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Crystallization and preliminary X-ray analysis of recombinant human transforming growth factor β2

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Recombinant human transforming growth factor β2 (TGF-β2) was cloned and expressed in *E. coli*. The protein was isolated from inclusion bodies, renatured and purified to a single component as judged by reversed-phase HPLC. The recombinant TGF-β2 was shown to have a biological activity equal to that of native TGF-β2 in a fibroblast migration assay. Pure, active recombinant TGF-β2 has been crystallized from polyethylene glycol 400. The trigonal crystals of spacegroup $P3_121$ or $P3_221$ have unit cell dimensions of $a=b=60.6$ Å, $c=75.2$ Å and diffract beyond 2.0 Å.

Transforming growth factor β2; Protein crystallization; Crystallography

1. INTRODUCTION

Transforming growth factor type-β (TGF-β) refers to a family of structurally conserved polypeptides which regulate a wide range of growth and differentiation processes. In addition to the five currently described TGF-β isoforms, which are highly conserved throughout different species, there also exist a number of other related proteins which show a widespread evolutionary distribution and which appear to play important roles during development [1,2].

TGF-β2, the second reported TGF-β isoform, has been purified from various natural sources including porcine platelets [3], human adenocarcinoma [4,5], human glioblastoma [6], simian epithelial kidney cells [7], bovine bone [8,9] and bovine milk [10,11]. TGF-β2 has a molecular weight of approximately 25 kDa and consists of two identical polypeptide chains covalently linked by one or more disulfide bonds [4]. TGF-β2 has been shown to have important roles in mouse embryogenesis [12], wound healing (Cox, D.A. et al., personal communication), bone formation [13], as well as in modulation of immune functions [6]. To date no studies have reported successful crystallization of any member protein of the TGF-β superfamily. Recent developments in expression and refolding from *E. coli* have now provided recombinant material of TGF-β2 to make such studies possible. The present work describes the crystallization and preliminary X-ray analysis of recombinant human TGF-β2.

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2. MATERIALS AND METHODS

2.1. *Expression, isolation and purification*

Recombinant human TGF-β2 was prepared and assayed for biological activity as will be described (Cerletti, N. et al., in preparation). Briefly, TGF-β2 cDNA encoding the mature 112 amino acid polypeptide was cloned into the expression plasmid, pPLMu via the *NcoI*/*SalI* restriction sites. Plasmid pPLMu is pPLMuSMCori (obtained from Biogen S.A., Cambridge, MA) [14] with the pUC8 polycloning site between the *NcoI* site which codes for the initiating AUG and the *BamHI* site. Plasmid pPLMuhTGF-β2 was transformed into the *E. coli* strain, SG936 (obtained from F. Goldberg), harbouring plasmid pCI_{857}, which codes for the thermolabile bacteriophage lambda c1 repressor and confers resistance to kanamycin [15]. The transformed cells were grown at 30°C to OD_{550} 1.0, whereafter the culture was heat-shocked to 42°C and grown for 5 h at this temperature. Monomeric denatured TGF-β2 was solubilized from inclusion bodies under acid conditions (pH 2.5) and purified by size-exclusion chromatography. Refolding into the biologically active, dimeric form was then performed at pH 8.0 in the presence of a redox system and the non-denaturing detergent 3-[(3-cholamidopropyl)-dimethylammonio]-propanesulfonate (CHAPS) [16]. Correctly folded dimeric TGF-β2 was separated from residual monomeric protein by cation-exchange chromatography and reversed-phase HPLC. The recombinant dimeric TGF-β2 was eluted as a single homogeneous peak which had the same retention time as the natural reference protein, and was shown to have the correct amino acid sequence. Biological activity of the recombinant protein was shown to be identical to that of natural protein in a previously described in vitro bioassay [10], which measures the level of BALB/c 3T3 fibroblast migration in response to natural and recombinant TGF-β2 (Fig. 1).

2.2. *Crystallization*

Crystals were grown using the hanging drop method [17] at room temperature. Lyophilized TGF-β2 was dissolved in 10 mM sodium acetate buffer at pH 3.0 (protein concentration 5 mg/ml). The drop, typically 2 μl of protein solution and 2 μl of reservoir solution, was equilibrated against a buffer consisting of 0.1 M sodium acetate buffer, pH 4.5–5.0, and 25–35% (w/v) polyethylene glycol 400 (PEG 400).

2.3. *Heavy atom derivative preparation*

For data collection, as well as for heavy atom derivative soaks,

crystals were transferred from the crystallization drop to a stabilizing solution consisting of 30–33% PEG 400 in 0.1 M sodium acetate buffer, pH 4.8. Heavy atom derivatives were prepared by soaking recombinant human TGF-β2 crystals in this stabilizing solution to which the appropriate heavy atom compound had been added.

2.4. *Crystal density measurements*

TGF-β2 crystals, from which all surrounding mother liquor had been removed by pipetting, were placed into a mixture of bromobenzene and xylene of known density [18]. The ratio of the two compounds was adjusted until the crystal remained suspended at the same place. The V_m was calculated as described by Matthews [19].

2.5. *X-Ray diffraction measurements*

X-Ray diffraction data were collected using a FAST area detector (Enraf-Nonius, Delft, The Netherlands). Graphite-monochromated Cu$K\alpha$ radiation was provided by an FR571 X-ray generator operated at 40 kV and 70 mA with an apparent focal spot of 0.3 × 0.3 mm^2. The capillary was mounted on a 4-circle κ goniostat and was rotated 90° about the ω-axis about an arbitrary crystal axis in steps of 0.1°/frame with an exposure time of 60 s/frame. The 2θ swing-out angle for the detector was set at −10° for data collection to 2.4 Å resolution or to −20° for collection to 1.8 Å resolution, with a crystal-to-detector distance of 50 mm. Crystal orientation determination, data collection and on-line data evaluation were performed using the program MADNES [20]. The measured intensity data were profile-fitted and scaled together using the program PROCOR [21]. Data reduction and merging were performed using the program package CCP4 (Daresbury Laboratory).

3. RESULTS

Optimal crystallization of recombinant human TGF-β2 occurred at pH 4.8 and 30% PEG 400 resulting in crystals of 0.2 × 0.2 × 0.15 mm^3 size that diffracted to beyond 2.0 Å. The space group was determined to be $P3_121$ or the enantiomorph $P3_221$. Systematic absences for the 001 reflections $1 \neq 3$ n indicated a 3-fold screw axis along c. The hk0 reflections showed clearly the 2-fold axis perpendicular to the c-axis. The unit cell edges were determined to be $a=b=60.6$ Å, $c=75.2$ Å. There is either one molecule (V_m = 3.13 Å^3/Da) or two (V_m=1.57 Å^3/Da) per asymmetric unit. Crystal density measurements gave a density of 1.07 g/cm^3 implying a V_m of about 3.2 Å^3/Da (data not shown). The first crystal, which was measured to 2.4 Å, provided a total of 29,107 reflections which were reduced and merged to 6,560 unique reflections (97.4% completeness in the last resolution range from 2.53–2.4 Å) with an R_{merge}, defined as $\Sigma(|I-\{I\}|)/\Sigma I$, of 0.094. A second crystal, measured to 1.8 Å, provided a total of 36,217 reflections which were reduced and merged to 14,995 unique reflections (94.5% completeness in the last resolution range from 1.9–1.8 Å) with an R_{merge} of 0.106. Potential heavy atom derivatives were prepared with different mercury, platinum, gold and uranyl compounds. Several derivative data sets were collected. Evaluation of heavy atom derivative sites and structure solution are currently under way.

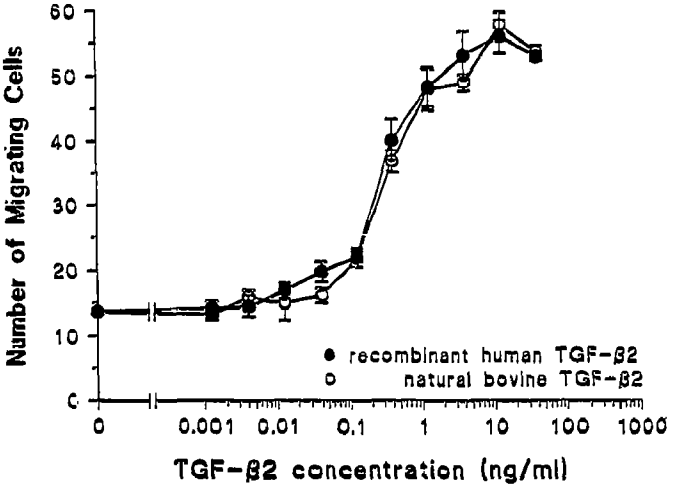

Fig. 1. Stimulation of BALB/c 3T3 fibroblast migration. Wounded confluent monolayers of BALB/c 3T3 fibroblasts were incubated for 22 h in serum-free medium containing varying amounts of either, (a) refolded and purified recombinant human TGF-β2 (solid circles), or (b) natural bovine TGF-β2 isolated from milk (open circles). Values represent the number of cells that have migrated across 1.2 mm of wound edge (one optical field of vision) at each concentration of TGF-β2. Each value represents the mean ± S.E.M. of six determinations from duplicate cultures.

4. DISCUSSION

The high-diffraction quality of the trigonal TGF-β2 crystals provides a good basis for the preparation of suitable heavy atom derivatives that should finally yield a complete structure of the TGF-β2 molecule. Considering the similarities between members of the TGF-β family, such a model may well be the basis for studies of other TGF-β isoforms and related proteins of the TGF-β family. These include Mullerian inhibitory substance (MIS) [22] which causes regression of the Mullerian duct during development of the male embryo; the inhibins/activins [23], proteins which regulate FSH secretion by pituitary cells; the decapentaplegic gene product dpp of *Drosophila* [24,25] which influences dorsoventral specification and morphogenesis of the imaginal discs; the Vg-1 gene product of *Xenopus* [26,27] which localizes to the vegetal pole of the egg; Vgr-1, the mammalian homologue of Vg-1 [28] which is expressed during mouse embryogenesis; OP-1, the predicted human homologue of a major component of bovine osteogenic protein [29]; GDF-1 [30] another Vg-1 related molecule which is expressed in early mouse embryos, and the bone morphogenetic proteins (BMPs) [31,32] which can induce de novo cartilage and bone formation. Besides their partial amino acid sequence homology to the TGF-β isoforms an outstanding feature of all these proteins is the strong positional conservation of cysteine residues, suggesting that these molecules may have similar 3D structures. Resolution of their structures will give useful insights into a better understanding of the actions and the biological roles of these important molecules.

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