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Crystallization of an antitumour antibody SM3 complexed with a peptide epitope

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Abstract

SM3 antibody binds to a tumour-associated epitope on polymorphic epithelial mucin (PEM). Crystals of the Fab fragment of SM3 in complex with a peptide antigen were obtained by vapour diffusion against mother liquor containing acetate buffer, pH 6.5, cadmium chloride and polyethylene glycol (PEG) 4000 as precipitating agent. Crystals belong to the monoclinic space group $P2_1$ with cell dimensions $a = 42.2$, $b = 83.9$, $c = 64.5$ Å and $\beta = 93.4°$. One Fab–antigen complex is present in the asymmetric unit. Diffracted intensities up to 1.95 Å resolution have been measured from a frozen crystal using synchrotron radiation.

1. Introduction

The monoclonal antibody, SM3, shows epithelial specificity and recognises tumour-associated antigens, the epitopes to which are found on high-molecular-weight mucin molecules. Mucins appear to be glycosylated differently by cancer cells, with the result that novel carbohydrate epitopes appear (Kjeldsen, Clausen, Hirohashi, Ogawa & Iijima, 1988). Therefore, core protein epitopes which are masked in the normal mucin can be exposed on cancer cells. One such exposed core epitope has been shown to be recognised by the SM3 antibody, which was developed using the chemically deglycosylated normal, polymorphic epithelial mucin (PEM) (Burchell *et al.*, 1987). The antibody reacts with more than 90% of breast cancer mucins and with the deglycosylated mucin, but shows little or no reaction with the normal breast mucins or the polyglycosylated mucin purified from milk (Burchell *et al.*, 1987; Girling *et al.*, 1989). The specificity of the SM3 antibody makes it a potentially useful tool in the diagnosis and treatment of breast cancer. It is, therefore, of significant interest to understand, at the atomic level, the specificity of the mucin–antibody interaction, as the techniques of rational antibody engineering (Rees *et al.*, 1994) could be applied to obtain perhaps even greater SM3–mucin binding affinity.

Much is already known about the structure of the mucin antigen including the amino-acid sequence of the immunogenic domain of the PEM mucin (Gendler, Taylor-Papadimitriou, Duhig, Rothbard & Burchell, 1988). PEM mucin is made up of repeating units 20 amino acids in length, where the number of repeats varies from individual to individual. A three-dimensional NMR structure for three multiple repeats reveals a 'knob-like' structure for each repeat connected by extended spacers (Fontenot *et al.*, 1995). The core epitope for SM3 has been shown to correspond to just five continuous amino acids, Pro-Asp-Thr-Arg-Pro (Burchell, Taylor-Papadimitriou, Boshell, Gendler & Duhig, 1989). These five residues form the 'knob-like' structures between repeats, and lie between potential serine and threonine glycosylation sites.

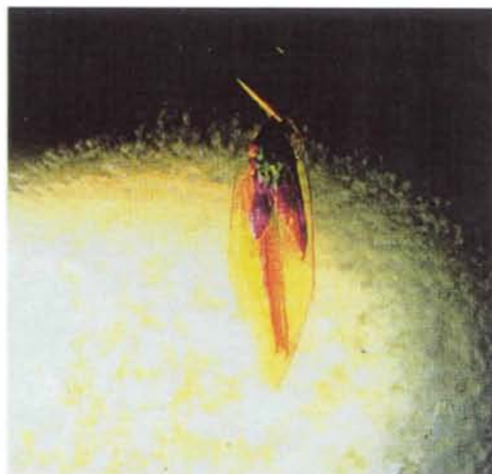

Fig. 1. The crystal of SM3–EP1 complex used for data collection.

In this report, we describe our preliminary crystallization and X-ray diffraction studies of the SM3 antibody bound to a 13-residue peptide epitope (EP1; Thr-Ser-Ala-**Pro-Asp-Thr-Arg-Pro**-Ala-Pro-Gly-Ser-Thr), encompassing the PEM mucin core.

2. Methods

2.1. *Preparation of SM3 Fab fragments*

SM3 at 4 mg ml^{-1} in 100 m*M* sodium acetate pH 6.0, 3 m*M* EDTA, 50 m*M* cysteine was incubated with 160 mg ml^{-1} of papain for 4 h at 310 K. Fabs were purified by gel filtration on Superose 6 in phosphate-buffered saline, followed by dialysis into 20 m*M* Tris pH 8.0 and then Mono Q ion-exchange chromatography. Fractions were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Fab fractions concentrated to 4 mg ml^{-1} in 10 m*M* Tris pH 8.0. The overall yield was 22%.

For crystallization purposes, the monoclonal antibody was mixed with concentrated solution of the peptide at a molar ratio 1:5, incubated for 3 h at 310 K, and then concentrated to 9.5 mg ml^{-1}. The crystallization trials were conducted using the hanging-drop method. A wide range of conditions were tested using commercial screens, namely Hampton Research I and II (Jancarik & Kim, 1991; Cudney, Patel, Weisgraber, Newhouse & McPherson, 1994). Although the initial screens did not give crystals, they indicated that polyethylene glycol (PEG) was the most promising precipitant. In subsequent trials we varied the molecular weight of PEG, buffer pH range (4–9) and effect of salts. Finally, a shower of tiny needles appeared in the drops

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Table 1. *Data statistics of SM3-EP1 complex*

D (Å)	No. of observed reflections	Completeness of data (%)	Multiplicity	Average $I/\sigma(I)$ in shell
6.06	1125	96.2	3.5	15.7
4.33	1994	98.8	3.6	14.7
3.54	2428	96.1	3.4	13.1
3.07	2985	98.1	3.5	10.8
2.75	3374	98.0	3.5	10.9
2.51	3718	98.0	3.5	8.0
2.33	3965	96.3	3.4	7.0
2.18	4299	97.3	3.1	3.4
2.06	4528	96.4	3.3	3.6
1.95	4623	93.2	3.2	3.1
Total	33039	96.6	3.4	8.1

equilibrated against a solution of PEG 4000 as a precipitant and acetate buffer, pH 5.0–7.0, with aggregated prisms appearing at higher pH 6.5–7.5. However, all of these original crystals were too small for data collection. As seeding attempts were unsuccessful, we tried other ways to improve the crystal morphology and size, including a metal ion screen. Addition of cadmium chloride gave larger crystals which were often twinned or aggregated. However, occasionally single crystals could be separated, one of which was used for data collection. The final conditions were: 2.5 µl of the antibody–antigen complex mixed with 2.5 µl of the well solution containing 0.2 *M* $CdCl_2$, 19%(*w*/*v*) of PEG 4000, and 0.1 *M* acetate buffer, pH 6.0.

3. Results and discussion

A monocrystal of dimensions 0.4 × 0.2 × 0.03 mm (Fig. 1) was used for the diffraction measurements at the X11 outstation of the DESY synchrotron in Hamburg. Analysis of the diffraction data showed that the crystal belongs to the monoclinic $P2_1$ space group with unit-cell dimensions $a = 42.2$, $b = 83.9$, $c = 64.5$ Å, and $\beta = 93.4°$ Assuming one complex molecule per asymmetric unit, the specific volume $V_m = 2.50$ Å^3 Da^{-1} of protein corresponds to a solvent content of 51% (Matthews, 1968). Since the number of available crystals was limited, data collection was carried out at 110 K. The crystal was transferred to a glycerol-enriched mother liquor (10% of the water in mother liquor was substituted by glycerol) and then flash frozen using standard techniques (Teng, 1990) in a stream of nitrogen gas produced by an Oxford Cryosystems Cryostream. A complete data set up to 1.95 Å resolution was collected using synchrotron radiation at wavelength 0.912 Å (Table 1). The data was collected in 1.0° oscillation frames over 180° oscillation range on an 18 cm MAR Research image plate from a single crystal. The frames were processed with *DENZO* software (Otwinowski, 1993) resulting in 111 363 observations with $I > 1.0\sigma(I)$. Further scaling with the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) gave 33 093 independent reflections with R_{merge} of 6.9%.

Although the core epitope for SM3 is only five amino acids in length (Burchell *et al.*, 1989), we have used a competitive binding assay to show that longer peptides which contain this core sequence can bind efficiently with high affinity to SM3 (Band & Snary, unpublished observations). Our initial molecular replacement studies indicate that there is substantial unaccounted electron density within the binding region of the SM3, suggesting that the crystals we report here do contain the bound peptide antigen. Once the structure has been fully determined, it will be of interest to compare the conformation of the bound antigen with that of the uncomplexed peptide as determined by NMR. These studies should enable us to understand the specificity of SM3 and perhaps provide further insights into antibody–antigen recognition.

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