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Cloning and Expression of a Murine Fab Fragment Recognizing a Defined Linear Epitope of *Chironomus thummi thummi* Major Allergen Chi t 1–9

Key Words

Antibody
Cloning
Recombinant Fab fragment
Allergen

Abstract

We have cloned and expressed in bacteria the genes coding for the Fab fragment of the monoclonal antibody (MoAb) M3. M3 is a murine IgG1 antibody reactive with the major allergen Chi t 1–9 of *Chironomus thummi thummi*. The major allergen Chi t 1–9 is known to be an aggressive inhalant allergen and causes type-I allergy. The immunoglobulin (Ig) fragment genes were cloned as a synthetic dicistronic operon. In this operon each gene is preceded by a bacterial signal sequence to direct the recombinant protein to the periplasmic space of the bacteria. The cloned genes were expressed in *Escherichia coli* using the strong T7-RNA-polymerase-based system. Sequence analysis revealed that the light and heavy chains of MoAb M3 belong to the V_κII and V_HIIC group of the Ig family, respectively. Genes of the V_κII and V_HIIC group are known to be used in response to haptens. The apparent affinity constants of the parent antibody M3 and the recombinant Fab fragment are nearly equivalent.

Introduction

Bacterial expression and secretion of cloned antibody Fv and Fab fragments has been demonstrated by Skerra and Plückthun [1] and Better et al. [2], and this technology has been rapidly utilized by other investigators. Cloned antibody fragments are especially valuable for therapeutic and diagnostic use [2–4] as well as for X-ray crystallography. One advantage in using engineered bacterial Fab fragments in crystallography is that the protein heterogeneity that results from nonspecific cleavages and differences in the susceptibility of antibodies to protease cleavage [5] will be obviated, making it possible to achieve a constant, homogeneous preparation [2]. We have used genetic engineering to generate a recombinant Fab fragment of the monoclonal an-

tibody M3 (MoAb M3). MoAb M3 reacts with the major allergen Chi t 1.01 of *Chironomus thummi thummi* [6]. *C. thummi thummi* belongs to the globally distributed family of nonbiting midges (Chironomidae). The non-cell-bound hemoglobin (Chi t 1–9) is known to be an aggressive inhalant allergen [6]. About 18% of employees from fish-food factories exposed to Chi t 1–9 suffer from type-I allergy, mainly from bronchial asthma, rhinitis, conjunctivitis, and urticaria [7]. Chi t 1–9 is composed of 3 monomeric and 9 homodimeric components [6]. MoAb M3 recognizes Chi t 1.01 [6], a monomer of 136 amino acids, which has been analyzed by X-ray crystallography [8]. Recently the epitope of M3 was determined as a linear epitope which comprises amino acids 13–17 of Chi t 1.01 [9]. Patients' immunoglobulin E (IgE) is reactive with a tryptic peptide which is comprised of amino

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acid residues 1–17 [7]. Although the B-cell epitope algorithm of Jameson and Wolf defines only one linear epitope in the N-terminal region of Chi t 1.01 [9], there is evidence that MoAb M3 recognizes a human IgE reactive epitope. In this study we describe the functional bacterial expression of the genes coding for the Fab fragment of MoAb M3. We have characterized the genes and the recombinant Fab fragment, established the appropriated culture conditions and determined the influence of different bacterial strains on the expression of the recombinant Fab fragment.

Materials and Methods

Lymphocyte RNA Preparation, Amplification and Cloning

The RNA of 1×10^8 hybridoma cells was prepared using the standard method [10]. For first-strand cDNA synthesis, 20 µg RNA in H₂O and 500 ng oligo-dT (Gibco BRL, USA) were heated at 65°C for 5 min and annealed for 45 min at ambient temperature. One unit RNase inhibitor (New England Biolabs, USA), 500 µM dNTPs (Boehringer-Mannheim, Germany), 10 µl reverse-transcriptase buffer, and 80 U M-MLV reverse transcriptase (Stratagene, USA) were added, the volume was increased to 100 µl and the solution was incubated for 90 min at 37°C. PCR amplification was performed in 100-µl reactions using 5 µl of the first-strand reaction with 200 µM dNTPs (Boehringer-Mannheim, Germany), 150 ng of 5' and 3' primer (Stratagene, USA) 0.2 mM MgCl₂, 10 µl *Taq* polymerase buffer (Perkin Elmer Cetus, USA), and 2.5 U *Taq* polymerase (Perkin Elmer Cetus, USA). The reaction mixture was overlaid with mineral oil and subjected to 40 cycles of amplification. Each cycle involved denaturation at 94°C for 1.5 min, annealing at 56°C for 2.5 min, and elongation at 72°C for 2 min with a final extension for 10 min at 72°C. Amplified DNA was purified using Gene Clean (Bio101, USA), digested with the appropriate restriction enzymes [11], and heavy- and light-chain cDNAs were cloned using the λ-ImmunoZAP system (Stratagene). Cloning of the combinatorial vector was done according to the manufacturer's instructions [11]. pM3.1, which contains the information for the fragment genes of MoAb M3 as a synthetic dicistronic operon in pBluescript was obtained by *in vivo* excision. Clones were sequenced using the T7 Sequencing kit (Pharmacia, Sweden).

Overexpression

For overexpression the synthetic dicistronic operon was cloned into pET-17b (Novagen, USA). For this a *Nde* I site was introduced directly in front of the *pelB* leader sequence by PCR. The mutated leader sequence was subcloned to pBluescript (Stratagene). The 5' primer was gag aca gtc cat atg aaa tac cta tgg cct and the 3' primer was tcc tcc aga ctc gag cag ttt cac ctg ggc. Italicized regions marked the recognition sequences for *Nde* I and *Xho* I, respectively. PCR conditions were 1 min at 94°C, 1 min at 56°C and 15 s at 72°C for 30 cycles with a 10-min final extension at 72°C. Correct junctions were verified by DNA sequencing as described above. Afterwards the *Nde* I and *Xho* I restricted leader sequence and the *Xho* I, *Xba* I cut Ig fragment gene construct were combined by subcloning into p11–25 (kindly provided by S. Hirsch, Botanisches Institut, Christian-Albrechts-Universität, Kiel, Germany). This provides a *Not* I site at the 3' end of the construct. Then the complete dicistronic operon was restricted by use of *Nde* I

and *Not* I and cloned into pET-17b, generating pM3.2. pM3.2 was expressed in *Escherichia coli* B strain BI21(DE3) (Novagen) in LB medium with shaking at 280 rpm. Cells were grown in LB containing 50 µg/ml carbenicillin at 30°C until the culture became slightly turbid, streaked on a plate containing 50 µg/ml carbenicillin (Serva, Germany) and incubated overnight at 37°C. A single colony from the former plate was grown in 2 ml LB with 200 µg/ml carbenicillin at 30°C to an OD₆₀₀ of 1.0. Cells were pelleted (4,000 g, 5 min, RT) and resuspended in fresh media with 200 µg/ml carbenicillin. This culture was diluted 1.5 in LB containing 500 µg/ml carbenicillin grown at 30°C to an OD₆₀₀ of 0.6–0.8 and subsequently collected (4,000 g, 5 min, RT). Cells were resuspended in LB with 500 µg/ml carbenicillin and 0.4 mM isopropyl thiogalactoside (IPTG) and grown at ambient temperature until harvested. Further bacterial strains used for expression of pM3.2 were λDE3 lysogens of the *E. coli* K12 strains HMS174 (Novagen) and RV308 (ATCC, USA).

Lysogenization

Lysogenization of bacteria was done using the λDE3 lysogenization kit (Novagen) according to the manufacturer's conditions.

Preparation of Periplasmic Extracts

Periplasmic extracts were prepared according to Minsky et al. [12]. One milliliter induced cells were pelleted (4,000 g, 5 min, RT) and then gently resuspended in 40 µl ice-cold spheroblast buffer (0.1 M Tris/HCl, 0.5 M sucrose, 0.5 mM EDTA, pH 8.0). Two microliters lysozyme solution (2 mg/ml in H₂O) was added, followed by the addition of 80 µl spheroblast buffer in 80 µl H₂O. The cells were gently mixed and incubated on ice for 20 min. Afterwards the cell debris was pelleted (8,000 g, 10 min, 4°C) and the supernatant consisting of the periplasmic fraction was collected.

SDS-PAGE and Immunoblot Analysis

Proteins were run on a 12.5% SDS-PAGE according to Laemmli [13] and transferred to nitrocellulose (Schleicher & Schuell, Germany) by Western blotting. Immunoblot analysis was done with alkaline-phosphatase-labeled goat antimouse IgG H+L (Dianova, Germany), 1:5,000 in PBS containing 0.05% Tween 20 (Merck, Germany).

Affinity Determinations

The apparent binding constants of MoAb M3 and the cloned Fab fragment were determined by inhibition ELISA [14]. Wells of Maxi-sorp microtiter plates (Nunc, Germany) were coated with 5 µM Chi t 1.01 overnight at 4°C followed by blocking with PBS containing 5% skimmed-milk powder (Uelzena, Germany) for 30 min. Dilutions of MoAb M3 and periplasmic extracts of bacterial hosts expressing the recombinant Fab fragment in PBS containing 2% skimmed-milk powder were added and incubated 2 h at 37°C. Binding was assayed using alkaline-phosphatase-conjugated goat antimouse IgG H+L (Dianova) followed by detection with paranitrophenylphosphate (Merck; 1 mg/ml in 1 M diethanolamine, 0.5 mM MgCl₂, pH 9.6). Washing between the different steps was done with PBS containing 0.05% Tween 20 (Merck). Plates developed for 1 h and read at OD₄₉₀. For determination of the apparent affinity, antibody dilutions corresponding to an OD₄₉₀ of about 1.5 were chosen and mixed with increasing amounts of antigen in PBS containing 2% skimmed-milk powder and incubated 2 h at 37°C before using in the ELISA as described above. Apparent affinities were determined as the reciprocal of the antigen concentration required to inhibit 50% maximal binding.

Results

Cloning of the Ig fragment genes required the amplification of the cDNA synthesized from the RNA isolated from hybridoma M3. Light-chain cDNA was amplified to the codon for amino acid residue 114, the natural last amino acid of κ light chains. The IgG1 heavy-chain cDNA was amplified in a truncated form ranging to the codon for amino acid residue 235, the first cysteine residue in the murine IgG1 hinge region. Amplified light- and heavy-chain cDNAs were at the expected size of about 700 bp each (data not shown). The Fab construct was cloned as a synthetic dicistronic operon by assembling distinct light- and heavy-chain vectors via a nonsymmetric *EcoRI* site [11]. In this dicistronic operon each gene is fused to the *pelB* signal sequence of *Erwinia carotovora* to direct protein secretion through the bacterial cytoplasmic membrane into the periplasmic space of the bacterial host. The oxidative environment of the periplasmic space permits the formation of the disulfide bridges of the Ig fragments.

The nucleotide and deduced amino acid sequence of MoAb M3 light- and heavy-chain variable regions are shown in figure 1. Comparison of the sequences of V_H and V_L domains with the Kabat database [15] revealed that the light-chain V gene belongs to the $V_{\kappa}II$ group of the Ig family, and the heavy-chain V gene to the V_HIIC group. The nucleotide sequence of the heavy- and light-chain V genes were screened against the GeneBank database using the program BlastN [16]. This showed that the sequence of the M3 V_H gene is most closely related to heavy-chain V genes from antibodies recognizing small epitopes, mainly haptens, as nitrophenyl phosphonate [17], GAT [18], the multiterminal peptide (T-G)_n-A-L [19], and 4-hydroxy-3-nitrophenyl-acetyl [20]. The identities are more than 90%. All of these antibodies are members of the V_HIIC group. The situation is the same for the light-chain V gene. Even the $V_{\kappa}II$ group is extensively used in response to haptens as (T-G)_n-A-L [19, 21], fluorescein [22], and chemically modified sweeteners [23], and the identities among these sequences are higher than 90%.

Due to poor expression from pM3.1 (data not shown) the Ig fragment genes were cloned into pET-17b, which generates pM3.2 pET-17b belongs to the strong T7 system. In this system, expression of the genes of interest is driven by T7 RNA polymerase cloned under control of the strong UV5 promoter inducible by IPTG [24]. pM3.2 was transformed into BI21(DE3) cells. Expression experiments revealed instability of the clones due to toxicity of the cloned genes. Stabilization of the clones was possible by the use of high amounts of carbenicillin and several changes of the growth

medium. The bacteria were grown in LB medium at 30°C and gene expression was induced at ambient temperature for 4 h by IPTG. Expression at 37 and 30°C revealed non-soluble protein, whereas at ambient temperature parts of the recombinant are soluble (data not shown).

Analysis of whole bacterial protein after induction using reducing conditions showed 2 bands of 24.3 and 24 kDa (fig. 2). This is consistent with the expected size of the truncated heavy-chain and the complete light-chain protein. The yield of the expressed recombinant light- and heavy-chain protein is estimated as 20–30 mg/l culture. The periplasmic protein of induced bacteria was obtained by cold osmotic shock. Analysis of the periplasmic protein using nonreducing conditions revealed a protein band of approximately 49 kDa, the size of an assembled Fab fragment. Assembled Fab fragments yielded a maximum 3 h after induction. No Fab fragments could be detected in the culture supernatant. The assembled protein is estimated as 1–1.5% of the whole recombinant protein.

To analyze the influence of the bacterial strain on the yield of transported and assembled protein we used λ DE3 lysogens of the *E. coli* strains HMS174 and RV308. While the whole expression of light- and heavy-chain protein in HMS174(DE3) was less compared to BI21(DE3), the amount of transported and assembled protein is in the same range. RV308(DE3) was not appropriate for the expression of pM3.2.

To determine the apparent binding affinities of MoAb M3 and the recombinant Fab fragment, we performed inhibition ELISAs. As shown in figure 3 the K_A of MoAb M3 was $1.6 \times 10^6 \text{ M}^{-1}$, whereas that of the recombinant Fab fragment was $1.1 \times 10^6 \text{ M}^{-1}$. The results demonstrate that the Fab fragment retained the same binding affinity as the parental antibody MoAb M3.

Discussion

For a better understanding of type-I allergy, detailed knowledge about the structure of allergens, particularly their IgE reactive epitopes, is necessary. We have cloned and expressed in *E. coli* the Ig fragment genes coding for the Fab fragment of MoAb M3. This is reactive with Chi t 1.01, the major allergen of *C. thummi thummi* [7, 9].

The V genes of M3 heavy and light chains belong to the murine V_HIIC and $V_{\kappa}II$ groups, respectively [15]. Sequence comparison with other heavy- and light-chain V genes shows strong similarities with hapten-specific antibodies [17–23]. These sequence similarities are consistent with the recent finding that MoAb M3 recognizes a linear epitope of

V_L

		FR I																	
		<u>Sac I</u>																	
1	1	GAG	CTC	GTG	ATG	ACC	CAG	TCT	CCA	CTC	TCC	CTG	TCT	GTC	AGT	CTT	GGA	48	
		E	L	V	M	T	Q	S	P	L	S	L	S	V	S	L	G	16	
		V _L -CDR I-----																	
49	17	GAT	CAA	GCC	TCC	ATC	TCT	TGC	AGA	TCT	AGT	CAG	AGC	CTT	GTA	CAC	ACT	96	
		D	Q	A	S	I	S	C	R	S	S	Q	S	L	V	H	T	27	
		-----FR II																	
97	28	AAT	GGA	AAC	ACC	TAT	TTA	CAT	TGG	TAC	CTG	CAG	AAG	CCA	GGC	CTG	TCT	144	
		N	G	N	T	Y	L	H	W	Y	L	Q	K	P	G	L	S	43	
		V _L -CDR II-----FR III																	
145	44	CCA	AAG	CTC	CTG	ATC	TAC	ATA	GTT	TCC	AAC	CGA	TTT	TCT	GGG	GTC	CCA	192	
		P	K	L	L	I	Y	I	V	S	N	R	F	S	G	V	P	59	
193	60	GAC	AGG	TTC	AGT	GGC	AGT	GGA	TCA	GGG	ACA	GAT	TTC	ACA	CTC	AAG	ATC	240	
		D	R	F	S	G	S	G	S	G	T	D	F	T	L	K	I	75	
		V _L -CDR III-----																	
241	76	AGC	AGA	GTG	GAG	GCT	GAG	GAT	CTG	GGA	GTT	TAT	TTC	TGC	TCT	CAA	AGT	288	
		S	R	V	E	A	E	D	L	G	V	Y	F	C	S	Q	S	91	
		-----FR IV																	
289	92	ACA	CAT	GTT	CCT	GGA	ACG	TTC	GGT	GGA	GGC	ACC	AAG	CTG	GAA	ATC	AAA	336	
		T	H	V	P	G	T	F	G	G	G	T	K	L	E	I	K	107	

V_H

		FR I																	
		<u>Xho I</u>																	
1	1	CAG	GTG	AAA	CTG	CTC	GAG	TCT	GGG	GCA	GAG	CTT	GTG	AAG	TCA	GGG	GCC	48	
		Q	V	K	L	L	E	S	G	A	E	L	V	K	S	G	A	16	
		V _H -CDR I																	
49	17	TCA	GTC	AAG	TTG	TCC	TGC	ACA	GCT	TCT	GGC	TTC	AAC	ATT	AAA	GAC	ACC	96	
		S	V	K	L	S	C	T	A	S	G	F	N	I	K	D	T	32	
		-----FR II																	
97	33	TAT	ATG	CAC	TGG	GTG	AAA	CAG	AGG	CCT	GAA	CAG	GGC	CTG	GAG	TGG	ATT	144	
		Y	M	H	W	V	K	Q	R	P	E	Q	G	L	E	W	I	48	
		V _H -CDR II-----																	
145	49	GGA	AGG	ATT	GAT	CCT	GCG	AAT	GGT	GAG	ATT	AAA	TAT	GAC	CCG	AAG	TTC	192	
		G	R	I	D	P	A	N	G	E	I	K	Y	D	P	K	F	64	
		--FR III																	
193	65	CAG	GGC	ACG	GCC	ACT	ATA	ACA	GCA	GAC	ACA	TCC	ACC	AAC	ACA	GCC	TAC	240	
		Q	G	T	A	T	I	T	A	D	T	S	T	N	T	A	Y	79	
241	80	CTG	CAA	CTC	AGC	AGC	CTG	ACA	TCT	GAG	GAC	ACT	GCC	GTC	TAT	TAT	TGT	288	
		L	Q	L	S	S	L	T	S	E	D	T	A	V	Y	Y	C	92	
		V _H -CDR III-----FR IV																	
289	93	GTT	AGA	AGG	GGC	TAT	GGT	AGT	AGC	CAG	GAG	CCT	TAC	TGG	GGC	CAA	GGC	336	
		V	R	R	G	Y	G	S	S	Q	E	P	Y	W	G	Q	G	106	
337	107	ACC	ACT	CTC	ACA	GTC	TCC	TCA	357										
		T	T	L	T	V	S	S	113										

Fig. 1. Nucleotide and deduced amino acid sequence of MoAb M3 light- and heavy-chain variable region genes. The numbering of the amino acids is according to Kabat et al. [15]. FR = Framework region; CDR = complementarity-determining region. The CDRs are marked by broken lines above the nucleotide sequence. The nucleotide sequences are deposited in the EMBL database under the accession numbers Z37299 (light chain) and Z37502 (heavy chain).

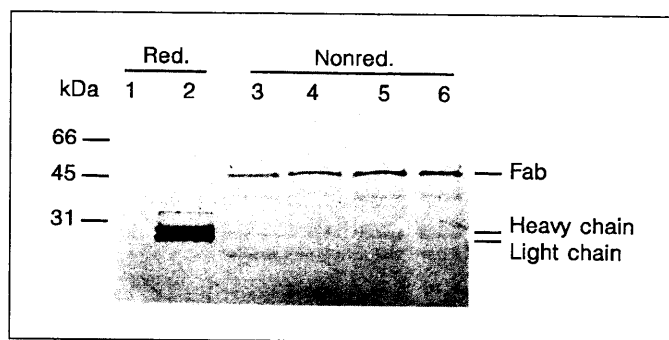


Fig. 2. Immunoblot analysis of MoAb M3 Fab fragment expressed in BI21(DE3) cells. Lanes 1, 2 = Whole bacterial protein of 50 μ l cells before and 4 h after induction with IPTG; lanes 3–6 = periplasmic extracts of 1 ml induces cells following the expression course for 1, 2, 3, and 4 h. The molecular weights are shown in the left-hand margin. Red. = Reduced protein; Nonred. = nonreduced protein. Proteins were run on 12.5% SDS-PAGE according to Laemmli [13], transferred to nitrocellulose and probed with alkaline-phosphatase-labeled goat anti-mouse IgG H+L.

5 amino acid residues in length. This epitope comprises amino acid residues 13–17 of Chi t 1.01 [9]. It remains to be elucidated whether the primary structure of the heavy- and light-chain V genes may in general be used to obtain information about the nature of an epitope.

The expression of the Ig fragment genes was strongly influenced by toxicity of the cloned genes to the bacterial hosts. Toxicity of Ig fragment genes that resulted in instability of the cloned DNA was also reported by other investigators [25]. Stabilization of the bacterial clones could be achieved by the use of high amounts of the selecting antibiotic and several changes of the growth medium. This provides a growing selection pressure. Thus bacteria which have lost the plasmid were not able to overgrow the culture.

Expression yielded 20–30 mg of the Fab protein per liter culture, comparable to amounts obtained by other authors [17, 26]. The growth temperature is important for the expression of functional Fab fragments. Expression at 37 and 30°C resulted in nonsoluble protein that is not in a transport-competent form. Expression at ambient temperature revealed partly soluble protein that assembled to the functional active Fab fragment.

Expression of the Ig fragment genes in different bacterial strains had no significant effect of the amount of transported protein. The amount of active Fab fragments can be increased by cotransformation of the bacterial hosts with chaperones such as DnaK and DnaJ as well as core components of the bacterial protein export machinery such as secY and secE [unpubl. data] or by cultivating the bacteria in a fermentor [3, 27].

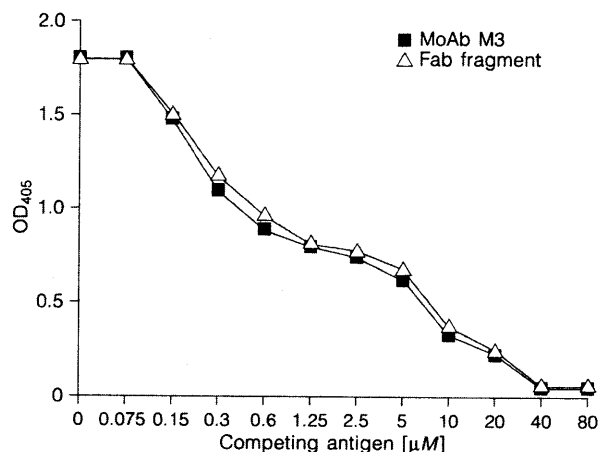


Fig. 3. Affinity determination by inhibition ELISA. The binding of MoAb M3 and the recombinant Fab fragment was inhibited by Chi t 1.01.

It is reported that cloned Fab fragments are released to the growth medium [2] from where they can be easily prepared for further experiments. Other authors describe that the expressed antibody fragments remain mainly within the bacterial periplasm [3]. In our experiments, no Fab fragments could be detected in the culture supernatant; thus further steps for purification from the periplasmic space of the bacteria are necessary in order to obtain the material for structural experiments. A different expression system might be helpful for this approach. Determination of the apparent binding affinities indicated that the recombinant Fab fragment had a similar K_A as the parent antibody, MoAb M3. These affinity constants are in the range typically seen for hapten-binding antibodies [25].

Since patients' IgE is reactive with amino acid residues 1–17 of Chi t 1.01 [7] and only amino acid residues 14–17 of this component show an increased antigenic index as determined by the method of Jameson and Wolf [9] there is a high chance that MoAb M3 recognizes a human IgE reactive epitope. In future experiments the recombinant Fab fragment should be used as a tool to elucidate the 3D structure of the antibody/antigen complex; thus, for the first time a human IgE reactive epitope in complex form may be shown.

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