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Cloning and expression in *Escherichia coli* of a human gelatinase B-inhibitory single-chain immunoglobulin variable fragment (scFv)

Naiming Zhou, Liesbet Paemen, Ghislain Opdenakker*, Guy Froyen

Laboratory of Molecular Immunology, Rega Institute for Medical Research, University of Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

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Abstract The murine monoclonal antibody REGA-3G12 selectively and specifically inhibits the activity of human gelatinase B. The cDNA fragments which encode the variable regions of the light and heavy chains were isolated by PCR-mediated cloning and sequenced. Single-chain Fv expression constructs for *Escherichia coli* were generated in which c-*myc* tag sequences were encoded. Inducible expression of the scFv and secretion to the periplasm were obtained with higher yields when the c-*myc* tag sequence was positioned at the amino-terminal side. The inhibitory activity of purified scFv on neutrophil gelatinase B was tested in a gelatin degradation assay and it was found to possess a similar specific activity as that of the intact monoclonal antibody and of the pepsin-clipped F(ab')$_2$ derivative. This shows for the first time that inhibition of soluble enzymes with scFv is possible and opens new perspectives for the treatment of diseases with excessive and detrimental enzyme production in the host.

Key words: Gelatinase B; Monoclonal antibody; Single-chain variable fragment; Matrix metalloproteinase; Inhibitor

1. Introduction

Matrix metalloproteinases (MMPs) have been recognized as key players in physiological tissue remodelling and in pathologies including cancer cell invasion, metastasis [1,2] and autoimmune diseases [3]. Several control levels of MMP activity exist in vivo: regulation of gene transcription, secretion, activation and specific inhibition [4]. Excessive production of the inducible gelatinase B has been observed in a number of diseases [5–8] and inhibition of gelatinase B may become a therapy for autoimmune disorders, such as rheumatoid arthritis and multiple sclerosis [3], and for invasive cancers [8]. The latter enzyme constitutes an essential step in the chain of matrix remodelling [3,9] and experiments in animals have shown that blockade of metalloproteinases has disease-limiting effects [10–12]. Inhibitory antibodies, however, have not yet been used in such blockade experiments.

Unfortunately, the so far identified low molecular weight inhibitors of gelatinase B (e.g. tetracyclines) possess low selectivity and specificity [13]. Even the natural tissue inhibitors of metalloproteinases (TIMPs) are not selective for one particular enzyme but may inhibit various collagenases, stromelysins, membrane-type metalloproteinases and gelatinases. Therefore, more specific and potent novel inhibitor types are currently being developed.

The most specific inhibitor of human gelatinase B known to date is a monoclonal antibody, REGA-3G12. It binds with high affinity ($K_d = 2.1 \times 10^{-9}$ M) to and inhibits the biological activity of gelatinase B, but not of gelatinase A [14]. The production of the inhibitory antibody REGA-3G12 and related monoclonals, and preferentially the engineering of derivatives, is essential for further biochemical and biological evaluation as specific inhibitors of gelatinase B in animal or human disease models.

For the application of gelatinase B-inhibitory monoclonals in patients, derivatization is necessary to minimize the human anti-mouse antibody (HAMA) response [15]. This may be achieved, for instance, by generating humanized antibodies or antibody fragments, by the production of human hybridomas or by in vitro immunization. Alternatively, transgenic human antibodies might be produced by the recently described expression of the megabase human light and heavy chain loci in the form of YAC clones in the mouse [16]. For particular immunodiagnostic and immunotherapeutic use in vivo, small antibody derivatives are desirable because of their shorter half-life (rapid clearance of diagnostic probes) and faster tissue penetration when compared to the intact humanized antibodies. In particular, the effector functions of the constant domains of the immunoglobulins, e.g. complement activation and the binding to Fc receptors, are superfluous for antibodies against soluble molecules (e.g. hormones, cytokines, neurotransmitters and enzymes). Indeed, the antigen-recognizing and inhibitory functions of the variable domains of the light and heavy chains, when appropriately folded and associated, may well replace the intact immunoglobulin. In addition, the avidity effect does not play a role in the binding to soluble antigens. Here, we describe the cloning, construction and characterization of a single-chain Fv with inhibitory activity against gelatinase B.

2. Materials and methods

2.1. Cell lines and expression vectors

REGA-3G12 is a murine hybridoma that secretes a monoclonal antibody against natural human neutrophil gelatinase B [14]. The MYC1-9E10.2 (ATCC CRL 1729) hybridoma produces a murine mAb (9E10) that recognizes part of the human c-*myc* protein. The mAb (9E10) facilitates the detection and purification of expression products containing the c-*myc* polypeptide. The expression vector pHEN1 [17] was kindly provided by Dr. G. Winter (MRC, Cambridge, UK). The vectors pscFv-D9D10 [18] and pscFv-D9D10-GF311 were constructed in our laboratory. The scFv genes are under the control of the *lacZ* promoter, the products are secreted into the periplasm with the help of the hydrophobic *pelB* signal peptide.

2.2. Cloning and sequencing of the variable heavy (VH) and light (Vκ) chain genes

Total cellular RNA was extracted from the hybridoma cells (REGA-3G12) by the TRIzol reagent (Life Technologies, Gaithersburg, MD) and 1 μg was used for first strand cDNA synthesis in a

*Corresponding author. Fax: (32) (16) 33 73 40.
E-mail: ghislain.opdenakker@rega.kuleuven.ac.be

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50 μl reaction mixture containing 1×RT buffer (0.14 M KCl, 8 mM $MgCl_2$, 50 mM Tris-HCl pH 8.1), 25 mM DTT, 0.15 mM dNTP, 1 μg random primers (Life Technologies), 50 U human placental ribonuclease inhibitor and 4 U RAV-2 reverse transcriptase (both from Amersham, Buckinghamshire, UK). The reaction mixture was kept at 23°C for 10 min followed by an incubation step of 60 min at 42°C. The enzyme was inactivated by a heating step of 5 min at 95°C.

For the amplification of VH and Vκ gene segments by PCR, 2.5 μl of the cDNA mixture was subjected to 35 cycles using the VH-specific primers VH1FOR-2 and VH1BACK [19] and the Vκ-specific primers VK2FOR and VK2BACK (sequences obtained from Dr. Winter, MRC. Cambridge, UK and also described in [18]), respectively. Each PCR cycle consisted of denaturation at 95°C for 35 s, annealing at 60°C (for VH) or 55°C (for Vκ) for 50 s, and extension at 72°C for 35 s, with a 10 min final extension using Ultma DNA polymerase (Perkin Elmer, Foster City, CA). The amplified VH and Vκ gene fragments were purified out of a 2.0% agarose gel with the QIAquick kit (Qiagen, Hilden, Germany). The purified VH and Vκ gene fragments were digested with *Bst*EII-*Pst*I and *Xho*I-*Sac*I, respectively, and inserted into the appropriate plasmid vectors (pEG04 and pBluescript, respectively). The sequences of VH and Vκ were determined with an ALF DNA sequencer (Pharmacia, Uppsala, Sweden). Each V-gene was cloned and sequenced in both directions from two independent PCRs.

2.3. Construction of scFv expression vectors

The VH gene segment, excised with *Bst*EII and *Pst*I, and the Vκ gene fragment, digested with *Xho*I and *Sac*I, were subcloned into pscFv-D9D10 vector which has the c-*myc* tag at the carboxy-terminus of the scFv gene. This construct was named p3G12T. For the construction of the scFv expression vector with the c-*myc* tag at the amino-terminus, the tag sequence was amplified by PCR with the primers (TAGNCOI: 5′-CCATGGCCGAACAAAAACTCATCTCAGAAGAGGATC-3′ and TAGPSTI: 5′-CTGCAGCTGCACCTGATTCAGATCCTCTTCTGAG-3′; indicated restriction sites are underlined), cloned in pGEM-T and, after DNA sequence analysis, subcloned as a *Nco*I-*Pst*I fragment into the *Nco*I/*Pst*I sites of the pHEN1 vector. Recombinant clones were screened by PCR and verified by DNA sequence analysis. The correct construct was cut by *Hin*dIII and *Pst*I and the fragment was subcloned into the pscFv-D9D10-GF311 vector which does not contain a c-*myc* tag sequence. Finally, the resulting expression vector was cut by *Pst*I and *Xho*I and ligated with the 3G12 scFv gene as a *Pst*I-*Xho*I fragment and the correct clone was confirmed by DNA sequence analysis. The latter plasmid was called pT3G12.

2.4. Expression, detection, purification and analysis of 3G12 scFv protein

Plasmid DNAs of p3G12T and pT3G12 were transformed in *Escherichia coli* JM83. The bacteria were grown at 30°C in LB medium (1% Bacto-tryptone, 0.5% yeast extract, 85 mM NaCl) with 100 μg/ml ampicillin and 1% glucose for 18 h. The culture was then diluted 1/20 in LB plus ampicillin and 0.1% glucose and further grown at 30°C for 2 h. Induction was performed with 0.1 mM IPTG before harvest 3 h later. The periplasmic extracts were prepared as described elsewhere [20]. The recombinant proteins were analyzed by SDS-PAGE and Western blotting and binding activity was assessed by ELISA. SDS-PAGE (15% acrylamide) was performed as described by Laemmli [21]. For immunoblot analysis, proteins were transferred to PVDF filters (Millipore, Bedford, MA) in 25 mM Tris-glycine, pH 8.2, containing 15% methanol. The filters were blocked with 1% casein in NT buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5) at 4°C, washed twice with NT buffer and incubated with the 9E10 mAb (10 μg/ml) for 2 h at 25°C. After washing the filters were incubated for 2 h at 25°C with an alkaline phosphatase-conjugated goat anti-mouse polyclonal IgG(H+L) (Jackson ImmunoResearch, West Grove, PA) as the second Ab. Binding to scFv proteins was visualized with BCIP and NBT. For quantitation of scFv expression, the periplasmic extracts obtained from equivalent cultures were serially diluted and analyzed by Western blotting and the amounts of scFv products estimated by comparison with a dilution series of purified scFv.

For the purification of scFv, periplasmic extracts were applied onto a 9E10 affinity column in equilibration buffer (50 mM Tris-HCl, 0.2 M NaCl pH 7.5). The column was washed with 0.1 M citrate pH 5.0 and the c-*myc* tag-containing Ig fragment was then eluted from the column with 0.1 M citrate pH 3.0. The eluted fractions were immediately neutralized with 1 M Tris-HCl pH 9.0. The purity of the fractions was analyzed by SDS-PAGE and the concentration determined with the Bradford method [22], bovine serum albumin was used as a standard.

2.5. ELISA for the determination of scFv binding to gelatinase B

Detection of scFv binding to human gelatinase B was performed by ELISA. Pretreated 96-well microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4°C with purified human gelatinase B [23] at 1 μg/ml in 50 mM Tris-HCl, pH 8.5 (50 μl/well). After blocking for 1 h at 37°C with 200 μl/well of blocking buffer (50 mM Tris-HCl, pH 7.4, 154 mM NaCl, 0.1% casein), the periplasmic extracts or purified scFv were incubated for 3 h at 37°C. After washing, the biotinylated anti-c-*myc* mAb 9E10 was added at 0.1 μg/ml in assay buffer (blocking buffer plus 0.05% Tween-20) and incubated for 2 h at 37°C. Binding of biotinylated Ab was detected with streptavidin-peroxidase (Sigma) 0.1 μg/ml in assay buffer (50 μl/well) and the substrate tetramethylbenzidine dihydrochloride (Aldrich; 1.8 mM in 100 mM NaAc:citric acid, pH 4.9, 0.003% H_2O_2) for 1 h at 37°C. The reaction was stopped by adding 2 M H_2SO_4 and the A_{450} was read spectrophotometrically.

2.6. Inhibition of biological activity

The inhibitory activity was determined as previously described [13,14]. Briefly, nasal septum collagen type II (Sigma) was heat-denatured at 60°C for 20 min and incubated for 1 h with the purified enzyme and scFv in buffer containing 50 mM Tris-HCl pH 6.9, 5 mM $CaCl_2$, 0.1% Triton X-100 and 1 mM Pefabloc (Boehringer Mannheim, Germany). The reaction was stopped by adding SDS-PAGE loading buffer to the mixture which was then run on a 7.5% SDS-PAGE gel under reducing conditions. Gels were stained with Coomassie brilliant blue. The neutralizing activity of scFv was detected by the presence of remaining intact type II collagen. As controls for gelatinase inhibition, the inhibitory REGA-3G12 monoclonal as well as a purified pepsin-treated $F(ab')_2$ derivative and 1,10-phenanthroline (Sigma Chemical Company, St. Louis, MO) were used.

3. Results

3.1. Cloning and sequencing of REGA-3G12 VH and Vκ genes

To obtain DNA fragments encoding the variable regions of the heavy and light chains of mAb REGA-3G12, total cellular RNA was isolated from hybridoma cells and transcribed into cDNA. The VH and Vκ fragments were subsequently amplified by PCR with primers covering the relatively conserved regions at the 5′ and 3′ ends of murine antibody variable domains and were inserted into pEG04 (*Pst*I/*Bst*EII) and pBluescript SK (*Sac*I/*Xho*I), respectively, and analyzed by DNA sequencing. The nucleotide and deduced amino acid sequences are shown in Fig. 1. Sequence comparison shows that the REGA-3G12 VH gene comprises 369 bp, encoding 123 amino acids, and is a member of subgroup II (B) according to the classification of Kabat [24] with a long CDR3 containing 14 amino acid residues. The Vκ gene, 321 bp in size encoding 107 amino acids, belongs to the mouse VL group V.

3.2. Expression of the scFv protein of REGA-3G12 in E. coli

Initially, the expression vector p3G12T (Fig. 2A) was constructed by replacing the VH and Vκ sequences in the vector pscFv-D9D10 [18] with those derived from REGA-3G12. Correct clones were selected and after induction of the cultures with 0.1 mM IPTG, the periplasmic fractions were prepared. SDS-PAGE and immunoblot analysis demonstrated an induced 31 kDa band (Fig. 2B) and detection in ELISA confirmed the expression of a gelatinase B-binding antibody fragment. However, the expression yields were < 0.2 mg/l of cul-

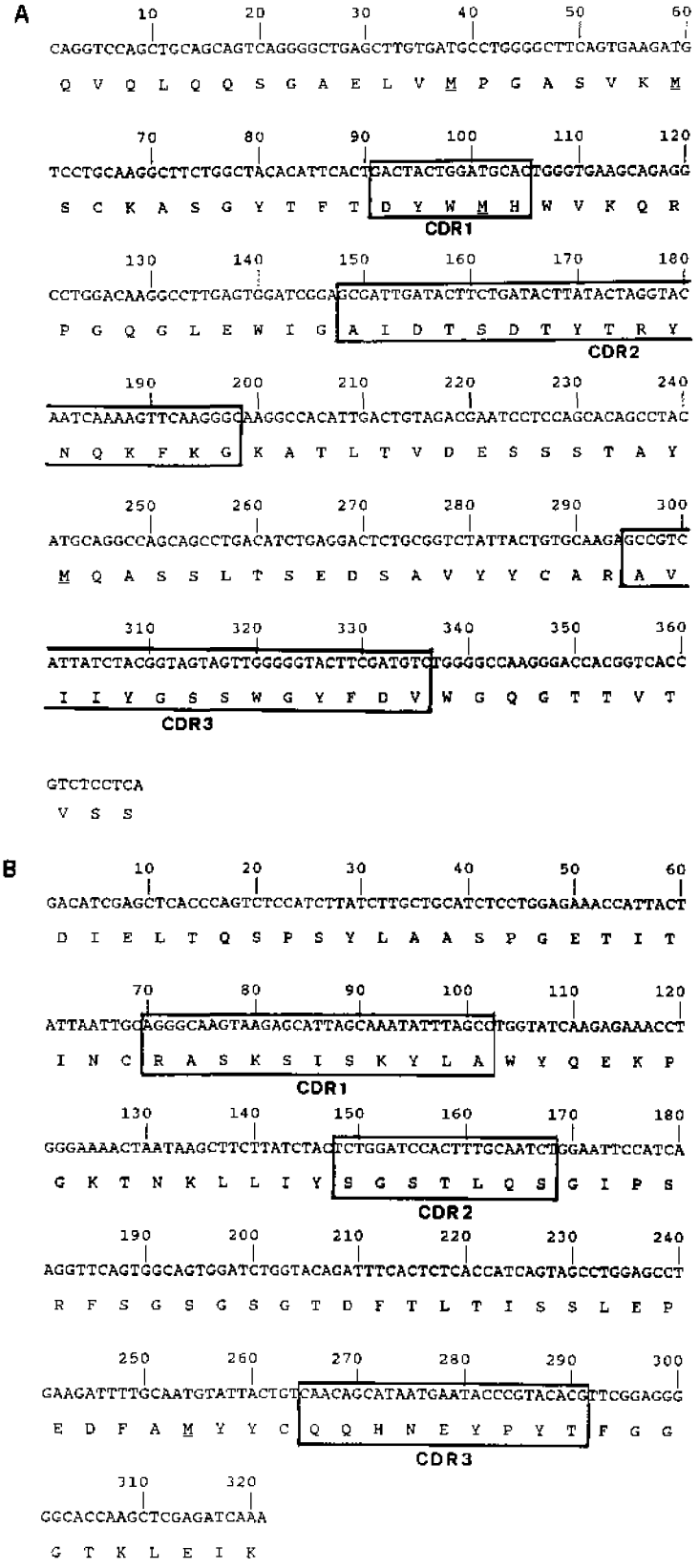

Fig. 1. Cloning of the variable fragments of the gelatinase B-inhibitory murine monoclonal REGA-3G12. Nucleotide and derived amino acid sequences of the VH (A) and the Vκ (B) encoding cDNA fragments of REGA-3G12 are shown. The hypervariable regions (CDR) are boxed and numbered.

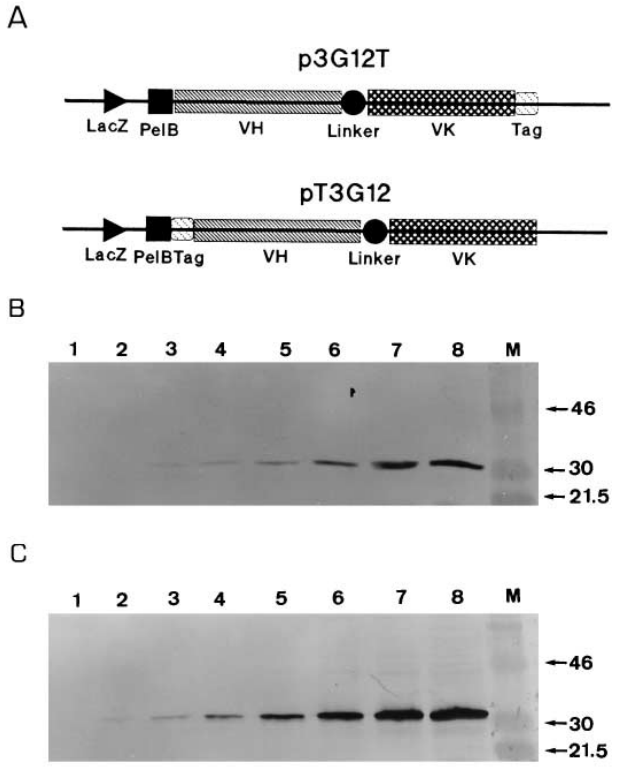

Fig. 2. Expression in *E. coli* of single chain Fvs derived from REGA-3G12. A: Construction of the expression plasmids p3G12T and pT3G12. In both instances the VH and Vκ (VK) regions (see Fig. 1) were fused by a linker. The c-*myc* tag cDNA sequence (Tag) was inserted in front or at the 3′-end of the variable gene segments. In all vectors the *lacZ* and *pelB* sequences were at the 5′-end. B and C: Western blot analysis of the recombinant product from IPTG-induced cultures p3G12T and pT3G12, respectively. Lanes 1–8 contain immunoreactive (anti-c-*myc*) scFv from the equivalent of respectively 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 and 25.6 μl periplasmic fraction. Lane M shows a molecular size standardization expressed in kDa.

ture. The VH-linker-Vκ sequence was then cloned into the pHEN1 expression vector in which the c-*myc* sequence was cloned so that the tag sequence is at the 5′ end of the scFv gene (Fig. 2C). More than 3-fold higher yields were obtained with the newly constructed pT3G12 vector as compared with p3G12T, as demonstrated by analysis of dilution series of the periplasmic fractions (Fig. 2C). However, the purification of the scFv-T3G12 by affinity chromatography did not yield significantly more scFv protein.

3.3. Inhibition of gelatinase B activity

The gelatinase B-inhibitory activity of the scFv of REGA-3G12 was compared with those of the intact monoclonal and a divalent $F(ab')_2$ derivative. The latter was prepared by pepsin treatment of the intact monoclonal antibody. On the basis of protein concentrations, the scFv derivative 3G12T inhibited the enzymatic activity of purified gelatinase B on denatured type II collagen equally well as REGA-3G12 and the $F(ab')_2$ derivative (Fig. 3).

4. Discussion

The variable regions of the light and heavy chain genes of the gelatinase B-inhibitory monoclonal antibody REGA-3G12 [14] were isolated by RT-PCR, cloned and sequenced. Both VH and Vκ genes showed the typical characteristics of mouse V domains, i.e. each contained two cysteines for intramolecular disulfide bridge formation and the distribution into framework (FR) and hypervariable (CDR) regions (Fig. 1). The relatively long CDR3 of the heavy chain (14 residues) indicates that the parent REGA-3G12 antibody is affinity matured. This is reflected in the high affinity of this antibody ($K_d = 2.1 \times 10^{-9}$ M). Two vectors were constructed for the inducible expression of scFv antibody fragments in *E. coli* and the recombinant products were purified and analyzed. The NH_2-terminal c-*myc* tag allowed for the expression of higher yields, when compared with the scFv containing this tag at the carboxy-terminus. Replacement of the c-*myc* tag from the COOH- to the NH_2-terminal end of the scFv gene was also observed to yield much higher antibody levels of a

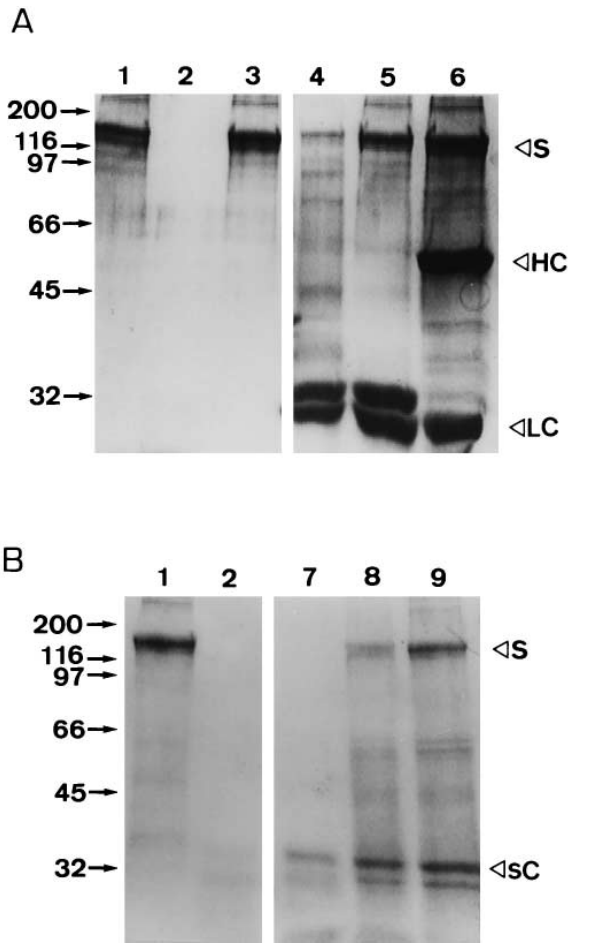

Fig. 3. Inhibition of gelatinase B activity by REGA-3G12 derivatives The gelatinase B inhibition assay consists in the degradation of denatured nasal septum type II collagen substrate (S) by purified neutrophil gelatinase B (E) and inhibition by various substances. A: Lane 1: S; lane 2: S digested by E; lane 3: S and E and 1,10-phenanthroline; lanes 4 and 5: S and E and, respectively 3 μg and 5 μg $F(ab')_2$ (these $F(ab')_2$ fragments were generated by pepsin-mediated clipping of the intact monoclonal REGA-3G12); lane 6: S and E and 5 μg intact REGA-3G12. HC and LC indicate light chain and heavy chain, respectively. The molecular standardization is indicated in kDa by arrows at the left side. B: Lanes 1 and 2 are the control reactions, as in A. Lanes 7, 8 and 9: S and E and, respectively, 1 μg, 3 μg and 5 μg of scFv 3G12T. sC indicates the single-chain variable fragment. The molecular standardization is indicated in kDa.

scFv directed towards another antigen (G. Froyen, unpublished results).

Production of scFv in inclusion bodies and secretion of scFv in the periplasmic compartment were monitored by spot blot, SDS-PAGE and Western blot analyses (Fig. 2 and data not shown), which showed that a considerable fraction is not secreted. Correct folding of the recognition domain of the secreted REGA-3G12 antibody derivative was evidenced by ELISA and by inhibitory activity against the natural substrate. Because the neutralization titers of the scFv resemble those of the original antibody, it is reasonable to accept that the glycine-serine linker enables the folding of the covalently linked VH and Vκ domains in a correct way (Fig. 3 and data not shown). Recombinant Fv proteins might mimic the antigen binding site but they dissociate in vivo. In contrast, recombinant scFv are more stable in vivo [25].

In most instances, immunoglobulins do not possess *N*-linked carbohydrates in the variable regions. Therefore, expression in bacteria will in such cases yield a product with unaltered antigenicity (due to *N*-linked sugars) when compared to expression in eukaryotic cells.

Many scFv proteins have been engineered into immunoconjugates which are directed against cell surface molecules, e.g. tumor cell markers. The scFv of our study is directed against a functional secreted enzyme. Recently, a scFv with inhibitory activity against the secreted cytokine, IFN-γ, has been developed [18] and, alternatively, catalytic antibodies and derivatives have been shown to possess intrinsic enzymatic activity. Our report demonstrates that an enzyme activity can readily be inhibited with scFv antibody fragments. In terms of metalloprotease-mediated catalysis, the availability of this scFv might enable further structural and functional studies of the active site of the enzyme. Additionally, the REGA-3G12 scFvs may prove to be useful for the diagnosis and therapy of diseases with excessive or detrimental gelatinase B production.

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