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Crystallization of the receptor-binding domain of parathyroid hormone-related protein in complex with a neutralizing monoclonal antibody Fab fragment

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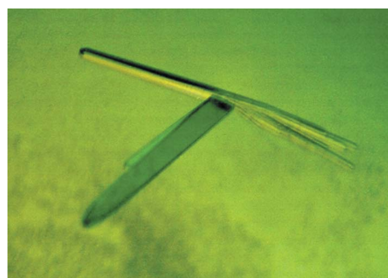

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Parathyroid hormone-related protein (PTHrP) plays an important role in regulating embryonic skeletal development and is abnormally regulated in the pathogenesis of skeletal complications observed with many cancers and osteoporosis. It exerts its action through binding to a G-protein-coupled seven-transmembrane cell-surface receptor (GPCR). Structurally, GPCRs are very difficult to study by X-ray crystallography. In this study, a monoclonal antibody Fab fragment which recognizes the same region of PTHrP as its receptor, PTH1R, was used to aid in the crystallization of PTHrP. The resultant protein complex was crystallized using the hanging-drop vapour-diffusion method with polyethylene glycol as a precipitant. The crystals belonged to the orthorhombic space group $P2_12_12$, with unit-cell parameters $a = 72.6$, $b = 96.3$, $c = 88.5$ Å, and diffracted to 2.0 Å resolution using synchrotron radiation. The crystal structure will shed light on the nature of the key residues of PTHrP that interact with the antibody and will provide insights into how the antibody is able to discriminate between PTHrP and the related molecule parathyroid homone.

1. Introduction

Parathyroid hormone-related protein (PTHrP) was originally identified as the causative factor for humoral hypercalcaemia of malignancy, a condition in which patients with various cancers have elevated levels of both serum calcium and urinary cAMP despite having low levels of parathyroid hormone (PTH), the key regulator of blood calcium levels (Stewart *et al.*, 1980; Kemp *et al.*, 1987; Moseley *et al.*, 1987; Suva *et al.*, 1987). Under physiological conditions, PTHrP is produced locally in a wide variety of tissues, where it acts as a paracrine or autocrine factor to regulate cellular and organ growth, development, migration, differentiation, survival and apoptosis, and calcium transport. For example, PTHrP regulates chrondrocyte growth and differentiation in the growth plates of developing long bones, stimulates branching morphogenesis in the mammary gland and is produced by both the mammary gland and placenta to promote calcium mobilization during normal homeostasis and foetal growth and development (reviewed in Gensure *et al.*, 2005).

PTHrP is a secreted polypeptide growth factor that can exist as three distinct isoforms of 141, 139 or 173 amino acids as a result of alternative mRNA splicing (Brandt *et al.*, 1994). PTHrP can also be regarded as a polyhormone since a family of peptides are generated by specific post-translational proteolysis, yielding an N-terminal receptor-binding region, a mid-region peptide and a C-terminal peptide with PTH-independent activities in placenta and bone.

Eight of the first 13 N-terminal amino-acid residues of PTHrP and PTH are identical and these residues form part of the N-terminal 34-amino-acid receptor-binding region which binds to and activates a class B seven-transmembrane G-protein-coupled receptor, PTH1R, that is shared by both hormones (Jüppner *et al.*, 1991). This receptor couples with the adenylate cyclase–protein kinase A and phospholipase C–protein kinase C signalling pathways. Studies with various PTH/PTHrP 1–34 peptide variants and receptor/ligand photo-cross-linking suggest that residues 1–14 bind to the transmembrane domain with low affinity (Luck *et al.*, 1999), while residues 15–34 bind to the N-terminal extracellular domain of the receptor to confer high affinity and specificity to the receptor (Dean *et al.*, 2006).

Numerous attempts to produce crystals of PTHrP have been made by our group and others over the years. NMR and secondary-structure prediction studies suggest that the PTHrP molecule has highly flexible regions that may impede the crystallization process. To determine the molecular detail of the receptor-binding domain of PTHrP, a neutralizing monoclonal antibody Fab fragment that recognizes the PTH1R-binding site on PTHrP was complexed with a PTHrP peptide ($PTHrP_{1-108}$) and subjected to crystallization trials. The crystals reported here will form the basis for understanding how PTHrP activity is neutralized by the antibody and provide insights into how the antibody is able to discriminate between PTHrP and PTH.

2. Experimental procedures and results

2.1. Fab production, purification and complex formation

The mouse anti-human PTHrP antibody was developed against a synthetic peptide directed to the first 34 residues of PTHrP. The resultant hybridoma #23-57-137-1 was established as a monoclonal cell line by Mitsubishi Kagaku BCL (Japan). The hybridoma cells were transplanted into the peritoneal cavity of Balb/c mice to produce ascites and the antibody present was purified by Protein A-Sepharose affinity chromatography (GE Biosciences, Uppsala, Sweden). Fab fragments were prepared from the monoclonal antibody using immobilized papain (Pierce, Rockford, USA). Briefly, 25 mg antibody was prepared at a concentration of 10 mg ml^{-1} in 10 m*M* phosphate buffer pH 7.4 containing 150 m*M* NaCl (PBS) containing 10 m*M* EDTA and 25 m*M* cysteine–HCl pH 7.0. Immobilized papain beads were added at an enzyme:antibody ratio of 1:50 and digestion was performed overnight at 295 K. The immobilized papain beads were removed by centrifugation and the Fab fragment was separated from residual intact antibody and Fc fragments by affinity chromatography on a 5 ml Protein-A agarose column (Pierce, Rockford, Illinois, USA) previously equilibrated in PBS. The Fab fragment eluted in the Protein-A agarose column flowthrough was concentrated using a Centricon-10 centrifugal concentrator (Millipore, Massachusetts, USA) and further purified by size-exclusion chromatography using a Superdex 75 prep-grade 2.6 × 60 cm column (GE Biosciences, Uppsala, Sweden) equilibrated in PBS.

Recombinant human PTHrP consisting of residues 1–108 ($PTHrP_{1-108}$) was expressed and purified from *Escherichia coli* as described previously (Hammonds *et al.*, 1989). A fivefold molar excess of $PTHrP_{1-108}$ was mixed with the Fab fragment and incubated at room temperature for 2 h. The $PTHrP_{1-108}$–Fab complex was separated from excess $PTHrP_{1-108}$ by size-exclusion chromatography using a Superdex 75 1.0 × 30 cm column (GE Biosciences, Uppsala, Sweden) previously equilibrated in 20 m*M* MES buffer pH 6.0 containing 150 m*M* sodium chloride, 0.02%(*v*/*v*) Tween-20 and 0.02%(*w*/*v*) sodium azide. The complex was further concentrated and buffer-exchanged into 10 m*M* MES buffer pH 6.0 containing 0.02%(*w*/*v*) sodium azide using a Centricon-10 centrifugal concentrator. The final protein concentration of the complex was 5 mg ml^{-1} as determined by SDS–PAGE analysis.

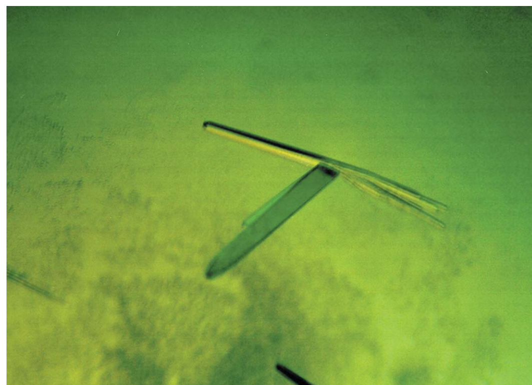

Figure 1
Crystals of the $PTHrP_{1-108}$–Fab complex grown at 295 K in 100 m*M* Tris–HCl pH 8.7 containing 38%(*v*/*v*) PEG 400. The maximum dimensions of a typical crystal are 0.16 × 0.06 × 0.06 mm.

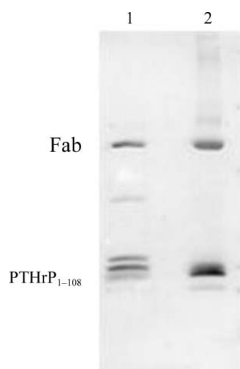

Figure 2
SDS–PAGE analysis of the $PTHrP_{1-108}$–Fab complex (10–20% polyacrylamide gradient gel, samples electrophoresed under nonreducing conditions, protein bands visualized by silver staining; lane 1) and the resulting protein crystals (lane 2).

2.2. Protein crystallization and data collection

Crystallization trials of the $PTHrP_{1-108}$–Fab complex were performed at 295 K using the hanging-drop vapour-diffusion method by

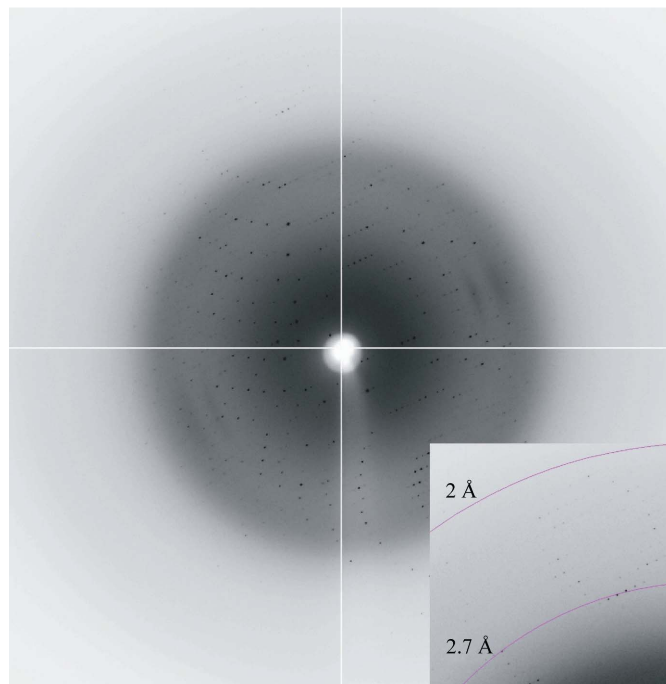

Figure 3
A representative 0.5° oscillation image of data collected from a crystal of the $PTHrP_{1-108}$–Fab complex using synchrotron radiation. The image was collected using an ADSC Q4 detector from a crystal frozen at 100 K on BioCARS beamline 14-BM-C, Advanced Photon Source, Chicago, USA.

Table 1
Crystal data and X-ray diffraction data-collection statistics.

Values in parentheses are for the highest resolution bin (approximate interval of 0.1 Å).

X-ray source	Beamline 14-BM-C, APS (Chicago, USA)
X-ray wavelength (Å)	0.99988
Temperature (K)	100
Space group	$P2_12_12$
Unit-cell parameters	
a (Å)	72.6
b (Å)	96.3
c (Å)	88.5
Maximum resolution (Å)	2.0 (2.0–2.07)
Total observations	331990
Unique reflections used	42014
Redundancy	7.8 (7.5)
Data completeness (%)	100 (100)
R_{merge}† (%)	8.3 (69.6)
Mean $I/\sigma(I)$	27.8 (4.1)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observation of reflection *hkl* and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations *i* of reflection *hkl*.

mixing 2 µl protein solution (protein concentration 5–7 mg ml^{-1}) with an equal volume of reservoir solution and equilibrating against 1.0 ml reservoir solution. The initial crystallization conditions were established using the crystallization screens described by Jancarik & Kim (1991) and Cudney *et al.* (1994). Protein crystals of the complex were obtained using a reservoir solution of 100 m*M* Tris–HCl buffer pH 8.7 containing 38%(*v*/*v*) polyethylene glycol 400. The crystals reached maximum dimensions of 0.16 × 0.06 × 0.06 mm over 10 d (Fig. 1). Crystals were washed, dissolved and precipitated with trichloroacetic acid and the sample was analyzed by SDS–PAGE, confirming that the crystals consisted of the $PTHrP_{1-108}$–Fab complex (Fig. 2). The triplet band observed on SDS–PAGE for the $PTHrP_{1-108}$ in the complex arised from proteolysis, to which the C-terminal region of $PTHrP_{1-108}$ is very susceptible. Importantly, the crystals of the complex have been enriched for a single species of $PTHrP_{1-108}$.

A complete X-ray diffraction data set was collected from a single crystal of the complex flash-frozen to 100 K with no additional cryoprotectant. The data were measured using an ADSC Q4 detector on the BioCARS beamline 14-BM-C (Advanced Photon Source, Chicago, USA). The data set extended to 2.0 Å resolution (Fig. 3). The crystals were found to belong to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 72.6, b = 96.3, c = 88.5 Å. The images were processed and scaled using the *HKL* program package (Otwinowski & Minor, 1997) and the statistics are shown in Table 1. A total of 42 014 unique reflections were measured with an average redundancy of 7.8. The merged data set is 100% complete to 2.0 Å resolution with an R_{merge} of 8.3% and a mean $I/\sigma(I)$ of 27.8 for all reflections and of 4.1 in the highest resolution bin. The calculated Matthews coefficient (V_M) of 2.6 Å^3 Da^{-1} suggested the presence of one $PTHrP_{1-108}$–Fab complex in the asymmetric unit, with a solvent content of about 52.2% (Matthews, 1968; Collaborative Computational Project, Number 4, 1994).

The availability of the crystal structure of $PTHrP_{1-108}$ in complex with the Fab fragment will provide important information as to how the antibody discriminates between PTH and PTHrP and this information will be used to develop novel agonists and antagonists against PTH1R.

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