex of two erythropoietin-receptor ECDs homodimerized by a dimeric agonist peptide, receptor–receptor interactions are virtually absent (Livnah *et al.*, 1996), despite these erythropoietin receptors being structurally highly homologous to the PRL-receptor and GH-receptor ECDs. It is unclear whether this is an artifact introduced by the non-native ligand (Livnah *et al.*, 1996), or whether active complexes can have highly variable requirements for efficient signaling.

In order to probe the structural requirements for receptor activation and mechanisms of cross-reactivity and specificity, we decided to determine the structure of a close prolactin homologue (about 40% sequence identity to mammalian prolactins), ovine placental lactogen (Jackson-Grusby *et al.*, 1988; Colosi *et al.*, 1989), in complex with two copies of the rat PRLR ECD (rPRLR ECD) (72% sequence identity to hPRLR ECD). In contrast to the transient homodimerization usually observed in prolactin–receptor complexes, these molecules form a stable 1:2 complex (Gertler *et al.*, 1996; Sakal *et al.*, 1997) that can be studied by X-ray crystallographic methods. Comparison of this complex to that between hGH and the hPRLR ECD will show the similarities and differences between the interactions of two different ligands with the same receptor, and enable elucidation of the structural reasons for the narrow specificity of prolactin. This comparison will also reveal whether the structural basis for the cross-reactivity of hGH is a prolactin-like interaction, or whether cross-reactivity is based on a mode of recognition that is distinct from prolactin and unique to the interaction between hGH and the PRLR. Furthermore, this structure will be only the second experimental example of a complex between a cytokine hormone and two homodimerized receptors, shedding further light on the

ISSN 0907-4449

Table 1. *Complexes of receptors in class 1 of the hematopoietic receptor superfamily for which crystal structures are published*

Complex	Reference
Endocrine receptors	
1:1 complex of hGH–hGHR ECD	Clackson *et al.* (1998); Sundstrom *et al.* (1996)
1:1 complex of hGH–hPRLR ECD	Somers *et al.* (1994)
1:2 complex of hGH–hGHR ECD	de Vos *et al.* (1992); Sundstrom *et al.* (1996)
Other receptors	
1:2 complex of peptide–EPOR ECD	Livnah *et al.* (1996)

possible homodimerization states that are compatible with efficient signaling.

2. Materials and methods

2.1. *Purification and complex formation*

Ovine placental lactogen (oPL) and the rPRLR ECD were purified as described previously (Sakal *et al.*, 1997; Sandowski *et al.*, 1995). Briefly, oPL was expressed in *E. coli* BL21 cells and purified from inclusion bodies solubilized in 4.5 *M* urea with 40 m*M* Tris base. The pH was brought to 11.3 and cysteine was added to 0.1 m*M*. After stirring at 277 K for 1 h, the solution was diluted and dialyzed against 10 m*M* Tris–HCl pH 9. The final step was purification on a Q-Sepharose column in the dialyzation buffer with elution by NaCl gradient. The rPRLR ECD was prepared from inclusion bodies from *E. coli* BL21/2 cells induced with IPTG. Protein was extracted from insoluble refractile bodies treated by sonication and solubilized in 4.5 *M* urea with Tris base. The pH was increased to 11.3, cysteine was added and the solution was stirred for 1 h at 277 K. The final step was identical to that used with oPL, except that the pH of the buffer was 8.6. Lyophilized proteins were solubilized in 50 m*M* Tris–HCl pH 7.5, 0.1 *M* NaCl, and dialyzed overnight against 3 volumes of the same buffer at 277 K. oPL and rPRLR ECD were mixed together in a 1:2.1 molar ratio and equilibrated for 30 min. The complex (total molecular weight 69978 Da) was then separated from excess oPL and aggregated receptor by size-exclusion chromatography (S-100, Pharmacia) at 277 K in 50 m*M* Tris–HCl pH 7.5, 0.1 *M* NaCl. Fractions containing the complex, as determined by SDS–PAGE on a 4–20% gradient gel, were pooled and concentrated in a Centricon 10000 (Amicon, Beverly, Massachusetts) such that the absorbence of the protein at a wavelength of 280 nm was 6.5 (corresponding to a concentration of about 9 mg ml^{-1}). The retention time on a size-exclusion column of the resulting complex was identical to that of a 1:2 complex between hGH and the hGHR ECD, showing that the composition of the complex was one molecule of oPL to two molecules of rPRLR ECD (Fig. 1). The complex was stored at 277 K until used in crystallization experiments.

2.2. *Crystallization and X-ray analysis*

All crystallization trials were set up at 294 K as hanging-drop vapor-diffusion experiments on Linbro tissue-culture plates. Initial screening was performed using the sparse-matrix method (Jancarik & Kim, 1991) with commercial crystal-screening kits (Hampton Research).

Crystallographic data were collected at two synchrotron-radiation sources. An initial 2.5 Å data set was collected on a frozen crystal at SSRL beamline 7–1 on a MAR scanner using radiation at a wavelength of 1.08 Å. 181 frames of data were collected in 1° oscillations at a crystal-to-detector distance of 190 mm. This data set was compromised by the presence of an ice ring, which obscured a shell of data between 3.4 and 3.9 Å resolution. A more complete data set to 2.3 Å resolution was collected at CHESS beamline A-1 with an ADSC 1K CCD detector using radiation at a wavelength of 0.908 Å. Using a single frozen crystal, 208 frames were collected in 1° oscillations at a crystal-to-detector distance of 82 mm and 149 frames at a crystal-to-detector distance of 90 mm. Both data sets were processed and scaled with *DENZO* and *SCALEPACK* (Otwinowski, 1993).

A potential mercury derivative was prepared by soaking a crystal for 8 h in a 1 m*M* solution of methylmercury iodide. The crystal was frozen under the same conditions used for the native crystals (below). A data set to 2.65 Å resolution was collected at CHESS using an ADSC 1K CCD detector and radiation at a wavelength of 0.908 Å. 179 frames of data were collected in 1° oscillations at a crystal-to-detector distance of 113 mm, and processed with *DENZO* and *SCALEPACK* (Otwinowski, 1993).

3. Results and discussion

Initial crystallization screens did not produce crystals; however, there were several conditions with PEG that produced crystalline precipitate. Optimization of crystallization parameters resulted in diffraction-quality crystals

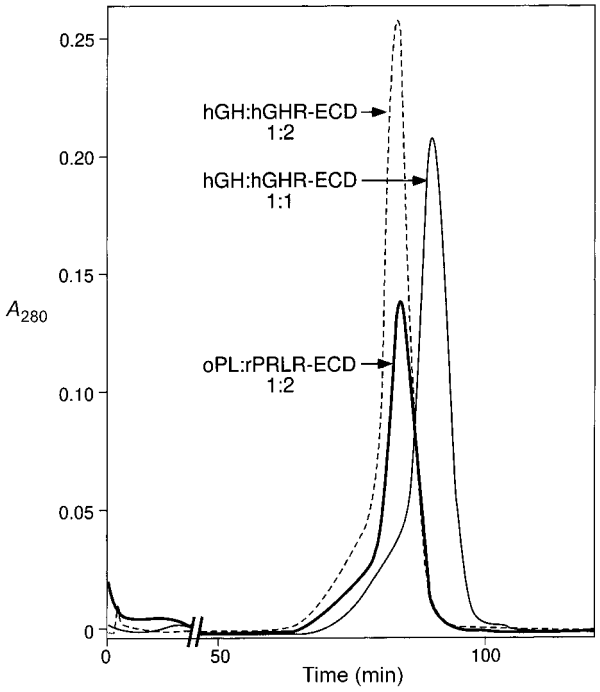

Fig. 1. Size-exclusion chromatography of related hormone-receptor complexes. Complexes were analyzed on tandem S-200 (Pharmacia) columns at a flow rate of 0.35 ml min^{-1} in 50 m*M* Tris–HCl pH 7.5 and 0.1 *M* NaCl. The complex between oPL and the rPRLR ECD is shown in bold, and the 1:1 and 1:2 complexes between hGH and the hGHR ECD in solid and dashed lines, respectively.

grown in drops of a mixture of 8 µl of protein solution with 1 µl of a reservoir solution of 15% PEG 4000, 15% 2-propanol, 1% MPD, 0.1 *M* MES pH 5.6, suspended over 1 ml of the reservoir solution. Long rod-shaped crystals (Fig. 2) appeared in 3–5 days and grew slowly over a 1–2 month period, reaching a size of 0.6 × 0.25 × 0.1 mm. Unfortunately, these conditions were not consistently reproducible, and only about a third of the drops yielded X-ray quality crystals. The concentration of 2-propanol in the mother liquor was adequate to protect the crystals from damage on freezing; however, the crystals proved to have a high mosaicity unless all manipulations were performed in a glove box saturated with 2-propanol to maintain the 2-propanol concentration in open drops. In all crystallization trials, a viscous film developed covering the surface of the drop, which was probably the result of denatured protein or of excess receptor dimers not removed during purification. Removal of the film from the crystals was difficult but essential, because its presence tended to result in ice rings in the diffraction pattern. Crystal handling was further complicated by the saturated 2-propanol atmosphere of the glove box. If the crystal manipulations were not sufficiently rapid, the mosaicity of the frozen crystals increased significantly, perhaps as a result of increased 2-propanol concentrations in the drop.

The crystals diffract beyond 2.1 Å and belong to space group *C*2 with cell parameters a = 168.2, b = 63.1, c = 88.4 Å and β = 118.6°. The crystals have a V_m (Matthews, 1968) of 2.6 Å^3 Da^{-1}, which is consistent with one 1:2 complex per asymmetric unit and a solvent content of 53%. Data statistics for both the low- and high-resolution data sets are listed in Table 2.

The extracellular domain of the rat prolactin receptor contains one free cysteine residue, which could be exploited in preparing a heavy-atom derivative with a mercury compound. A non-isomorphous derivative resulted from soaking a crystal for 8 h in a 1 m*M* solution of methylmercury iodide. Statistics describing this data set are shown in Table 2. Even though the *c* axis of this crystal had changed by over 3%, a difference Patterson map calculated using this derivative and the high-resolution native data set clearly revealed one heavy-atom site per PRLR ECD. The severe non-isomorphism of this data set has precluded direct heavy-atom phasing attempts, but will allow phase improvement by cross-crystal averaging after initial phases have been obtained. We are now attempting to solve the structure with molecular-replacement methods, using the structure of the 1:1 complex between hGH and the hPRLR ECD (Somers *et al.*, 1994) as a model.

Table 2. *Crystal parameters and data statistics*

	Native 1 (SSRL)	Native 2 (CHESS)	Hg derivative
Space group	*C*2	*C*2	*C*2
Unit-cell parameters			
a (Å)	168.2	168.2	168.5
b (Å)	63.1	63.1	61.8
c (Å)	88.4	88.4	85.5
β (°)	118.6	118.6	118.6
Measured reflections	83014	92119	75164
Unique reflections	23560	33034	21200
Resolution (Å)	2.50 (2.59–2.50)†	2.30 (2.38–2.30)	2.65 (2.74–2.65)
Completeness (%)	85.5 (84.9)	90.7 (70.4)	97.2 (76.5)
$R_{\rm merge}$‡	0.072 (0.35)	0.060 (0.13)	0.041 (0.065)
Average $I/\sigma(I)$	18.7 (2.9)	16.2 (4.6)	20.6 (10.7)
$R_{\rm scal}$§			0.374

† Values in parentheses refer to the highest resolution shell. ‡ $R_{\rm merge} = \sum_{hkl} |I - \langle I_{hkl}\rangle| / \sum_{hkl} \langle I_{hkl}\rangle$, where the average is over all symmetry-related observations of reflection *hkl*, and the summation is over all unique reflections. § $R_{\rm scal}$ is the R value between the Native 2 and the Hg-derivative data sets. $R_{\rm scal} = \sum_{hkl} |F_{\rm PH} - F_P| / \sum_{hkl} F_P$, where $F_{\rm PH}$ and F_μ are structure-factor amplitudes of the derivative and native data sets, respectively.

We thank the staff at SSRL and at CHESS for help with beamlines 7–1 and A-1, respectively; Mike Randal, Charles Eigenbrot, Nancy Gerber, Yves Muller and Christian Wiesmann for help with data collection and Wayne Anstine for help with the figures. Part of this research was supported by the USA–Israel Binational Science Foundation, grant No. 9500327.

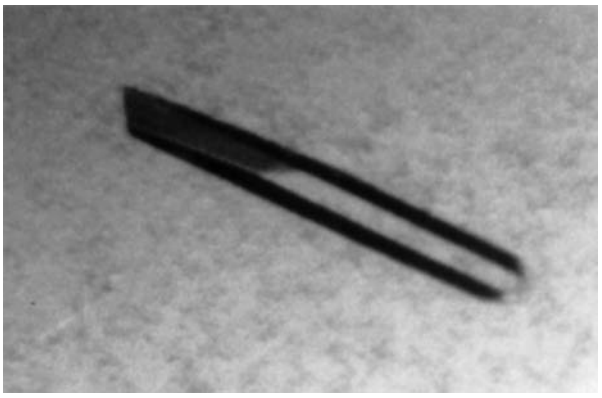

Fig. 2. Crystal of the complex of ovine placental lactogen with two copies of the extracellular domain of the rat prolactin receptor. The crystal shown has dimensions of approximately 0.050 × 0.075 × 0.250 mm.

References

Clackson, T., Ultsch, M. H., Wells, J. A. & de Vos, A. M. (1998). *J. Mol. Biol.* **277**, 1111–1118.
Colosi, P., Thordarson, G., Helmiss, R., Singh, K., Forsyth, I. A., Gluckman, P. & Wood, W. I. (1989). *Mol. Endocrinol.* **3**, 1462–1469.
Fuh, G., Cunningham, B. C., Fukunaga, R., Nagata, S., Goeddel, D. V. & Wells, J. A. (1992). *Science*, **256**, 1677–1680.
Gertler, A., Grosclaude, J., Strasburger, C. J., Nir, S. & Djiane, J. (1996). *J. Biol. Chem.* **271**, 24482–24491.
Jackson-Grusby, L. L., Pravtcheva, D., Ruddle, F. H. & Linzer, D. (1988). *Endocrinology*, **122**, 2462–2466.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Kelly, P. A., Djiane, J., Postel-Vinay, M. C. & Edery, M. (1991). *Endocr. Rev.* **12**, 235–251.
Kossiakoff, A. A., Somers, W., Ultsch, M., Andow, K., Muller, Y. A. & de Vos, A. M. (1994). *Protein Sci.* **3**, 1697–1705.
Livnah, O., Stura, E. A., Johnson, D. L., Middleton, S. A., Mulcahy, L. S., Wrighton, N. C., Dower, W. J., Jolliffe, L. K. & Wilson, I. A. (1996). *Science*, **273**, 464–471.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Otwinowski, Z. (1993). *Proceedings of the CCP4 Study Weekend*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 55–62. Warrington: Daresbury Laboratory.

Sakal, E., Bignon, C., Grosclaude, J., Kantor, A., Shapira, R., Leibovitch, H., Helman, D., Nespoulous, C., Shamay, A., Rowlinson, S. W., Djiane, J. & Gertler, A. (1997). *J. Endocrinol.* **152**, 317–327.

Sandowski, Y., Nagano, M., Bignon, C., Djiane, J., Kelly, P. A. & Gertler, A. (1995). *Mol. Cell. Endocrinol.* **115**, 1–11.

Somers, W., Ultsch, M., de Vos, A. M. & Kossiakoff, A. A. (1994). *Nature (London)*, **372**, 478–481.

Sundstrom, M., Lundqvist, T., Rodin, J., Giebel, L. B., Milligan, D. & Norstedt, G. (1996). *J. Biol. Chem.* **271**, 32197–32203.

Vos, A. M. de, Ultsch, M. & Kossiakoff, A. A. (1992). *Science*, **255**, 306–312.

Wells, J. A. & de Vos, A. M. (1996). *Annu. Rev. Biochem.* **65**, 609–634.