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Expression, characterization and crystallization of the Fv fragment of mouse antibody 3B62 from the secondary immune response

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Affinity of antibodies increases in the course of the immune response. Mouse anti-nitrophenol antibody 3B62 from the secondary immune response shows higher affinity than the primary-response antibodies. An expression system for the 3B62 Fv fragment was constructed by introducing coding regions for the V_L and V_H into the genome of the methylotrophic yeast *Pichia pastoris*. Each of the coding regions was placed downstream of the coding region for the secretion signal of the yeast α-factor. The α-factor signals were cleaved off from the expressed proteins and the Fv was secreted as a heterodimer consisting of the V_L and V_H domains. The binding constant of the expressed Fv against the (4-hydroxy-5-iodo-3-nitrophenyl)acetate ligand was comparable to that of the Fab fragment. Crystals of the Fv were obtained in the presence of the ligand and diffracted X-rays to 1.8 Å resolution. The crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 46.48 (9), b = 34.99 (4), c = 77.76 (17) Å, β = 101.47 (14)°, and contain one Fv molecule per asymmetric unit.

1. Introduction

The affinity of antibodies against specific antigens increases during the time course of the immune response; this phenomenon is referred to as the affinity maturation of antibodies (Milstein & Neuberger, 1996). Studies on the sequences of the mouse antibodies raised against the (4-hydroxy-3-nitrophenyl)-acetate (NP) ligand have revealed (i) that the primary-response antibodies from the strain C57BL/6 exhibit frequent use of the V_λ1-V186.2 germ-line repertoire which is rarely combined with somatic mutations (Cumano & Rajewsky, 1985) and (ii) that the secondary-response antibodies encoded by the same germ-line repertoire show somatic mutations and often exhibit increased affinities (Cumano & Rajewsky, 1986). In most of these secondary-response antibodies, a replacement of Trp33H (H denotes the heavy chain, L the light chain) by Leu is frequently observed. The three-dimensional structural studies on anti-NP antibodies N1G9 (Mizutani *et al.*, 1995) and 88C6/12 (Yuhasz *et al.*, 1995) revealed that the affinity maturation of the anti-NP antibodies is mainly ascribable to the mutation of this residue.

Antibody 3B62 from the secondary response retains this Trp33H but shows higher affinity than the secondary-response antibodies bearing Leu33H (Cumano & Rajewsky, 1986). Hence, it has been suggested that the structural mechanism of the affinity maturation of antibody 3B62 is different from that of the antibodies with Leu33H. Here, the expression, purification and crystallization of the Fv fragment of antibody 3B62 are reported in order to examine the structural mechanism of the affinity maturation.

2. Materials and methods

2.1. Construction of the expression system of the 3B62 Fv

Complementary DNAs were prepared by reverse-transcribing mRNAs of hybridoma cell line 3B62 (Cumano & Rajewsky, 1986) using the First-Strand cDNA Synthesis Kit (Pharmacia) and random hexamer primers. Each of the V_L and V_H coding regions was amplified from the cDNAs by the polymerase chain reaction (PCR) with *KOD* polymerase (Toyobo). Primers used in the PCR were: L-sense, 5′-ATG GCC TGG ATT TCA CTT ATA-3′; L-antisense, 5′-AGA GGA AGG TGG AAA CAG GGT-3′; H-sense: 5′-ATG GGA TGG AGC TGT ATC ATG-3′; H-antisense, 5′-AGA TCC CTT GGC CAG TGG ATA-3′. The sense primers are for sense strands of the V_λ1 and V186.2 signal sequences. The antisense primers are for antisense strands of the C_λ1 and C_γ1 sequences. The PCR-amplified coding regions were directly sequenced using the same primers.

Each of the V_L coding regions corresponding to the amino-acid residues 1–107 (the numbering scheme follows Kabat *et al.*, 1991) and the V_H coding region corresponding to

residues 1–112 was reamplified by the PCR for insertion between the *Xho*I and *Eco*RI restriction sites of the pPIC9 vector of the *P. pastoris* Expression System (Invitrogen). The constructions of the inserted regions are schematically shown in Fig. 1. The primers used for the PCR were: V_L-sense, 5′-TCT **CTC GAG** AAA AGA CAG GCT GTT GTG ACT CAG GAA-3′; V_L-antisense, 5′-AGG **GAA TTC** CTA GCC TAG GAC AGT CAG TTT GGT-3′; V_H-sense, 5′- TCT **CTC GAG** AAA AGA CAG GTC CAA CTG CGG CAG CCT-3′; V_H-antisense, 5′-AGG **GAA TTC** CTA GGA GAC TGT GAG AGT GGT GCC-3′ (bold sequences denote the restriction sites). The sense primers introduce the *Xho*I sites followed by the coding regions of the Lys-Arg recognition sequences that are designed for post-translational removal of the secretion signal of the yeast α-factor. The antisense primers introduce the *Eco*RI sites preceding the stop codons. The pPIC9 vectors carrying the V_L and V_H coding regions were referred to as pPIC9-V_L and pPIC9-V_H. An equimolar mixture of the pPIC9-V_L and pPIC9-V_H were linearized with *Sac*I and were then co-transformed into the *P. pastoris* KM71 cell line (*his4*) with the spheroplast method. Transformed recombinants were isolated as *HIS*$^+$ transformants.

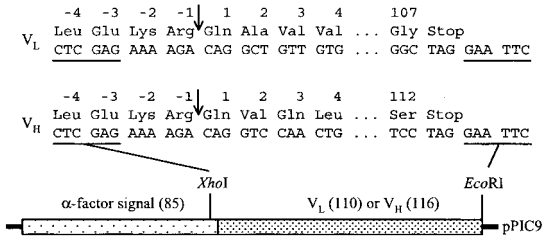

Figure 1
Schematic diagram for the vector constructions. Each of the V_L and V_H strands was inserted into the pPIC9 vector between its *Xho*I and *Eco*RI sites. Arrows indicate the polypeptide cleavage sites by the *KEX*2 endoprotease. The parenthesized numbers indicate the numbers of the amino acids corresponding to the coding regions.

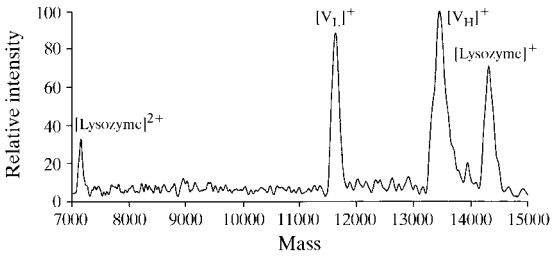

Figure 2
Mass spectrum of the 3B62 Fv. The V_L and V_H polypeptides of the 3B62 Fv were calibrated with an internal standard of hen egg-white lysozyme with a mass of 14 303 Da.

2.2. Screening of transformants secreting the Fv

Following the brochure of the *P. pastoris* Expression System, 75 transformants were screened for the secreted 3B62 Fv. Each transformant was grown for 48 h in 2 ml of the culture medium buffered with minimal methanol yeast extract (BMMY). The supernatant of the culture broth (200 µl) was applied to 50 µl of an NP-coupled agarose gel and the gel was washed with phosphate-buffered saline (PBS). Bound protein fractions were eluted with 50 µl of PBS containing 500 µ*M* (4-hydroxy-5-iodo-3-nitrophenyl)acetate (NIP, purchased from Sigma). The eluates corresponding to the 3B62 Fv were analyzed for expression levels by SDS–PAGE.

2.3. Large-scale production and purification of the Fv

The transformant expressing the 3B62 Fv in larger quantities was cultured in 7 l of buffered minimal glycerol yeast extract (BMGY) medium. After approximately 2 d, when the dissolved oxygen concentration increased, methanol was added into the medium at a rate of 3.5 ml h^{-1}. After methanol induction at 303 K for 24 h, the culture broth was centrifuged and the resultant supernatant was loaded onto a 5 ml NP-agarose gel column. The Fv protein was eluted with 100 ml of PBS containing 500 µ*M* NIP and then applied to a CM Sepharose cation-exchange column (Pharmacia) equilibrated with 50 m*M* sodium acetate buffer pH 5.0 containing 100 µ*M* NIP. The Fv complexed with NIP was eluted with a 0–50 m*M* NaCl gradient. The mass spectra of the Fv protein were analyzed with Kratos Kompact MALDI IV (Shimadzu). The amino-terminal sequences of the V_L and V_H domains were analyzed with the automated Edman degradation sequencer of the Applied Biosystems Model 477A.

2.4. Affinity measurements

The NIP ligand bound to the affinity-purified Fv was removed by two-step dialyses against 100 m*M* sodium citrate buffer pH 4.0 and against 50 m*M* sodium phosphate buffer pH 7.0 containing 150 m*M* NaCl. The dialysate was concentrated to 5 mg ml^{-1} and loaded onto a Superdex75 gel-filtration column (Pharmacia). The main peak fraction was pooled for measurement of the affinity toward the NIP ligand.

The Fab fragment was prepared from monoclonal antibody 3B62 as described previously (Mizutani *et al.*, 1995). The binding constants of the Fv and Fab were determined for the NIP ligand by the fluorescence-quenching method (Azuma *et al.*, 1987) using 50 n*M* solutions pH 7.0 of the antibody fragments. The values for the binding constant and maximum quenching were determined from non-linear least-squares fitting based on the equation describing ligand–protein binding (Eftink, 1997).

2.5. Crystallization and collection of X-ray diffraction data

The purified Fv complexed with the NIP ligand was concentrated to 6 mg ml^{-1} using a buffer consisting of 50 m*M* Tris pH 7.2, 0.02%(*w*/*v*) NaN_3 and 1 µ*M* NIP. Crystallization attempts were performed with the sitting-drop vapour-diffusion method at 293 K using Crystal Screen kits (Hampton Research) as reservoir solutions. A 1 µl droplet of the Fv protein was mixed with 1 µl of a reservoir solution and was equilibrated against 100 µl of the reservoir using CrystalClear Strips (Douglas Instruments). X-ray diffraction data were recorded at 277 K on an R-AXIS IV imaging-plate detector (Rigaku) using graphite-monochromated Cu *Kα* radiation generated from a rotating-anode X-ray generator with an effective focus of 0.3 × 0.3 mm operated at 40 kV and 134 mA (MacScience). Diffraction data were collected from one crystal with an oscillation range of 2.0° around the *b* axis and an exposure time of 15 min per exposure frame. Integration, scaling and post-refinement of diffraction intensities from 91 frames were performed with the programs *DENZO* and *SCALEPACK* (Otwinowski, 1993).

3. Results and discussion

The sequence of the 3B62 V_L coding region, as determined by the direct sequencing, shows that mutations from the germ-line $V_\lambda 1$ exist at codons 18L (**G**CA, mutation in the codon is in bold), 21L (**T**TC), 27bL (**T**CT), and 55L (G**TT**). These mutations cause amino-acid replacements of Thr18L with Ser, Leu21L with Phe, Ala27bL with Ser and Ala55L with Val. The 3B62 V_L sequence at the codons 21L and 27bL are

Table 1
Diffraction data-collection statistics.

Values in parentheses are for the highest resolution range, 1.86–1.80 Å.

Resolution range (Å)	30–1.8
No. of observed reflections	101878
No. of unique reflections	21737
Completeness	0.945 (0.905)
$R_{\rm merge}(I)$†	0.067 (0.305)

† $R_{\rm merge}(I) = \sum_h \sum_i^{N_h} |I_j(h) - \bar{I}(h)| / \sum_h \sum_i^{N_h} I_j(h)$, where intensity $I_j(h)$ is measured N_h times for a reflection h.

inconsistent with the mRNA sequence of 3B62 V_L (Cumano & Rajewsky, 1986). These inconsistencies are ascribable either to mutations after the hybridoma establishment or to presumed low accuracy of the mRNA sequencing. On the other hand, the sequence of the 3B62 V_H coding region is identical to that of the genomic DNA (Allen *et al.*, 1988).

In the co-transformation of pPIC9-V_L and pPIC9-V_H into the *P. pastoris* genome, generation of transformants carrying both the V_L and V_H genes is limited to the chance of multiple insertion events (Clare *et al.*, 1991). The SDS–PAGE analysis of the transformants showed that 15 out of the tested 75 transformants (20%) expressed the 3B62 Fv.

Figure 3
Crystal of the 3B62 Fv. The crystal has dimensions of 0.3 × 0.1 × 0.03 mm and is yellow coloured owing the bound NIP ligand.

Using one of the transformants, 30 mg of the affinity-purified Fv was obtained from the 7 l culture. The molecular masses obtained from the Fv specimen were 11 615 Da for the V_L (calculated value of 11 601 Da) and 13 444 Da for the V_H (calculated value 13 363 Da) as measured by mass spectroscopy (Fig. 2). The results obtained by the amino-terminal sequencing indicate that the V_L and V_H polypeptides possess the amino-acid sequences Gln1-Ala2-Val3-Val4 and Gln1-Val2-Gln3-Leu4, respectively. These demonstrate that the secreted V_L and V_H polypeptides were homogeneously cleaved off at the Arg-Gln junction to the α-factor secretion signal. The binding constant of the Fv is $6.4 \times 10^7\ M^{-1}$, comparable with the value of $1.7 \times 10^7\ M^{-1}$ obtained for the Fab. Therefore, the three-dimensional structure of the expressed 3B62 Fv is presumably identical to the Fv portion of the 3B62 antibody and hence is applicable to the structural study of the affinity maturation.

The 3B62 Fv crystals appropriate for crystallographic analysis were obtained in the presence of NIP, using a reservoir solution consisting of 20%(*w*/*v*) PEG 6000, 0.1 *M* HEPES pH 7.5 and 10 m*M* $SrCl_2$. The crystals grew to dimensions of 0.3 × 0.1 × 0.03 mm in a few weeks (Fig. 3). They belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 46.48 (9), b = 34.99 (4), c = 77.76 (17) Å, β = 101.47 (14)° (values in parentheses are the standard uncertainty for the NIP-liganded form). Assuming one Fv molecule per asymmetric unit, a V_M value (Matthews, 1968) of 2.48 $Å^3\ Da^{-1}$ and a solvent content of 0.50 were obtained. A total of 101 878 reflections were collected from one crystal to 1.8 Å resolution, as summarized in Table 1. Molecular replacement using the Fv portion of the N1G9 antibody Fab structure (Mizutani *et al.*, 1995) as a search model has revealed an NIP-liganded Fv structure with a high Patterson correlation maximum of 0.351 for the 8–4 Å data by use of the *X-PLOR* program (Brünger, 1992); crystallographic refinement is now in progress.

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