Sequence analysis and bacterial production of the anti-c-*myc* antibody 9E10: the V_H domain has an extended CDR-H3 and exhibits unusual solubility

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Abstract The cDNAs for the two variable domains of the antibody 9E10 were cloned from the hybridoma cell line. A chimeric 9E10 F_{ab} fragment was produced in *E. coli* under control of the tightly controlled tetracycline promoter. The functional F_{ab} fragment was isolated in a single step via a His_6-tag, which also served for its recognition by a nickel chelate-alkaline phosphatase conjugate. Thus, the recombinant F_{ab} fragment permitted the immunochemical detection of the *myc* tag in a sandwich ELISA. The dissociation constant for the interaction with the *myc* tag peptide was determined as 80 ± 5 nM by fluorescence titration. In an attempt to produce the smaller 9E10 F_v fragment it was found that its V_H domain alone can be readily isolated from *E. coli* as a soluble protein. This unusual behaviour may be explained by the 18 amino acid-long CDR-H3 and could be of value in the design of 'single domain' antibodies.

Key words: Antibody engineering; *E. coli* secretion; Immunoglobulin; F_{ab} fragment; *Myc* tag

1. Introduction

The murine anti-c-*myc* antibody 9E10 [1] serves as a widespread immunochemical reagent in cell biology and in protein engineering. The IgG1/κ monoclonal antibody was originally raised against a synthetic peptide immunogen derived from the human c-*myc* proto-oncoprotein [1]. Since then it has been utilized in cancer research for detecting and studying the distribution of the c-*myc* oncogene product by immunohistochemistry (see e.g. [2,3]).

Soon after its development it was discovered that the epitope of the antibody, comprising the amino acid sequence 'Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn', can be expressed in a different protein context and still confers recognition by the 9E10 immunoglobulin [4]. Thus, the so-called *myc* tag was created, the prototypic example for an increasing number of short affinity tags, which have nowadays gained considerable popularity as tools for the detection and/or purification of recombinant proteins [5].

In the past years numerous publications appeared where 9E10 was employed for the immunochemical identification or separation and biochemical characterization of a variety of proteins that had been genetically fused with the *myc* tag sequence. By means of different experimental techniques the monoclonal antibody was used, e.g. for monitoring expression of recombinant polypeptides in their foreign hosts and for investigating the intracellular targeting of proteins (for some topical applications see [6–10]).

In addition, 9E10 proved to be of considerable value in the field of antibody engineering, where the *myc* tag was utilized during the cloning [11,12], bacterial production [13,14], and phage display [15,16] of antibody fragments. Thus, in conjunction with the antibody 9E10 differing recombinant F_v, scF_v, and F_{ab} fragments can be applied for the detection of specific antigens in immunochemical assays.

In order to make 9E10 itself amenable to protein engineering, we describe here the full length sequences of its variable domains and we demonstrate that its bacterially produced F_{ab} fragment is directly suited for identifying a *myc* tag fusion protein.

2. Materials and methods

2.1. Cloning procedures

DNA manipulations were performed according to standard methodology [17]. *E. coli* K-12 strain JM83 [18] was used for cloning and production of the recombinant antibody fragments. Cloning and expression vectors were from the author's collection (see Table 1).

2.2. Purification and sequencing of the monoclonal antibody 9E10

9E10 hybridoma cells were cultured in a 1:1 mixture of Iscove's Modified Dulbecco's and Ham's F12 medium containing 10% fetal calf serum. After 2 to 3 days 5 to 10 ml of the supernatant was applied to a 1 ml column with protein A agarose (Sigma). The column was washed with PBS buffer (4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl) and the antibody was eluted with 0.1 M glycine/HCl pH 2.8, followed by immediate neutralization with 1 M Tris/HCl pH 8.0 (80 μl per 1 ml fraction) and dialysis against 100 mM Tris/HCl pH 8.0. SDS-PAGE revealed that the antibody preparation was pure and consisted of a single light and a single heavy chain. The two chains were then separated by SDS-PAGE with a borate buffer system and electro-transferred to an Immobilon P membrane (Millipore). After staining with Coomassie Brilliant Blue R250 the bands were cut out and subjected to N-terminal sequencing in an Applied Biosystems Procise Sequencer.

2.3. Amplification of the variable domain genes

PCR was performed after oligo-dT-primed first strand cDNA synthesis from total RNA of the Myc1-9E10 cell line [1] using *Pfu* DNA polymerase and phosphorothioate primers as previously described [19,20]. V_H was amplified with the primers V_Hc3-T, 5′-GAG GTS MAR CTG CAG SAG TCW GG, and V_Hr2-T, 5′-TGA GGA GAC GGT GAC CGT GGT SCC TTG GCC CC. V_L was amplified with V_κc1-T, 5′-GAC ATT GAG CTC ACM CAG WCT CCA KYC TCC CTG KCT G, and V_κr1-T, 5′-CCG TTT CAG CTC GAG CTT GGT SCC WSC WCC GAA CGT. The amplification products were purified by agarose gel electrophoresis and cut with the restriction

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Abbreviations: BSA, bovine serum albumin; IMAC, immobilized metal affinity chromatography; OmpA, outer membrane protein A; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PhoA, alkaline phosphatase

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enzymes *Pst*I and *Bst*EII in the case of V_H and *Sst*I and *Xho*I in the case of V_L (cf. underlined recognition sites in the primer sequences). The V_H and V_L genes were separately inserted into the F_{ab} fragment expression vector pASK85 (Table 1) that had been cut with the same enzymes. Plasmid DNA from several clones was analysed by restriction digest and, for some of them, by double strand dideoxy-sequencing (2 for V_L and 6 for V_H) using primers as described [20] and a T7 sequencing kit (Pharmacia). The variable domain genes from one V_L clone and one V_H clone (with a continuous reading frame; see text) were combined into a single plasmid utilizing the *Xba*I and *Nco*I restriction sites on the vector, yielding pASK85-9E10.

2.4. Site-directed mutagenesis

The primers 5′-GTC TCC CCC AGA CTC AAC CAG ATC TAC TTC GGC CTG CGC T and 5′-AGA GAT ACA GCC AGG CTA GCT GGA GAC TGT GTG AGT ACG ATG TCG GCT T were used for correcting the N-terminal coding regions of the cloned V_H and V_L genes, respectively, according to the amino acid sequences that had been determined for the hybridoma immunoglobulin (see Results). During this step the *Pst*I site at the 5′ end of V_H was replaced by *Bgl*II and the *Sst*I site at the 5′ end of V_L was replaced by *Nhe*I (cf. Fig. 1). Site-directed mutagenesis was performed with single-stranded deoxyuridin-containing pASK85-9E10 plasmid DNA [21] using both oligodeoxynucleotides. The plasmid from one positive clone, whose proper assembly was confirmed by restriction analysis and by DNA sequencing, was designated pASK85-9E10a. The resulting V_H and V_L nucleotide sequences were deposited at the EMBL Database with accession codes AJ000488 and AJ000489, respectively.

2.5. Production of the recombinant antibody fragments

For the bacterial production of the 9E10 F_{ab} fragment the corrected V_H and V_L genes were transferred from pASK85-9E10a via the restriction sites *Xba*I and *Bst*EII and *Nco*I and *Xho*I, respectively, into the vector pASK88 (Table 1). The F_{ab} fragment thus encoded on pASK88-9E10a carried human constant domains instead of those from mouse so that its detection by commercially available, chain-specific antibodies was easier. Furthermore, the two immunoglobulin chains were better separated during SDS-PAGE [22] in this case. A 2 l *E. coli* culture of JM83 transformed with pASK88-9E10a was grown in a shaking flask at 22°C to an optical density at 550 nm of 0.5 and induced by the addition of 200 μl of a 2 mg/ml solution of anhydrotetracycline (ACROS Chimica) in dimethylformamide [23]. After further shaking for 2.5 h at 22°C periplasmic cell fractionation and IMAC purification of the F_{ab} fragment was performed as described [24].

The periplasmic cell fraction containing the D1.3 scF_v fragment [25] with a $(Gly_4Ser)_3$ linker, which was used as antigen in the ELISA (see below), was prepared according to the same procedure. In this case the *tet* promoter vectors pASK98-D1.3 and pASK101-D1.3 were employed for the synthesis of scF_v fragments with the C-terminally fused *Strep*-tag or *myc* tag, respectively (see Table 1).

For the bacterial secretion of the 9E10 V_H domain the vector pASK90 was used (Table 1), which carried the generic cloning cassette for F_v fragments from pASK68 [14] inserted between the *Xba*I and *Hin*dIII sites of pASK75 [23]. The V_H gene was transferred from pASK85-9E10a to pASK90 via the restriction sites *Xba*I and *Bst*EII, yielding pASK90-9E10-VH. Bacterial cell growth and induction was performed as above and the soluble V_H domain was affinity-purified from the periplasmic protein fraction on immobilized engineered streptavidin [27] via its C-terminal *Strep*-tag [26]. Chromatography was carried out in the presence of 100 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA. 2.5 mM desthiobiotin (Sigma) dissolved in the same buffer was used for competitive elution [27].

2.6. ELISA

ELISA was performed in a 96 well microtitre plate (Becton Dickinson) with an incubation time of 45 min at ambient temperature, followed by washing three times with PBS/Tween (PBS containing 0.1% v/v Tween 20), unless otherwise stated. The wells were coated with 3 mg/ml lysozyme (Sigma) dissolved in 50 mM $NaHCO_3$ pH 9.6 over night and blocked with 3% w/v BSA, 0.5% v/v Tween 20 in PBS for 1 h. After washing with PBS/Tween the wells were incubated in the first step with 50 μl of the periplasmic protein fraction that contained the D1.3 scF_v fragment fused with either the *myc* tag or the *Strep*-tag. For the second incubation a dilution series of the purified 9E10 F_{ab} fragment in 50 μl PBS/Tween was applied. Its concentration was determined with a calculated absorption coefficient at 280 nm of 1.41 mg ml^{-1} cm^{-1}. PBS/Tween was used as a negative control during these two incubations (see legend to Fig. 3). Bound F_{ab} fragment was detected in the third incubation with a nickel chelate-alkaline phosphatase conjugate (Qiagen), diluted 1:1000 in PBS/Tween. After finally washing twice with PBS/Tween and twice with PBS, 100 μl of a solution of 0.5 mg/ml --nitrophenylphosphate in 1 M Tris/HCl pH 8.0, 1 mM $ZnSO_4$, 5 mM $MgCl_2$ was added and enzymatic activity was measured at 25°C as the change in absorbance at 405 nm per min with a SpectraMAX 250 instrument (Molecular Devices). Curve fitting was performed by non-linear least squares regression using an equation of the type $y = a \cdot x/(b+x)$ [27].

2.7. Peptide synthesis and fluorescence titration

The *myc* tag peptide comprising the amino acid sequence Abz-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn-COOH was assembled on a PS3 synthesizer (RAININ Instrument Co.) using solid phase Fmoc chemistry, except for the 2-aminobenzoyl group (Abz), which was coupled as the Boc-protected derivative (Bachem Biochemica). The molecular mass of the HPLC-purified peptide was confirmed by ESI mass spectrometry. Fluorescence titration of the 9E10 F_{ab} fragment or the V_H domain was carried out as previously described [27]. Protein concentrations were adjusted using calculated extinction coefficients [28]. The dissociation constant for the F_{ab} fragment was determined from a non-linear least-squares fit of the binding isotherm according to bimolecular complex formation after the fluorescence data measured in the presence of the *myc* tag peptide had been corrected for the small effect that was observed in an independent titration with a solution of recrystallized 2-aminobenzamide (MERCK-Schuchardt).

3. Results

3.1. Analysis of the variable domain sequences

The coding sequences for the two variable domains of 9E10 were amplified after first strand cDNA synthesis from total RNA of the cell line and separately cloned in the vector pASK85 [23]. Degenerate PCR primers which annealed to the partly conserved regions at both ends of the variable do-

Table 1
$Tet^{p/o}$ expression vectors used in this study

Plasmid	Expression cassette[a]	Reference
pASK85	OmpA-(V_H)-$C_H1\gamma1$-His_6; PhoA-(V_κ)-C_κ	[23]
pASK85-9E10	OmpA-V_H-$C_H1\gamma1$-His_6; PhoA-V_κ-C_κ	this study
pASK85-9E10a	OmpA-V_H-$C_H1\gamma1$-His_6; PhoA-V_κ-C_κ	this study
pASK88	OmpA-(V_H)-$huC_H1\gamma1$-His_6; PhoA-(V_κ)-huC_κ	[29]
pASK88-9E10a	OmpA-V_H-$huC_H1\gamma1$-His_6; PhoA-V_κ-huC_κ	this study
pASK90	OmpA-(V_H)-Strep; PhoA-(V_κ)-myc	A. Skerra, unpuplished
pASK90-9E10-VH	OmpA-V_H-Strep; PhoA-(V_κ)-myc	this study
pASK98-D1.3	OmpA-V_H-sc-V_κ-Strep	A. Skerra, unpuplished
pASK101-D1.3	OmpA-V_H-sc-V_κ-myc	A. Skerra, unpuplished

[a]OmpA and PhoA denote the bacterial signal sequences, whereas His_6, Strep, and myc represent the different affinity tags used. V_H and V_κ in parentheses mean that the corresponding vector just carries the cloning site instead of a variable domain gene.

main sequences and *Pfu* DNA polymerase with proofreading activity were used for this purpose (for details, see Section 2). Only one type of amplification product was found each for V_H and for V_L (Fig. 1), although in roughly half of the V_H sequences a point deletion was observed. The substitution of the nucleotides 'AG' (position 260/261 in the V_H sequence) by a single 'C' led to a frameshift within framework region 3 in these clones.

In order to confirm whether the correct sequences had been amplified, the antibody 9E10 was isolated from the culture supernatant of the hybridoma cell line and its chains were subjected to N-terminal amino acid sequencing. The stretch

V_H:

```
          BglII
  1 gaagtagatctggttgagtctggGGGAGACTTAGTGAAGCCTGGAGGGTCCCTGAAACTC  60
         ta a   cag
    GluValAspLeuValGluSerGlyGlyAspLeuValLysProGlyGlySerLeuLysLeu

 61 TCCTGTGCAGCCTCTGGATTCACTTTCAGTCACTATGGCATGTCTTGGGTTCGCCAGACT 120
    SerCysAlaAlaSerGlyPheThrPheSerHisTyrGlyMetSerTrpValArgGlnThr
                                  ==== CDR-H1 ===

121 CCAGACAAGAGGCTGGAGTGGGTCGCAACCATTGGTAGTCGTGGTACTTACACCCACTAT 180
    ProAspLysArgLeuGluTrpValAlaThrIleGlySerArgGlyThrTyrThrHisTyr
                               ====================== CDR-H2 ====

181 CCAGACAGTGTGAAGGGACGATTCACCATCTCCAGAGACAATGACAAGAACGCCCTGTAC 240
    ProAspSerValLysGlyArgPheThrIleSerArgAspAsnAspLysAsnAlaLeuTyr
    ==================

241 CTGCAAATGAACAGTCTGAAGTCTGAAGACACAGCCATGTATTACTGTGCAAGAAGAAGT 300
    LeuGlnMetAsnSerLeuLysSerGluAspThrAlaMetTyrTyrCysAlaArgArgSer
                                                      ======

301 GAATTTTATTACTACGGTAATACCTACTATTACTCTGCTATGGACTACTggggccaaggc 360
    GluPheTyrTyrTyrGlyAsnThrTyrTyrTyrSerAlaMetAspTyrTrpGlyGlnGly
    ================== CDR-H3 ======================

         BstEII
361 accacggtcaccgtctcctca 381
    ThrThrValThrValSerSer
```

V_L:

```
                          NheI
  1 gacatcgtactcacacagtctccagctagcctggctgTATCTCTAGGACAGAGGGCCACC  60
        ag         a      tctc
    AspIleValLeuThrGlnSerProAlaSerLeuAlaValSerLeuGlyGlnArgAlaThr

 61 ATCTCCTGCAGAGCCAGCGAAAGTGTTGATAATTATGGCTTTAGTTTTATGAACTGGTTC 120
    IleSerCysArgAlaSerGluSerValAspAsnTyrGlyPheSerPheMetAsnTrpPhe
          ================== CDR-L1 ====================

121 CAACAGAAACCAGGACAGCCACCCAAACTCCTCATcTaTGCTATATCCAACCGAGGATCC 180
    GlnGlnLysProGlyGlnProProLysLeuLeuIleTyrAlaIleSerAsnArgGlySer
                                       ====== CDR-L2 =======

181 GGGGTCCCTGCCAGGTTTAGTGGCAGTGGGTCTGGGACAGACTTCAGCCTCAACATCCAT 240
    GlyValProAlaArgPheSerGlySerGlySerGlyThrAspPheSerLeuAsnIleHis

241 CCTGTAGAGGAGGATGATCCTGCAATGTATTTCTGTCAGCAAACTAAGGAGGTTCCGTGG 300
    ProValGluGluAspAspProAlaMetTyrPheCysGlnGlnThrLysGluValProTrp
                                     ========== CDR-L3 ======

                        XhoI
301 acgttcggagctggcaccaagctcgagatcaaa 333
    ThrPheGlyAlaGlyThrLysLeuGluIleLys
    ===
```

Fig. 1. Nucleotide and encoded amino acid sequences for the variable domain genes of the antibody 9E10 cloned on pASK88-9E10a. The sequences that were part of the degenerate PCR primers at each 5′ and 3′ end are depicted in lower case letters. The single nucleotides that are shown below the consecutive sequences correspond to those sequences which had initially been cloned and include a *Pst*I (nucleotides 10 to 15 in V_H) and a *Sst*I (nucleotides 7 to 12 in V_L) restriction site, respectively. The singular *Bgl*II and *Nhe*I restriction sites that are shown were introduced during repair via site-directed mutagenesis of the differences compared with the experimentally determined primary structure. The amino acid sequences encoded by the corrected genes (starting with the first residue of each mature antibody chain) are given below with the CDRs labelled [33]. The N-terminal regions that were confirmed by amino acid sequencing of the native antibody are underlined.

of 18 amino acids that was determined for V_H (underlined in Fig. 1) corresponded to the cloned sequence, except for Lys H3 and Gln H5, whose coding sequences were part of the vector. The 20 N-terminal amino acids that were sequenced for V_L were also in accordance with the amplified cDNA. In this case the codons for Glu L3, Thr L7, and Val L9, which were either part of the vector or introduced by the PCR primer, were different. Based on these findings and on the antigen-binding activity of the subsequently produced recombinant F_{ab} fragment (see below) it was concluded that the cloned genes indeed represented the variable domains of 9E10.

3.2. *Bacterial production of 9E10 as a functional F_{ab} fragment*

For the production as a recombinant F_{ab} fragment and its biochemical characterization the differences between the cloned N-terminal coding regions and the experimentally determined amino acid sequences were first corrected by site-directed mutagenesis (cf. Fig. 1). The two variable domain genes were then combined into a generic vector for the bacterial secretion of F_{ab} fragments with human IgG1/κ constant domains [29], yielding the plasmid pASK88-9E10a. Gene expression was under control of the tightly regulated and chemically inducible *tet* promoter/operator in this case [23]. The two encoded polypeptide chains ended each with a Cys residue, giving rise to the interchain disulfide bond in the F_{ab} fragment. In addition, the heavy chain had a His_6 tag appended [24].

The chimeric F_{ab} fragment was produced in *E. coli* at the shaker flask scale according to our standard protocol [20,29]. The recombinant protein was purified from the periplasmic cell fraction in a single step via IMAC with Zn(II) [24], whereby the 9E10 F_{ab} fragment was eluted as a homogeneous protein fraction by means of an imidazole concentration gradient. SDS-PAGE revealed the presence of both of its chains and the correct formation of the interchain disulfide bond (Fig. 2).

Binding activity for the *myc* tag was determined with the purified 9E10 F_{ab} fragment in an ELISA (Fig. 3). For this purpose the scF_v fragment derived from the anti-lysozyme

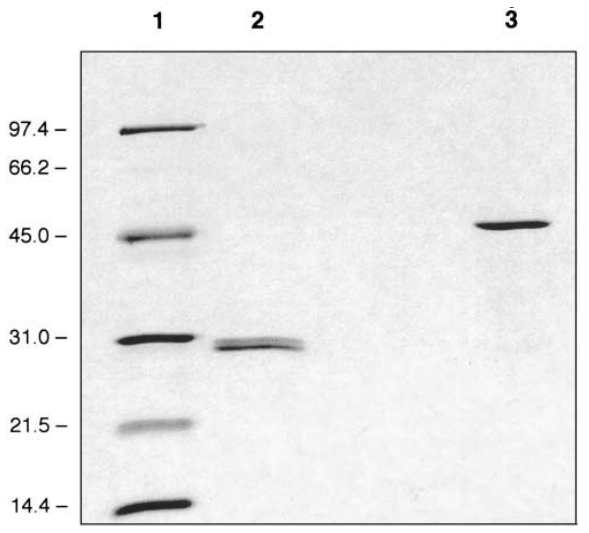

Fig. 2. Twelve procent SDS-PAGE of the bacterially produced 9E10 F_{ab} fragment after IMAC purification from the periplasmic cell fraction. Lanes: 1, molecular size standard (numbers are given in kDa); 2, purified F_{ab} fragment; 3, as in lane 2, without reduction of the disulfide bonds.

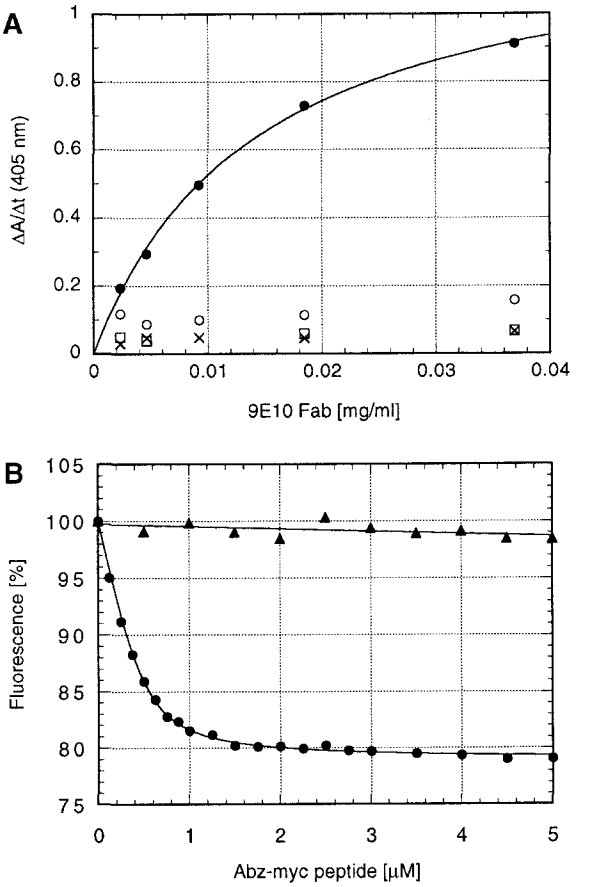

Fig. 3. Determination of antigen-binding activity for the recombinant 9E10 F_{ab} fragment. (A) Detection of the *myc* tag in an ELISA. Microtitre plate wells coated with lysozyme were incubated with the scF_v fragment of the D1.3 antibody carrying either the *myc* tag (filled circles and crosses) or the *Strep*-tag (open circles). Lysozyme and scF_v fragment were omitted in a control (open squares). A dilution series of the purified 9E10 F_{ab} fragment was then added (except for a further control, crosses), followed by detection with a nickel chelate-alkaline phosphatase conjugate. (B) Fluorescence titration of the purified 9E10 F_{ab} fragment (0.5 μM, circles) and the isolated V_H domain (1 μM, triangles) with the synthetic *myc* tag peptide. Protein tryptophan and tyrosine fluorescence was excited at 280 nm and measured at 345 nm (F_{ab} fragment) or 340 nm (V_H domain). The *myc* tag was equipped with an Abz group at its amino terminus, effecting resonance energy transfer and thus strong fluorescence quenching upon complex formation with the protein [27].

antibody D1.3 [25], which had been equipped with the *myc* tag at its C-terminus, was used as antigen. When this scF_v fragment was bound to a microtitre plate that had been coated with lysozyme a clear concentration-dependent signal was obtained upon addition of the 9E10 F_{ab} fragment. No signal was detected when the same scF_v fragment carrying the *Strep*-tag [13] instead of the *myc* tag was applied. The precise affinity between the 9E10 F_{ab} fragment and the synthetic *myc* tag peptide was determined by fluorescence titration, yielding a K_d value of 80 ± 5 nM (Fig. 3B).

3.3. *Expression studies with the recombinant 9E10 F_v fragment and its V_H domain*

For the production of the smaller F_v fragment of 9E10 a vector was constructed based on earlier plasmids [14,23,30] that directed secretion of the two variable domains into the

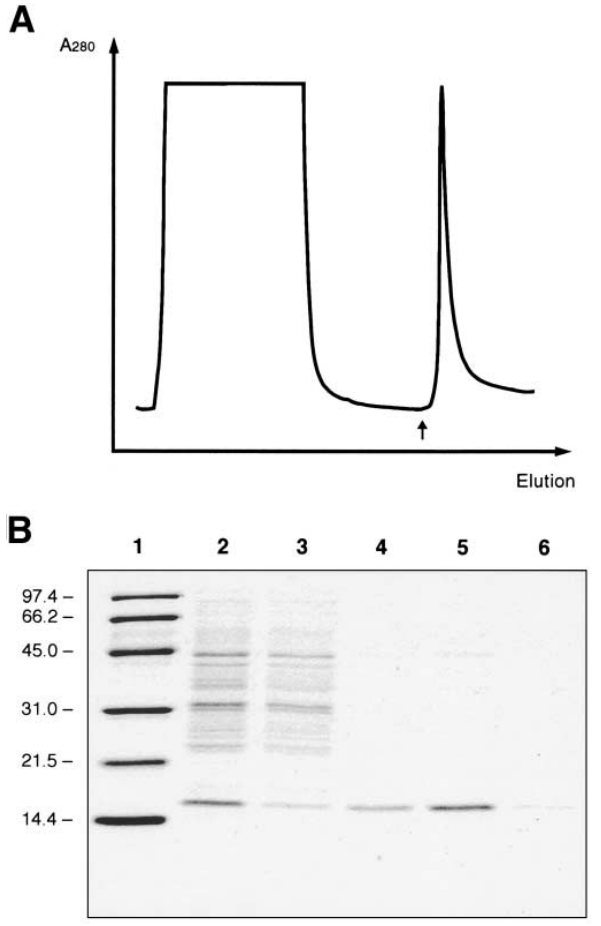

Fig. 4. Purification of the 9E10 V_H domain carrying the *Strep*-tag as a soluble protein from the bacterial periplasmic fraction. (A) Elution profile of the streptavidin affinity chromatography (see Section 2). The pure V_H domain was obtained in a single peak by competitive elution (arrow). (B) 15% SDS-PAGE of the purified V_H domain. Lanes: 1, molecular size standard (numbers are given in kDa); 2, periplasmic fraction containing the V_H domain; 3, flow through; 4 to 6, fractions from the peak in (A).

periplasm of *E. coli*. In this case the V_H domain carried the *Strep*-tag [13] and the V_L domain carried a His_6-tag at its C-terminus. When the corresponding recombinant F_v fragment was purified via streptavidin affinity chromatography [26,27] from the fraction of the soluble periplasmic proteins the V_H chain remarkably appeared in significant excess (not shown).

In all cases so far investigated by us F_v fragments had been isolated with stoichiometric composition of the two chains. This was owing to the gentle elution conditions for *Strep*-tag fusion proteins under which the non-covalent domain association is retained [13,26]. Although an excess of the V_L domain, which is known for its tendency to form Bence Jones-like dimers [31], can often be detected in the periplasmic protein fraction, it gets lost during the affinity chromatography as long as it does not carry the same tag. The V_H domain, on the other hand, aggregates if it is not correctly assembled with V_L and, therefore, any excess of it normally remains associated with the bacterial spheroplasts.

In order to further investigate the present phenomenon the vector pASK90-9E10-VH was constructed, which was devoid of the V_L gene. As in the case of the F_v fragment the vector encoded the V_H domain with an N-terminal *Omp*A signal sequence and with the C-terminal *Strep*-tag. The corresponding periplasmic cell fraction was applied to a streptavidin column and bound protein was eluted in a physiological buffer that contained desthiobiotin as competitor for the *Strep*-tag/streptavidin interaction [27]. Surprisingly, a pronounced peak was obtained, which contained the pure immunoglobulin chain (Fig. 4). Thus, the 9E10 V_H domain can be individually produced as a soluble protein in *E. coli*. However, a specific binding activity for the *myc* tag was not detected in the case of this immunoglobulin fragment (Fig. 3B).

4. Discussion

The full length cDNA sequences have been elucidated for the variable regions of the monoclonal anti-c-*myc* antibody 9E10 by means of a polymerase chain reaction with primers that hybridize in the FR1 and J region, followed by cloning of the amplified V genes. Recently, partial sequences were described for the same antibody, which had been obtained by direct sequencing of one-side PCR products [32]. However, although an elaborate strategy had been employed for the efficient amplification of the V genes via C region primers, the first 42 codons (i.e. including CDR-H1) were missing in the published V_H sequence and the V_L sequence was only reported beginning with codon 19. An alignment of those sequences (EMBL accession numbers X79789 and X79788, respectively) with the cDNA sequences described here revealed moreover 2 differences within the amplified V_H region and 27 differences within V_L.

From a comparison of the sequences shown in Fig. 1 with the Kabat database of immunoglobulin sequences ([33]; the 6th edition was accessed via the World Wide Web) it was concluded that V_L belongs to the mouse immunoglobulin kappa chain subgroup III whereas V_H belongs to the heavy chain subgroup IIID. CDR-L1 in the light chain carries a 4 amino acid insertion (Ser-Val-Asp-Asn, corresponding to the Kabat residues 27A–D), which probably forms a solvent exposed loop in the tertiary structure. CDR-H3 consists of 18 amino acids and comprises thus one of the most extended murine hypervariable loops known so far.

CDR-H3 of 9E10 is furthermore remarkable as it possesses two stretches of three consecutive Tyr residues. Together with an additional Tyr residue at its downstream end and one Phe residue the fraction of aromatic residues in this hypervariable region amounts to 44%. An analysis with the program DNAPLOT (accessible through the World Wide Web: http://www.genetik.uni-koeln.de/dnaplot/) revealed that CDR-H3 most likely originated from the DFL16.1 diversity segment (used in reading frame 2) and the JH4 joining region, whereas the V_H gene segment was derived from the 7183 subfamily. Taken together, the two protruding loops formed by CDR-H3 and CDR-L1 constitute an interesting structural feature, which might imply a peculiar binding mechanism of this antibody for its peptide antigen.

The observation that the isolated V_H domain of 9E10 can be purified as a soluble entity was unexpected. The notion of employing V_H domains as 'single domain' antibodies was first raised by Ward et al. [11] using the anti-lysozyme antibody D1.3 [25] as an example. However, this concept was not much further pursued due to experimental difficulties regarding the solubility of V_H domains (see literature quoted in [34]). Generally, it was recognized that exposure of the hydrophobic

surface region in the V_H domain which comprises the interface in the association with V_L normally provokes aggregation in the absence of this subunit.

Recently however, the V_H domain of a camel immunoglobulin that is devoid of a light chain was structurally analysed [35]. The property of the isolated camel V_H domain as a well soluble, monomeric protein was attributed to the substitution of some hydrophobic surface residues by hydrophilic side chains. Furthermore, this immunoglobulin carried an extremely long CDR-H3 with an additional disulfide bond, which partially covered the remainder of the hydrophobic surface area. Large-sized CDR-H3 loops seem to constitute a general characteristic of V_H domains from camel antibodies that lack light chains [36].

A similar structural mechanism might therefore account for the observed solubility of the 9E10 V_H domain, where the partially hydrophobic and probably flexible extended CDR-H3 could accidentally bury some exposed hydrophobic groups in the interface region when the V_L domain is absent. Structural analysis will have to be done to support this hypothesis but, nevertheless, this finding might stimulate current attempts to construct soluble monomeric antibody domains by protein engineering [37].

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