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Crystallization and preliminary crystallographic study of HIP/PAP, a human C-lectin overexpressed in primary liver cancers

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Human HIP/PAP is an adhesion protein expressed in normal pancreatic and Paneth cells and overexpressed in hepatocellular carcinoma. HIP/PAP was crystallized using the Hampton Research Crystal Screen and *SAmBA* software to define the optimal crystallization protocol. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 30.73, b = 49.35, c = 92.15 Å and one molecule in the asymmetric unit. Flash-frozen crystals diffract to 1.78 Å resolution using synchrotron radiation. A molecular-replacement solution was obtained using the human Reg/lithostathine structure and the *AMoRe* software.

1. Introduction

The differential screening of a human hepatocellular carcinoma (HCC) complementary DNA library using subtracted probes enabled us to identify and characterize a gene named hepatocarcinoma-intestine-pancreas (*HIP*) (Lasserre *et al.*, 1992). The *HIP* gene encodes a protein which is identical to the human pancreatic associated protein (PAP-H) overexpressed during acute pancreatitis (Orelle *et al.*, 1992). The amino-acid sequence derived from human HIP/PAP showed a 49% identity with Reg/lithostathine, another human protein expressed in the pancreas. The three-dimensional structure of the O-glycosylated Reg/lithostathine isoform is known (Bertrand *et al.*, 1996). HIP/PAP and Reg/lithostathine contain only one carbohydrate-recognition domain (CRD) linked to a signal peptide and thus belong to group VII of the C-type lectin family, according to Drickamer's classification (Drickamer, 1993). In this group, three PAPs (PAP I or peptide 23, PAP II and PAP III) have been described for the rat (Frigerio *et al.*, 1993), whilst three Reg/lithostathine genes have been identified in the mouse (I, II and III; Itoh & Teraoka, 1993; Narushima *et al.*, 1997). One PAP gene (*HIP*/*PAP*) and one Reg/lithostathine gene have been described in humans (Lasserre *et al.*, 1992; Orelle *et al.*, 1992; Watanabe *et al.*, 1994).

Our previous investigations had shown that HIP/PAP was expressed in normal subjects in the intestine (Paneth and neuro-endocrine cells) and the pancreas (acinar pancreatic cells and the islets of Langerhans), while in the liver its expression was triggered in the event of primary liver cancer (Christa *et al.*, 1996). Recently, the rat PAP I gene has also been found to be expressed in regenerating motor neurons, and the protein exhibits mitogen-like activity *in vitro* on Schwann cells (Livesey *et al.*, 1997). Interestingly, motor neurons are the only adult mammalian neurons of the central nervous system which regenerate following injury. Thus, the same protein has been identified using several independent approaches and exhibits marked tissue-specific expression.

We showed previously that HIP/PAP CRD was specific for lactose (Christa *et al.*, 1994). We also showed that HIP/PAP acted as an adhesion molecule for rat hepatocytes in primary culture and that this lectin interacted with extracellular matrix proteins such as laminin-1 and fibronectin (Christa *et al.*, 1996).

We have produced soluble pure recombinant HIP/PAP protein from the milk of transgenic mice carrying the *HIP*/*PAP* gene under the rabbit *WAP* gene regulatory region (Christa *et al.*, in preparation).

In this study, we report the crystallization of the human HIP/PAP C-type lectin. Knowledge of the HIP/PAP protein structure will provide new insight into its functional properties as well as extending the three-dimensional structural homology with the other C-type lectins.

2. Results and discussion

2.1. Purification of the HIP/PAP protein

Milk (100 ml) was collected at day 13 post-parturition from anesthetized HIP/PAP transgenic mice previously injected with 0.05 U oxytocin to stimulate milk letdown production. Mouse milk was diluted (1/10) in 10 m*M* Tris–HCl pH 7.5, 100 m*M* $CaCl_2$ and was centrifuged for 30 min at 40000*g*. The pellet was

discarded and the supernatant lactoserum was acidified by sodium acetate pH 4.6 to precipitate caseins. Supernatant was loaded onto a Mono-S HR 5/5 cation-exchange column equilibrated with 70 m*M* sodium acetate buffer pH 4.8 and elution was performed with a 0–500 m*M* gradient of NaCl in the working buffer. Details of the purification are described in detail elsewhere (Christa *et al.*, in preparation).

The protein migrated to an apparent molecular weight of 16 kDa on 12.5% sodium dodecyl sulfate polyacrylamide gels. The different forms of HIP/PAP protein produced by the mammary gland of the transgenic mice, by the human intestine, pancreas (Christa *et al.*, 1996) and serum (data not shown) migrated similarly, indicating that the HIP/PAP protein is secreted through a similar pathway in the transgenic mouse mammary gland and in human tissues. The Bio-Rad Immun-blot kit for glycoprotein detection showed the absence of glycosylation of the purified HIP/PAP protein from milk. By using the Edman method, the NH_2-terminal region was sequenced (Glu27–Glu26–Pro25–Gln24), indicating that a 26 amino-acid signal peptide was eliminated from the pre-HIP/PAP.

2.2. Crystallization

The HIP/PAP protein was concentrated to 3 mg ml^{-1} in 10 m*M* sodium acetate buffer pH 4.5, 0.2 *M* NaCl using a centrifugal filter device (Ultrafree Biomax 30 K, Millipore, Bedford MA, USA). The NaCl was required to avoid protein aggregation and to keep the protein as a monomer. Both the Hampton Research Crystal Screen and the experimental protocol designed by the *SAmBA* software (Audic *et al.*, 1997) were used to investigate the HIP/PAP crystallization conditions. Crystallization trials were performed at 293 K by hanging-drop vapour diffusion using 24-well culture plates. Each hanging drop was prepared by mixing 0.5 µl 3 mg ml^{-1} HIP/PAP with an equal volume of the reservoir solution. The hanging drop on the cover glass was vapour equilibrated against 1 ml of the reservoir solution in each well of the tissue-culture plate. Two solutions of the Hampton Research crystal screen produced promising results. Both solutions (solution 45 from Crystal Screen 1 in 10 m*M* zinc sulfate and solution 27 from Crystal Screen 2 in 0.2 *M* zinc acetate) contained zinc ions. We used the optimized design provided by *SAmBA* (Audic *et al.*, 1997) to refine the crystallization conditions, and the best crystals were obtained at 0.1 *M* sodium cacodylate pH 6.0, 20%(*w*/*v*) PEG 8000, 5% glycerol and 0.2 *M* zinc acetate. The drops contained 1.0 µl of protein in 0.5 µl of reservoir.

2.3. Data collection and processing

Two crystals were used. The first crystal was sealed in a thin-walled glass capillary and subjected to X-ray diffraction. A complete data set was collected on an 18 cm MAR Research imaging-plate detector on a Rigaku RU-200 rotating-anode generator running at 40 kV and 80 mA with a copper target. The data collection was carried out with oscillation angles of 2.0° and a crystal-to-detector distance of 130 mm. The total oscillation range collected was 92°. This data set was processed using the *DENZO* package (Otwinowski, 1993), and programs from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) were used for scaling. Space-group determination was performed using the autoindexing option in *DENZO*. The crystal diffracted to 2.45 Å and 23 854 reflections were measured in the resolution range 2.45–19.8 Å. The data were reduced to 5679 unique reflections with an $R_{\rm sym}$ value of 8.2. This represents a completeness of 98.6%, with a multiplicity of 3.2 and an average $I/\sigma(I)$ of 5.7. For the highest resolution shell, 2343 reflections were measured in the resolution range 2.45–2.58 Å, corresponding to 828 unique reflections, an $R_{\rm sym}$ value of 27.5, an average $I/\sigma(I)$ of 2.6, a completeness of 98.6 and a multiplicity of 2.8.

The second crystal was collected in a Hampton Research 0.3 mm^3 loop, flash-frozen in liquid nitrogen and subjected to X-ray diffraction. This data set was collected at a wavelength of 0.976 Å on a 30 cm MAR Research imaging-plate detector at the LURE synchrotron-radiation facility. The data collection was carried out with oscillation angles of 2.0° and a crystal-to-detector distance of 120 mm. The total oscillation range collected was 100°. The native data set was processed using the *MOSFLM* package (Kabsch, 1993; Campbell, 1995; Steller *et al.*, 1998; Leslie, 1990), and the *SCALA* program from the *CCP*4 package was used for the scaling and data reduction of the native data set. The crystal diffracted to 1.78 Å and 92 104 reflections were measured in the resolution range 1.78–22.7 Å.The data were reduced to 11 751 unique reflections with an $R_{\rm sym}$ value of 8.2. This represents a completeness of 84.3%, with a multiplicity of 4.3 and an average $I/\sigma(I)$ of 6.3. For the highest resolution shell, 6913 reflections were measured in the resolution range 1.78–1.88 Å, corresponding to 1557 unique reflections, an $R_{\rm sym}$ value of 26.3, an average $I/\sigma(I)$ of 2.9, a completeness of 78.1 and a multiplicity of 4.4.

The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 30.73, b = 49.35, c = 92.15 Å. The packing density (or V_m) of a monomer of HIP/PAP (16.5 kDa) in the asymmetric unit of these crystals (volume 140 279 Å^3) is 2.16 Å^3 Da^{-1}, a reasonable value for globular proteins, indicating an approximate solvent content of 43% (Matthews, 1968). These crystals are suitable for a complete three-dimensional structure determination by the molecular-replacement method using the human Reg/lithostathine structure. A solution was found using the *AMoRe* software (Navaza, 1994) and the data set collected on the Rigaku RU-200 rotating-anode generator. The estimated correlation is 41% and the initial R factor is 45%. Refinement is in progress using the data set collected at the synchrotron radiation facility.

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