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Crystallization and Preliminary X-Ray Diffraction Analysis of the Extracellular Domain of the Cell Surface Antigen CD38 Complexed with Ganglioside[1]

Mutsuko Kukimoto,[*] Osamu Nureki,[†] Mikako Shirouzu,[*,‡] Toshiaki Katada,[§] Yoshio Hirabayashi,[‖,2] Hiroshi Sugiya,[¶] Shunsuke Furuyama,[¶] Shigeyuki Yokoyama,[*,†,‡] and Miki Hara-Yokoyama[¶,3]

[*]Cellular Signaling Laboratory, [‡]Genome Sciences Center, and [‖]Laboratory for Glyco-Cell Biology, Frontier Research Program, RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako-shi, Saitama, 351–0198; [†]Department of Biophysics and Biochemistry, Graduate School of Science, and [§]Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113–0033; and [¶]Department of Physiology, Nihon University School of Dentistry at Matsudo, 2-870-1 Sakae-cho Nishi, Matsudo 271–8587

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The cell surface antigen CD38 is a multifunctional ectoenzyme that acts as an NAD$^+$ glycohydrolase, an ADP-ribosyl cyclase, and also a cyclic ADP-ribose hydrolase. The extracellular catalytic domain of CD38 was expressed as a fusion protein with maltose-binding protein, and was crystallized in the complex with a ganglioside, G_{T1b}, one of the possible physiological inhibitors of this ectoenzyme. Two different crystal forms were obtained using the hanging-drop vapor diffusion method with PEG 10,000 as the precipitant. One form diffracted up to 2.4 Å resolution with synchrotron radiation at 100 K, but suffered serious X-ray damage. It belongs to the space group $P2_12_12_1$ with unit-cell parameters of a = 47.9, b = 94.9, c = 125.2 Å. The other form is a thin plate, but the data sets were successfully collected up to 2.4 Å resolution by use of synchrotron radiation at 100 K. The crystals belong to the space group $P2_1$ with unit-cell parameters of a = 57.4, b = 51.2, c = 101.1 Å, and β = 97.9°, and contain one molecule per asymmetric unit with a V_M value of 2.05 Å^3/Da.

Key words: crystallization, CD38, ganglioside, maltose-binding protein, X-ray crystallography.

The leukocyte cell surface antigen CD38 is an ectoenzyme of NAD$^+$ glycohydrolase, and is capable of both generating and degrading cyclic ADP-ribose (cADPR) (*1*). The expression of CD38 is widely observed in various tissues in addition to lymphocytes (*2*, *3*). CD38 is considered to mediate a variety of cellular events: lymphocyte activation (*4*), apoptosis (*5*), cytokine secretion (*6*), and adhesion (*7*). In addition to its role as a regulatory molecule in the immune system, CD38 acts as an ectoenzyme that catalyzes the glycohydrolysis of NAD$^+$, the synthesis of cADPR from NAD$^+$, and also the hydrolysis of cADPR (*8–10*). cADPR was identified as a second messenger that induces the release of Ca^{2+} from intracellular stores and functions widely in Ca^{2+}-mediated cell signaling (*11*, *12*). The involvement of CD38 in the regulation of humoral immune responses and glucose-dependent insulin secretion has been recently demonstrated by studies involving CD38-deficient mice (*13*, *14*).

The extracellular part of CD38 shows significant (30%) sequence identity to *Aplysia* ADP-ribosyl cyclase, a soluble protein that converts NAD$^+$ to cADPR (*15*, *16*). The 3-D structure of *Aplysia* ADP-ribosyl cyclase has been shown to be a homodimer enclosing a cavity at the dimer interface, and provides a model structure of the CD38 extracellular domain (*17*). To understand the precise function of CD38, however, structural analysis of CD38 itself is essential. We previously showed that gangliosides, one of the lipid components of cell membranes, act as inhibitors of the enzyme activity of an extracellular domain of CD38 expressed as a fusion protein with maltose-binding protein (MBP-CD38) (*18*). Through an experiment in which CD38 was expressed in lymphocyte cells, it has also been shown that gangliosides inhibit the CD38-catalyzed NAD$^+$ glycohydrolase activity on the cell surface (Hara-Yokoyama *et al.*, submitted for publication). Since gangliosides did not inhibit the NAD$^+$ glycohydrolase activity of the extracellular domain of CD38 that was not fused to MBP (data not shown), it is likely, in the MBP-CD38/gangliosides complex, that MBP serves as a scaffold for gangliosides to interact with CD38. Here we report the crystallization and preliminary X-ray

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[2] Present address: Laboratory for Memory and Learning, RIKEN Brain Science Institute.

[3] To whom correspondence should be addressed. Phone: +81–47–360–9326, Fax: +81–47–360–9327, E-mail: yokoyama@mascat.nihon-u.ac.jp

crystallographic analysis of MBP-CD38 complexed with a ganglioside, G_{T1b}. Structural information of the CD38 catalytic domain bound with its inhibitor will be important for understanding how the activity of this ectoenzyme is regulated on the cell surface.

We previously constructed pMAL-cR1/ext CD38, an expression plasmid for MBP-CD38 under the control of the *tac* promoter (*19*). Upon induction by IPTG, MBP-CD38 was overexpressed in both soluble and insoluble forms in *Escherichia coli* (Fig. 1A). However, the yield of the MBP-CD38 fusion protein was about 1% with the use of a refolding procedure (as described below). For the large-scale preparation of MBP-CD38, we used a coproduction system with bacterial thioredoxin, since it has been reported that coproduction of thioredoxin increases the solubility of foreign proteins (*20*). An expression plasmid for *E. coli* thioredoxin under the control of the T7 promoter, pT-Trx, was kindly supplied by Dr. S. Ishii (Laboratory of Molecular Genetics, RIKEN). With the coproduction of thioredoxin, the solubility of MBP-CD38 was significantly increased, leading to an increase in the yield by about 5-fold (Fig. 1A).

E. coli JM109 (DE3) cells carrying both pMAL-cR1/ext CD38 and pT-Trx were cultured in 3 liters of LB medium containing 25 μg/ml ampicillin and 33 μg/ml chloramphenicol at 37°C. After the culture reached an absorbance value at 600 nm of 0.7, IPTG was added to 0.8 mM, and the cultivation was continued for 5 h at 30°C. The cells were harvested, suspended in 70 ml of 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA (buffer A) containing 0.8 mM PMSF, and then disrupted by sonication. The cellular debris was removed by centrifugation at 25,000 ×*g*, and the resulting supernatant was applied to an amylose column (25 × 40 mm; New England Biolabs, Beverly, USA) equilibrated with buffer A. The adsorbed proteins were eluted with buffer A containing 10 mM maltose. Since the MBP-CD38 eluted from the amylose column showed little enzyme activity, denaturation-renaturation treatment was performed to reactivate the MBP-CD38, as previously described by Kukimoto *et al.* (*19*) with minor modifications. In this treatment, the inactive MBP-CD38 was denatured once with 6 M guanidine hydrochloride and then refolded by dialysis against 20 mM Tris-HCl (pH 7.5) containing 0.10 M NaCl (buffer B). The dialysate was concentrated with a Centriprep 30 unit (Millipore, Bedford, USA) and then loaded onto a Superdex 200 column (Amersham-Pharmacia Biotech., Tokyo) equilibrated with buffer B. As shown in Fig. 1B, MBP-CD38 with high NAD^+ glycohydrolase activity was eluted separately from the aggregated MBP-CD38 with no activity. The active fractions were pooled, desalted in Centriprep tubes, and then applied to a ResourceQ column (Amersham-Pharmacia Biotech.). The

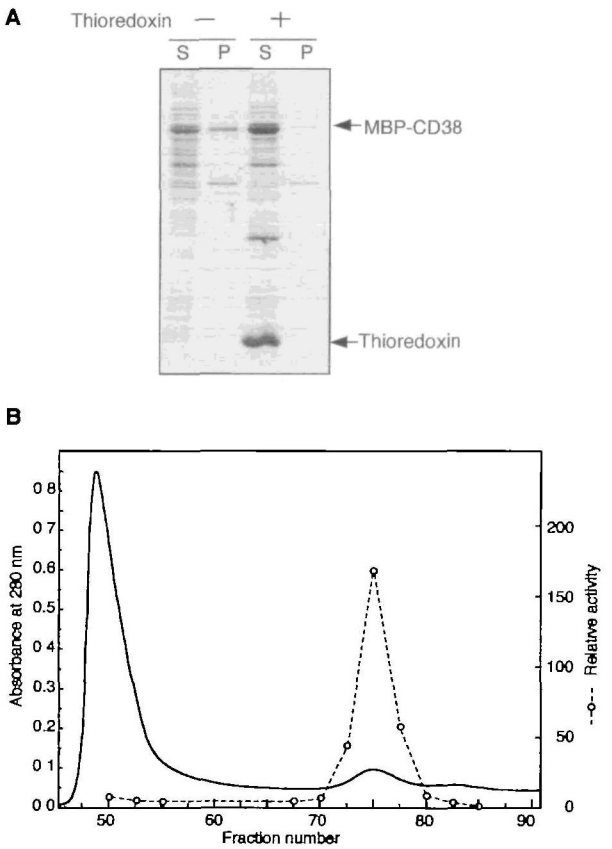

Fig. 1. **Expression and purification of MBP-CD38.** A: SDS-PAGE of an *E. coli* lysate, MBP-CD38 being produced alone (–) or coproduced with thioredoxin (+). S indicates supernatants of the *E. coli* lysates, and P precipitates of the lysates. B: Elution profile and relative NAD^+ glycohydrolase activity of MBP-CD38 on gel filtration chromatography. The enzyme activity was measured by a fluorometric method, as previously described by Kontani *et al.* (*24*).

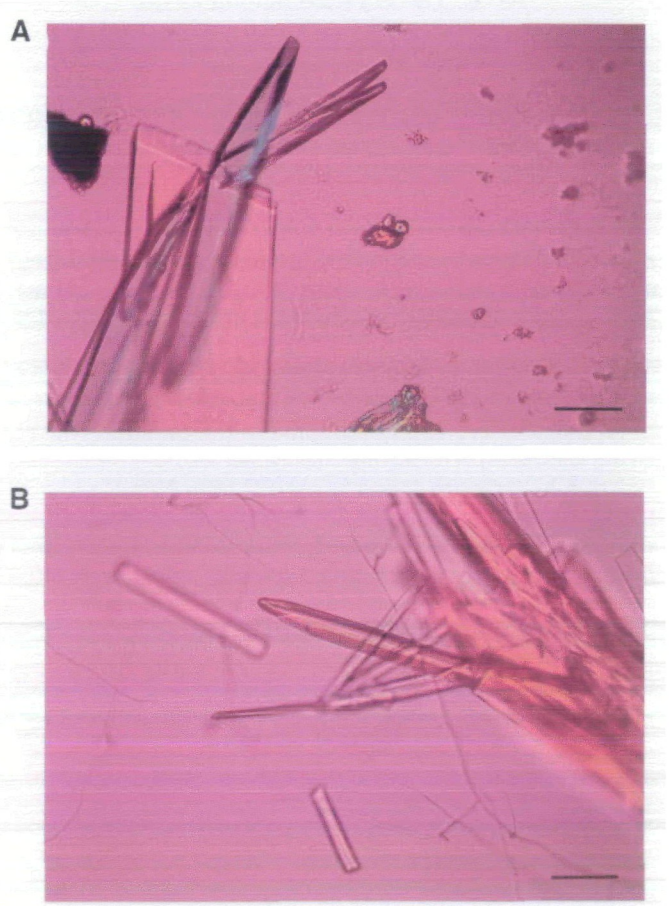

Fig. 2. **Crystals of MBP-CD38 complexed with G_{T1b}.** A: Form I crystals. B: Form II crystals. The scale bars correspond to 0.1 mm.

adsorbed proteins were eluted with a linear gradient of 20–400 mM NaCl in 20 mM Tris-HCl (pH 7.5). MBP-CD38 was eluted at about 150 mM NaCl and gave a single band on SDS-PAGE (data not shown). Approximately 0.5–1 mg of MBP-CD38 was purified from 3 liters of culture. MBP-CD38 was concentrated up to 10 mg/ml and then mixed with 0.15 mg of ganglioside G_{T1b}, purified from bovine brain as described previously (*21*, *22*).

The crystallization conditions for MBP-CD38 complexed with G_{T1b} were screened using a Crystal Screen kit (Hampton Research, Laguna Hills, USA) by the hanging drop vapor diffusion method. The protein solution (1 μl of 10 mg/ml) was mixed with an equal volume of the reservoir solution, and each hanging drop was then vapor-equilibrated against 0.5 ml of the reservoir solution at 293 K. Condition No. 35 in Screen kit II, 10–20% PEG 10,000 and 100 mM hepes (pH 7.5), yielded very thin polycrystals. Further refinement of the crystallization conditions, using an Additive Screen kit (Hampton Research), produced two crystal forms (Form I and Form II). Form I crystals with a plate-like morphology were obtained when 100 mM NaI was added to the hanging drops (Fig. 2A), and Form II crystals with a rod-like morphology were obtained when 100 mM glycine was added (Fig. 2B). Possibly due to a slight difference in the refolding conditions, the reproduction of these crystals was quite difficult. Micro and macro seeding techniques were tried, but did not work.

Form II crystals diffracted up to 3.0 Å resolution at room temperature, with synchrotron radiation on BL6A at the Photon Factory (Tsukuba). They belong to the orthorhombic space group $P2_12_12_1$ with unit-cell parameters of a = 47.9, b = 94.9, c = 125.2 Å. Since the diffraction decayed very fast on exposure to X-rays, we collected data sets for Form II crystals at 100 K with ethylene glycol as a cryoprotectant, using synchrotron radiation on BL41XU at SPring-8 (Harima). Although the crystals diffracted initially to 2.4 Å resolution, significant X-ray decay was also observed during this data collection. Correspondingly, the mosaicity of the crystals increased along with the data collection up to 1.5°. We therefore changed to Form I crystals for X-ray analysis. Since cryoprotection with either MPD, PEG400, or glycerol resulted in significant damage to the Form I crystals, ethylene glycol was used as the cryoprotectant. To minimize the damage to the crystals by cryoprotection, they were sequentially dialyzed against harvest buffers containing 5 and 10% ethylene glycol for 30 min at each concentration, using Spectra/Por dialysis membranes (Spectrum Labs, Laguna Hills, USA) with a molecular weight cut off of 25,000. Finally, data sets for Form I crystals were successfully collected up to 2.4 Å resolution at 100 K with a wavelength of 1.00 Å, using BL45XU at SPring-8 (Fig. 3). X-ray diffraction data were recorded on an R-AXIS IV imaging plate (Rigaku, Tokyo), and processed with the DENZO and SCALEPACK programs (*23*). The space group of the Form I crystals was determined to be monoclinic $P2_1$ with unit-cell parameters of a = 57.4, b = 51.2, c = 101.1 Å, and β = 97.9°. An asymmetric unit contains one MBP-CD38 molecule (M_r = 71,900; MBP and the extracellular domain of CD38, with M_r of 40,600 and 29,600, respectively, are connected by a short spacer peptide with a M_r of 1,700), corresponding to a crystal volume per protein mass of V_M = 2.05 Å^3/Da with a solvent content of 40.0%. 52,161 reflections were measured in the resolution range of 50–2.4 Å

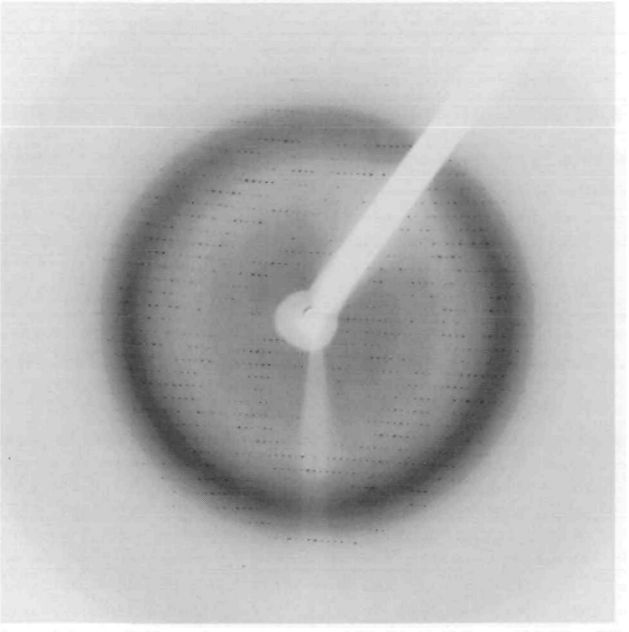

Fig. 3. **X-ray diffraction pattern of MBP-CD38 at 100 K, with synchrotron radiation on BL45XU at SPring-8.** The resolution of the highest-order reflection is 2.2 Å.

with 20,610 unique reflections, which represents a multiplicity of 2.531. The average $I/\sigma(I)$, R_{sym} value ($\Sigma(|I-\langle I\rangle|)/\Sigma I$, summed over symmetrically equivalent observations), and completeness are 8.7, 7.6, and 85.1%, respectively. For the highest resolution shell (2.43–2.40 Å), the R_{sym} value is 23.2% with 77.7% completeness. Since the crystal growth along the c axis is not good, the crystal morphology becomes a plate as thin as 5 μm. Therefore, the diffractions are weak when the crystal a-b face is exposed to X-rays, resulting in the relatively low overall completeness. Molecular replacement using either MBP or a CD38 homologue, ADP-ribosyl cyclase, as the search model failed to yield a clear solution. This suggests that conformational changes of CD38 or MBP may occur upon binding with gangliosides. Therefore, it will be necessary to solve the phase problem by means of multiple isomorphous replacement or multiple wavelength anomalous dispersion methods. Data collection for heavy-atom derivatives as well as the selenomethionine derivative is now underway.

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