

Crystallization and preliminary X-ray analysis of leukemia inhibitory factor

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Leukemia inhibitory factor (LIF) is a polyfunctional molecule with significant and diverse biological activities. LIF is a glycoprotein secreted by a number of different cell types *in vitro*. It is induced in fibroblasts, lymphocytes, monocytes and astrocytes by various inducers such as serum, TNF, interleukin-IP and EGF. Due to extensive and variable glycosylation the molecular weight can range from 38 to 67 kDA. The biological functions of LIF are mediated through a receptor and a signal transducer, gp130, which is also used by factors like interleukin-6 (IL-6), ciliary neurotropic factor (CNTF), and oncostatin M (OSM). Here, we report the crystallization of the non-glycosylated human-like LIF expressed in *E. coli*. The present crystals diffract to 2.0 Å using synchrotron radiation. They belong to the monoclinic space group C2, and the cell dimensions are $a = 61.5$ Å, $b = 45.3$ Å, $c = 77.7$ Å and $\beta = 112.3^\circ$.

Cytokine; Isoelectric focusing; Crystal; Synchrotron radiation

1. INTRODUCTION

Leukemia inhibitory factor (LIF), also known as differentiation-inducing factor (Dfactor), human interleukin for DA cells (HILDA), cholinergic neuronal differentiation factor (CDF), hepatocyte-stimulating factor III (HSF III), is a polyfunctional molecule [1]. LIF is a pluripotent glycoprotein with important regulatory physiological activities (for reviews, see [2–4]). These include its ability to induce maturation of murine and human leukemia cells, to direct phenotype expression in neuronal tissue, to induce changes in calcium metabolism, to promote acute phase protein synthesis in hepatocytes, and to increase platelet numbers.

LIF is produced by human epithelial cells. Cell lines such as Kreb's ascites, Ehrlich ascites, STO, L929, buffalo rat liver, H4 hepatoma, bladder cell carcinoma, osteoblastoma and lung adenocarcinomas produce LIF in detectable amounts. It is induced by mitogens in spleen cells, and in alloreactive T lymphocytes upon antigen binding. During embryonic development, LIF is expressed in blastocysts, egg cylinders, and may play a crucial role in the implantation process [5,6]. LIF is a generalized stem cell factor as many of its actions appear to be mediated via stem cells or progenitor cells rather than by a direct effect on differentiated tissues [2–5]. Receptors for LIF are present on cells of monocytic and macrophagic series, on embryonic stem cells,

embryonic carcinoma cells, on a majority of lymphocytes, myoblast, hepatocytes, osteoblasts, adipocytes and adrenal cortical cells. Some effects of LIF on these cells appear to be similar to the action of interleukin-6 (IL-6) [7]: both trigger the immediate early response upon induction of differentiation of murine leukemic cells [8]. However, the differentiation inhibitory effect of LIF on embryonic stem cells is not shared by IL-6.

Although there is no overall amino acid sequence homology between LIF, IL-6, and other cytokines which use gp130 for signal transduction (IL-11, oncostatin M, ciliary neurotropic factor [9]), they have a number of biological effects in common. Therefore, it is speculated that these cytokines may have similar tertiary structures [10]. We have crystallized recombinant human LIF as a prerequisite of the structure analysis. The three-dimensional structure of LIF should help understand the relationship of its structure and various functions.

2. MATERIALS AND METHODS

2.1. Crystallization of LIF

A chemically synthesised gene for human LIF was expressed in *E. coli* under the heat-inducible pL promotor. LIF protein was purified from inclusion bodies using multiple chromatography steps [11]. This preparation of endotoxin-free recombinant human LIF has been found to be active in a number of biological assays [12–14]. Purified LIF was suspended in PBS and frozen in aliquots at -80°C . For the crystallization experiments, LIF was dialyzed extensively against water and lyophilized. Crystals of LIF were obtained by sitting drop vapour diffusion [15]. For the crystallization experiments, a concen-

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tration of 10 mg/ml was used. Crystallization set-ups were performed at 16°C using 200 mM sodium acetate, 100 mM cacodylate buffer, pH 6.0, with 30% w/v polyethylene glycol 8000 as precipitant. Plate-like crystals grew within about 20 days.

2.2. Isoelectric focusing

Gels ($25 \times 10 \times 0.05$ cm³) were prepared from 5% T/3% C acrylamide and 0.2 M Immobiline solutions (Pharmacia). The mixtures of Immobiline species were calculated as in [16] to give a linear gradient from pH 6 to 11. Gels were equilibrated for 30 min in 2% glycerol, dried in air and re-swollen in 8 M urea containing 0.1% Tween 20 and 0.5% ampholine (pH 3–10; Pharmacia). Protein samples were applied in surface wells of the gradient gel mounted on a LKB multipore apparatus. Focusing was performed for 17 h at 5,000 V after an initial period of 2 h at 500 V. Staining was carried out in Coomassie brilliant blue in the presence of Cu²⁺ [16].

3. RESULTS AND DISCUSSION

The purity of recombinant LIF was assessed by polyacrylamide gel electrophoresis. Fig. 1A shows one single protein band migrating to 21 kDa which is in accordance with the molecular size of the LIF protein core derived from the cDNA sequence. Incomplete recombinant expression or proteolytic degradation can thus be excluded. Isoelectric focusing of LIF preparations, however, revealed a considerable charge heterogeneity: about 6 major and some minor components in the range between pH 8 and 10 were separated on immobilized pH gradients (Fig. 1B). Charge heterogeneity is not uncommon for preparations of 'pure' proteins, since post-translational modifications like deamidation or oxidation of thiol residues might have occurred. In spite of the presence of multiple LIF species crystallization of this preparation was possible. The sitting drop vapour diffusion technique yielded crystals of up to 0.3 mm diameter (Fig. 2) which grew in the form of plates and tended to stick together. Inspection of the crystals

under the microscope suggested a major component with one or more small satellites (or twins). As the crystals were small, it was not possible to separate the individual sections for diffraction experiments. For X-ray studies a crystal with a small satellite was mounted in a glass capillary. The dimensions of the crystal are $0.3 \times 0.3 \times 0.1$ mm³. This crystal was investigated using synchrotron radiation from the wiggler station D32 at the storage ring DCI, LURE, Orsay [17], using an EMBL Image Plate Scanner. The storage ring was operating at 1.85 GeV and about 310 mA. The diffraction extended to about 2.0 Å resolution. From one sample, native data to 3.5 Å were collected. The crystal-to-detector distance was 350 mm. At a wavelength of 0.9 Å, 34 images were collected with a rotation range of $\Delta\phi$ 3.0° per image. The pattern showed one major lattice accompanied by some weak reflections from a smaller satellite (Fig. 2). The cell dimensions were preliminarily assigned using the program MOSFLM [18]. The space group was assigned to be C2 with unit cell dimensions $a = 61.5$ Å, $b = 45.3$ Å, $c = 77.7$ Å and $\beta = 112.3^\circ$. These yield a unit cell volume of 2.0×10^6 Å³ and a packing density parameter, V_M , of 2.5 Å³/Da assuming 1 molecule in the asymmetric unit [19]. The lattice is pseudo I222. The data were integrated using the program DENZO [20]. The completeness of the native data is 99.4% and the R_{sym} (I) is 3.5%. Attempts to solve the structure using molecular replacement with the model of G colony stimulating factor from [21] are in progress and, in parallel, a heavy atom search has been initiated. Also, single LIF isoforms of discrete pI values will be purified in novel multicompartiment electrolyzer with isoelectric membranes [16]. The limiting size and quality of LIF crystals obtained so far may be due to charge heterogeneity of the protein which is known to impair the crystallization

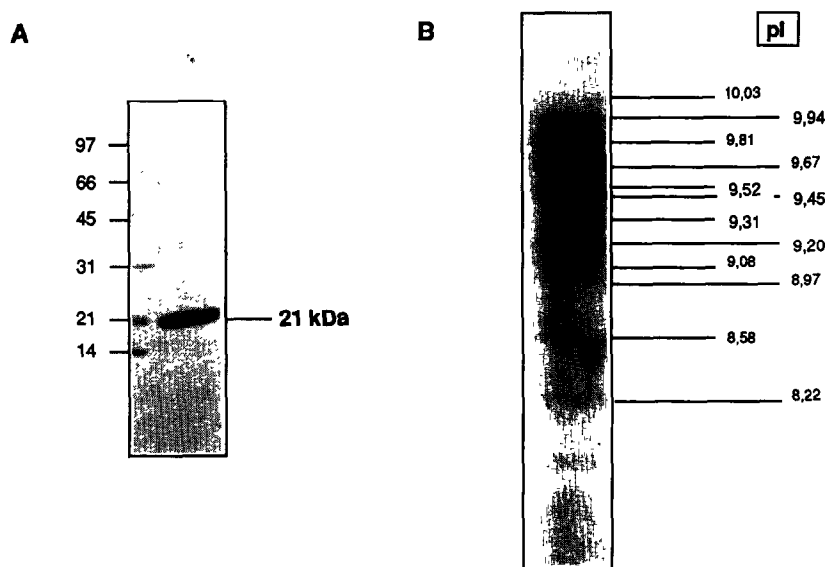


Fig. 1. Gel electrophoretic analysis and isoelectric focusing of LIF protein. (A) PAGE of 30 µg LIF (as well as protein markers) in the presence of dodecyl sulfate on a gel containing 9% acrylamide (routine protocol). (B) 40 µg LIF in an immobilized pH gradient (pH 6.0–11.0); for details see section 2.

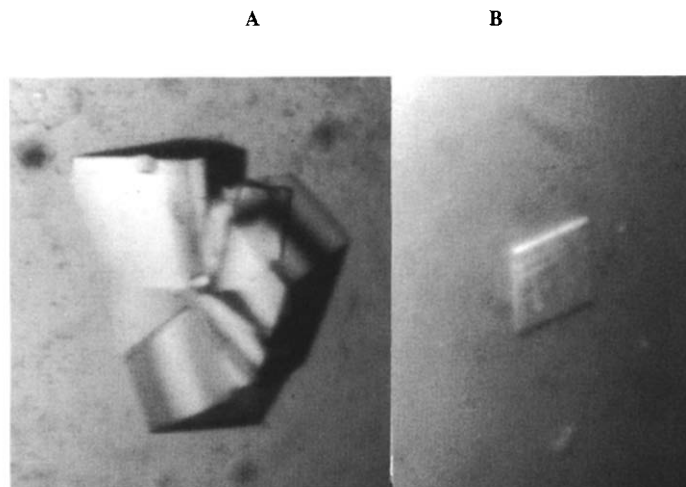


Fig. 2. Crystals of LIF. (A) A cluster of LIF crystals. (B) The plate-like crystal of LIF.

process. As found for the crystallization of another protein (EGF receptor [16]; and manuscript in preparation) the structure analysis of LIF is also expected to benefit from the purification to single isoforms.

As discussed above, LIF is a multifunctional molecule which shares biological activities and signal transduction pathway with other cytokine molecules. As the glycosylation is not actually required for biological activity *in vitro* or *in vivo*, this study will reveal an active conformation and will shed light on potential receptor binding sites as well as its mode of action. A structural comparison with other cytokines, such as granulocyte colony stimulating factor (GCSF) and IL-6, will be important to understand certain effects of LIF in signal transduction. Structure analysis of GCSF [21] and GM-CSF [22] had been extremely useful in under-

standing the mechanism of action of these two cytokines. Information obtained from the three dimensional structure of LIF will also make it possible to design analogs and antagonists which will help to elucidate its pluripotent activities.

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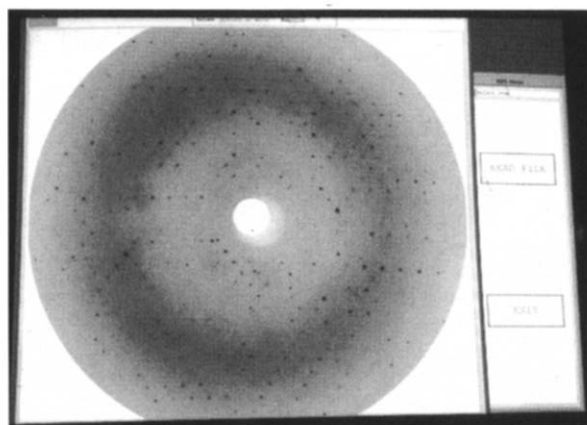


Fig. 3. Diffraction pattern of a LIF crystal recorded with an EMBL Image Plate Scanner, photographed from the screen of a VAXstation 3100. The resolution extends to 3.5 Å at the edge of the image. The exposure time was set to 7 min. The oscillation angle is 3°. The maximum resolution observed from this crystal was 2.0 Å. The satellite diffraction is visible but was successfully allowed for during the data reduction.