Mammalian cell expression of dimeric small immune proteins (SIP)

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We have designed and expressed bivalent small immune proteins (SIP) based on scFv fragments connected through a short linker of four amino acids to the CH3 domain of the human immunoglobulin γH-chain. Three different versions have been designed and expressed in mammalian cells. In one construct a cysteine residue was included in the last amino acid of the flexible 15-amino acid long linker connecting the VL and VH domains, thus creating a disulphide bond stabilized molecule. A version with a shorter (five amino acids) VL/VH linker was also produced. The 6C6 antibody VH and VL regions were PCR amplified in Western blotting and having a comparable functional affinity (avidity) as determined by ELISA.

Keywords: dimerization/eukaryotic cell expression/linker design/recombinant antibodies/single chain Fv

Introduction

The genes encoding antibody variable domains can be derived from hybridomas (Orlandi et al., 1989) or from filamentous bacteriophages displaying antibody fragments (McCaffery et al., 1990). The antibody genes can be manipulated to construct a variety of new molecules. A major breakthrough in the technology of antibody engineering was the derivation of single-chain molecules (scFv fragments, Bird et al., 1988; Huston et al., 1988) that retained the antigenic specificity of the original VH/VL association.

The antibody fragments are preferred to intact antibodies for the treatment of solid tumours because of their enhanced tissue penetration. Also, their rapid clearance in vivo is ideal for diagnostic imaging of cancerous, diseased or other specific tissues (Milenic et al., 1991; Yokota et al., 1992).

Recombinant Fab and Fv fragments of antibodies can be secreted from bacteria (Skerra and Plückthun, 1988), however, these fragments carry a single antigen-binding site. Recombinant fragments with two binding sites have been made in several ways; for example, by chemical cross-linking of the hinge cysteine residues (Shalaby et al., 1992), by including C-terminal peptides that promote dimerization (Kostelny et al., 1992; Pack and Plückthun, 1992), by using short linkers between VH and VL that do not allow the interaction of VH and VL domains from the same chain and therefore promote the formation of bivalent scFv dimers (diabodies, Holliger et al., 1993), or by making bispecific scFv able to chelate two adjacent epitopes on the same antigenic molecule (CRAbs, Neri et al., 1995). The dimerization, or a higher order of polymerization, of antibody fragments results in an increase of the total apparent affinity (or avidity) of the molecule, by means of an overall increase in the valency (Pack et al., 1995).

Here we describe the production of bivalent small immune proteins built by selection and modification of relevant domains of the antibody molecule that can be expressed in lymphoid and non-lymphoid mammalian cells.

Materials and methods

Cell lines and media

Mouse myeloma Sp2/0, Chinese hamster ovary (CHO) and human breast cancer MCF7 cells are all available from the American Type Culture Collection (Rockville, MD, USA). Sp2/0 and MCF7 cells were grown and maintained in RPMI-1640 medium supplemented with 10% fetal calf serum; CHO cells were grown in α-MEM + 10% fetal calf serum + 40 μM deoxy-ribonucleosides and 40 μM ribonucleosides.

Construction of recombinant SIP genes

The 6C6 antibody VH and VL regions were PCR amplified from the hybridoma RNA and sequenced. The sequences have been submitted to the EMBL Data Bank under accession numbers X98537 and X98538.

The three different linkers used to connect the VH with the VL were obtained by cloning three pairs of complementary oligonucleotides (SIP-L1, 5’-TGACGGCAGCACTAGTGGTAGCCGGCAAAACCGAGTTCCGGCGAAGGTTCTACCA-3’; and LSh-2, 5’-CTTCGCCCTTTGTGTAAGACCTTTCGGCGAATCCACTTGGTTGAGGTGCAGCGTACCACCTAAGCTGTCGCG-3’; and LSh-2, 5’-GCTGACCACTCGAACACCTAGGGTCCGAATCGCCGCGCGAAGGTTCTACCA-3’). The linker ‘long’, encoded by LC-1, 5’TAGCCGAGGTGCAGCTGCA-3’TGCACGGCAGCACTAGTGGTAGCCGGCAAAACCGAGTTCCGGCGAAGGTTCTACCA-3’; and LSh-2, 5’-GCTGACCACTCGAACACCTAGGGTCCGAATCGCCGCGCGAAGGTTCTACCA-3’TGCACGGCAGCACTAGTGGTAGCCGGCAAAACCGAGTTCCGGCGAAGGTTCTACCA-3’. The linker ‘short’, encoded by LC-1, 5’TAGCCGAGGTGCAGCTGCA-3’TGCACGGCAGCACTAGTGGTAGCCGGCAAAACCGAGTTCCGGCGAAGGTTCTACCA-3’; and LSh-2, 5’-GCTGACCACTCGAACACCTAGGGTCCGAATCGCCGCGCGAAGGTTCTACCA-3’TGCACGGCAGCACTAGTGGTAGCCGGCAAAACCGAGTTCCGGCGAAGGTTCTACCA-3’. The linker ‘cys’, encoded by LC-1, 5’TAGCCGAGGTGCAGCTGCA-3’TGCACGGCAGCACTAGTGGTAGCCGGCAAAACCGAGTTCCGGCGAAGGTTCTACCA-3’TGCACGGCAGCACTAGTGGTAGCCGGCAAAACCGAGTTCCGGCGAAGGTTCTACCA-3’. The linker ‘cys’, encoded by LC-1, 5’TAGCCGAGGTGCAGCTGCA-3’TGCACGGCAGCACTAGTGGTAGCCGGCAAAACCGAGTTCCGGCGAAGGTTCTACCA-3’TGCACGGCAGCACTAGTGGTAGCCGGCAAAACCGAGTTCCGGCGAAGGTTCTACCA-3’.

The pUT-SEC vector was designed to provide the recombinant SIPs with a secretion signal, the leader peptide required for the secretion of proteins in the extracellular medium. It is a tetracycline-selectable vector, based on pUC-19, that contains the 163 bp genomic sequence encoding a mouse heavy-chain immunoglobulin secretion signal.

All the three linker-coding sequences contain an internal SpeI restriction site. The linker ‘long’, encoded by the SIP-L1/SIP-L2 oligonucleotide pair, has the sequence GSGKPGSPEGST (according to Whitlow et al., 1993), the linker ‘cys’, encoded by LC-1, 5’TGCACGGCAGCACTAGTGGTAGCCGGCAAAACCGAGTTCCGGCGAAGGTTCTACCA-3’TGCACGGCAGCACTAGTGGTAGCCGGCAAAACCGAGTTCCGGCGAAGGTTCTACCA-3’. The pUT-SEC vector was designed to provide the recombinant SIPs with a secretion signal, the leader peptide required for the secretion of proteins in the extracellular medium. It is a tetracycline-selectable vector, based on pUC-19, that contains the 163 bp genomic sequence encoding a mouse heavy-chain immunoglobulin secretion signal.

The cloned VL region was amplified with primers L5V (5’-
GTGTGCACTCGGATTTGTGA TG-3

E. Li et al.

The V_L–V_H linker was constructed and subjected to 10 ps of MD and 1000 cycles of CGM, keeping the rest of the structure fixed. The CH3/CH3 dimer was added in an orientation that allows the small V_L–CH3 linker to span the distance between the two domains.

Cell transfection

About 10^7 mouse myeloma Sp2/0 cells or Chinese hamster ovary (CHO) cells were resuspended in 0.5 ml cold PBS (10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, pH 7.2) and put in a cuvette for electrophoresis with an electrode gap of 0.4 cm; 10 µg Bcl/I-linearized plasmids (either pcDNA3-SIP-long, pcDNA3-SIP-cys or pcDNA3-SIP-short) were added to the cells and electrophoresis was performed with a single pulse at 960 µF, 290 V, in a Bio-Rad Gene Pulser equipped with a capacitance extender. After electrophoresis cells were kept 5 min on ice, washed, resuspended in 30 ml culture medium and seeded in 10 cm dishes at a density of ~4x10^5 cells/dish. After 24 h selective medium containing G418 (Geneticin, Gibco-BRL, Gaithersburg, MD, USA) at a final concentration of 400 µg/ml was added. Selected clones were screened by ELISA of supernatants on plates coated with anti-human IgG to detect the presence of secreted SIPs.

Metabolic labelling and immunoprecipitation

Transfected Sp2/0 and CHO cells were cultured in 60 mm dishes until they were ~70% confluent. After washing with PBS, RPMI medium without methionine was added and cells were incubated for 20 min at 37°C, to deplete the intracellular pool of methionine, then [^35]S]-methionine (10 µCi/ml, 1000 Ci/mmol; Amersham, Little Chalfont, UK) was added to a final concentration of 100 µCi/ml. After 2 h labelling, cells were washed and chased with unlabelled methionine for 3 h, then supernatants were collected and cells washed with cold PBS, lysed in 40 µl TNN buffer (50 mM Tris–HCl pH 8.0, 250 mM NaCl, 0.5% NP-40, 1 mM phenyl-methyl-sulfonyl fluoride) and centrifuged 10 min at 10 000 g at 4°C. The supernatants and the cell lysates were mixed with 1 µl of anti-human IgG (Dako), incubated for 2 h at 4°C and immune complexes precipitated with 25 µl Protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden); beads were washed with 4 ml TNN, 4 ml TNNB (TNN + 1% BSA), 4 ml RIPA buffer (0.1 M Tris–HCl pH 8.0, 0.1 M NaCl, 5 mM MgCl_2, 1% NP-40, 1% deoxycholate, 0.1% SDS) and 4 ml PBS. Immune complexes bound to Protein A were eluted with 50 µl SDS sample buffer (30 mM Tris–HCl pH 6.8, 1.5% SDS, 10% glycerol, 0.1 mg/ml bromophenol blue) either reducing (with 2.5% β-mercaptoethanol) or not reducing (without β-mercaptoethanol) and loaded onto 10% SDS–polyacrylamide gels. After electrophoresis gels were fixed in 10% acetic acid, 10% methanol, soaked for 30 min in Amplify fluorographic radioactivity enhancer (Amersham), dried and exposed for autoradiography.

Size-exclusion chromatography

Supernatants (0.5 ml) from transfected clones secreting the recombinant SIPs were applied to a Superdex-75 HR 10/30 column (Pharmacia), equilibrated in PBS and eluted with 30 ml (approximately one bed volume) of PBS at a flow rate of 0.7 ml/min. Fractions (0.35 ml) were collected and tested in an indirect ELISA on recombinant 6C6 antigen coated plates. The column had previously been calibrated by the separation, in the same conditions, of the molecular weight markers: hen ovaltransferrin (77 kDa), hen ovalbumin (42 kDa) and bovine erythrocyte carbonic anhydrase (30 kDa).
Western blotting

Human breast cancer MCF7 cells were lysed in TNN buffer, as described above. The cell lysate was resuspended in reducing SDS sample buffer and loaded onto a 12% SDS–polyacrylamide gel. After electrophoresis the gel was transferred to nitrocellulose, the membrane was cut in slices and incubated for 2 h with culture supernatants from SIP secreting cells or from 6C6 hybridoma cells. Detection was with alkaline phosphatase-conjugated antibodies, anti-mouse immunoglobulins (for 6C6 hybridoma) or, for SIPs, anti-human IgG (Dako, Glostrup, Denmark). Substrate for alkaline phosphatase was 0.33 mg/ml NBT (nitro-blue-tetrazolium chloride), 0.165 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate) in 0.1 M Tris–HCl pH 9.5, 0.1 M NaCl, 5 mM MgCl2.

Immunofluorescence analysis

MCF7 cells grown on glass slides (TC Chamber Slides, Nunc, Roskilde, Denmark) were washed twice with PBS and fixed for 20 min in 3% paraformaldehyde (Sigma, St Louis, MO, USA) in PBS. The fixed cells were blocked with 0.25% gelatin (Sigma) in PBS and incubated for 1 h at room temperature with SIP or 6C6 culture supernatants. After several washes with PBS–gelatin, cells were incubated for 1 h with FITC-conjugated antibodies anti-mouse or anti-human IgG (Dako). The slides were mounted in Fluorescent Mounting Medium (Kirkgaard & Perry Laboratories, Gaithersburg, MD, USA) and observed with a microscope Leitz Biomed (Wild Leitz, Wetzlar, Germany).

Competitive ELISA

A polystyrene ELISA plate (Nunc Maxisorp) was coated overnight at 4°C with the recombinant 6C6 antigen fused with β-galactosidase, dissolved at 1 µg/ml in 50 mM Na2CO3/NaHCO3 buffer, pH 9.5. The plate was washed three times in PBS containing 0.01% Tween-20 (Merck, Darmstadt, Germany) and blocked with 10 µg/ml BSA (Boehringer, Mannheim, Germany) in PBS–Tween (PBS–BSA–Tween). Supernatants of the different SIPs and of the original 6C6 hybridoma were diluted in PBS–BSA–Tween and incubated for 2 h at room temperature with the recombinant 6C6 antigen at concentrations ranging from 100 pM to 10 nM. The dilution of the supernatants were determined in a previous experiment to give a comparable O.D. in the absence of competitor antigen. The SIP–antigen complexes were subsequently applied to the plate and incubated at room temperature for 15 min, then the plate was washed three times with PBS–Tween and immunoproteins bound to the plate were detected with peroxidase-conjugated antibodies anti-mouse IgG (for 6C6 hybridoma) or anti-human γ-chains (for SIPs) (Dako). Substrate for peroxidase was 2 mg/ml o-phenylenediamine (Sigma) in 100 mM K2HPO4/KH2PO4 buffer, pH 6; the reaction was stopped with 50 µl of 2 M H2SO4 and O.D. at 492 nm was read with a microplate reader Bio-Rad 450. The dissociation constant, KD, was determined, according to Friguet et al. (1985), using the equation:

$$\frac{A_0}{A_0 - A} = 1 + \frac{K_D}{[a]}$$

where A represents the absorbance at 492 nm after incubation with the antigen, A0 is the absorbance in the absence of incubation with the antigen and [a] is the antigen concentration. In particular, when A = 1/2A0, then K_D = [a].

Results

We used as a model a murine monoclonal antibody, 6C6 (Li et al., 1987), which was originally obtained from a mouse immunized with human breast cancer cell membranes. 6C6 IgG recognizes a 28 kDa transmembrane protein expressed on many breast cancer cells and in human cell lines like MCF7 and K562. The murine antibody reacts strongly against the 6C6 antigen protein in Western blots (Li et al., 1996).

To obtain a small immune protein (SIP) with the same antigenic specificity as the original antibody, we constructed a single chain molecule containing the two V region domains of the antibody (V_L and V_H) and one domain (CH3) of the human IgG (Dako). The V_L domain was linked to the CH3 domain in all cases was separated from the V_H by a short 4 aa linker of the sequence GGSG (Figure 1). The SIP–antigen complexes were then cloned into an expression vector containing the CMV promoter sequence, filled boxes the genomic sequence of the signal secretion leader peptide and white boxes the V_L, V_H and CH3 domain sequences. The length and sequence of the V_L/V_H and V_H/CH3 linkers are indicated.

Fig. 1. Schematic representation of SIP constructs. Hatched boxes represent the CMV promoter sequence, filled boxes the genomic sequence of the signal secretion leader peptide and white boxes the V_L, V_H and CH3 domain sequences. The length and sequence of the V_L/V_H and V_H/CH3 linkers are indicated.
Fig. 3. Immunoprecipitation of SIPs from [35S]methionine labelled cells and supernatants. Reducing (a) and non-reducing (b) SDS–PAGE analysis of immunoprecipitated secreted (a) and secreted and intracellular (b) [35S]methionine labelled SIPs. The position of the SIP-cys dimer in the non-reducing gel is indicated by an arrowhead.

interactions. In the second case (SIP-cys), a modified version of the linker was used (GSTSGSKPGSGEGSTGC), where a cysteine residue was introduced in the terminal position of the linker, in order to covalently stabilize the dimeric molecule through a disulfide bond. The location of this cysteine was decided after molecular modelling of the SIP-long molecule that showed spatial proximity of the last residues of the V_{L} / V_{H} linker (Figure 2).

In the third construct (SIP-short) we decided to investigate an alternative strategy of achieving dimerization, by including a short linker of five residues (GSTSG) between the two V region domains. In bacteria it has been shown that a short linker does not allow interactions between the V_{L} and V_{H} domains of the same molecule and forces interactions between domains located on different chains (Holliger et al., 1993), thus favouring dimerization or even multimerization of the scFv (Alfthan et al., 1995). In our case, dimerization would be driven by interchain interactions of three pairs of domains (V_{Lb}/V_{Hb}, V_{Ha}/V_{Lb} and CH_{3a}/CH_{3b}), producing a more stable bivalent molecule. Even though the orientation of the three domains could impose limitation to the assembly of the complete bivalent molecule, the molecular modelling (Figure 2c) showed that such construction was possible.

The expected molecular weights of the monomeric SIPs are around 37 kDa. This was indeed the size obtained in all three cases, as revealed by immunoprecipitations of the secreted [35S]methionine labelled material analysed on reducing SDS–PAGE (Figure 3a). In the case of the SIP-cys, a band of about 80 kDa was also obtained when the analysis was performed under non-reducing conditions (Figure 3b), indicating that a substantial amount (estimated at about 80%) of the secreted SIP was covalently dimerized through a disulfide bond. Figure 3 also shows that all the three SIPs are actively secreted from the different transfectomas: after a 3 h chase period,

Fig. 2. Molecular models of SIP-long, SIP-cys and SIP-short. In each single chain the V_{L}, V_{H} and CH_{3} domains are shown in red (chain a) or green (chain b). Linkers are shown in yellow. Figure produced with the program MOLSCRIPT (Kraulis, 1991) and Raster3d (Merrit and Murphy, 1994).
Mammalian cell expression of dimeric SIPs

Fig. 4. Size-exclusion chromatography profile of the cell culture supernatants of SIP-producing clones. Fractions from the column were analysed in an ELISA assay to detect the presence of SIPs.

Fig. 5. Western blot analysis on cellular extracts from the human breast cancer MCF7 cell line. Membranes were incubated with culture supernatants from transfected cells (SIPs) or 6C6 hybridoma and detected with peroxidase labelled anti-human IgG (SIPs) or anti-mouse IgG (mAb). Following a 2 h pulse labelling, almost all the labelled SIP proteins were recovered from the culture supernatants.

The dimeric character of the SIP-long and SIP-short molecules, that cannot form covalent disulphide bonds, was assessed by FPLC gel filtration analysis of the secreted material. As shown in Figure 4, in both cases the material eluted at a retention time corresponding to globular proteins of ~70–80 kDa, indicating that these two SIPs are dimers resembling the bivalent immunoglobulin. A small fraction was also recovered in the position of the monomers.

To compare the binding properties of the SIP molecules with the model 6C6 antibody, we performed western blot analysis on extracts of the MCF-7 cell line that express the SIP-long 6C6 antigen. Figure 5 shows that all three SIPs are as specific as the original monoclonal antibody in detecting the 28 kDa protein. In addition, immunofluorescence analysis on MCF7 cells also showed the same reactivity of the three SIPs and mAb 6C6 (Figure 6).

A comparison of the apparent affinities (avidities) was carried out using an ELISA based method (Friguet et al., 1985). The soluble competitor protein used in this assay was the cloned 6C6 antigen expressed as a β-galactosidase fusion protein in Escherichia coli (Li et al., 1996). The results obtained with the three SIPs (Figure 7 and Table I) showed that they had similar dissociation constants, ranging from 1.3 to 3.3 x 10⁻⁹ M, while the kD value obtained for the original mAb 6C6 was 0.4 x 10⁻⁹ M (corresponding to an avidity of 2.8 x 10⁹ M⁻¹). These results indicate that no major constraints were added in either construct.

Table I. Dissociation constants and corresponding affinity values of the 6C6 mAb and the different SIPs

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Dissociation constant (K_D)</th>
<th>Affinity (1/k_D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb 6C6</td>
<td>0.4 ± 0.1 x 10⁻⁹ M</td>
<td>2.8 ± 0.7 x 10⁹ M⁻¹</td>
</tr>
<tr>
<td>SIP-long</td>
<td>1.3 ± 0.4 x 10⁻⁹ M</td>
<td>7.7 ± 0.9 x 10⁸ M⁻¹</td>
</tr>
<tr>
<td>SIP-short</td>
<td>3.1 ± 1.0 x 10⁻⁹ M</td>
<td>3.3 ± 1.2 x 10⁸ M⁻¹</td>
</tr>
<tr>
<td>SIP-cys</td>
<td>3.3 ± 0.6 x 10⁻⁹ M</td>
<td>3.1 ± 0.7 x 10⁸ M⁻¹</td>
</tr>
</tbody>
</table>
Discussion

We have shown here that a dimeric small immune protein can be produced in mammalian cells by including a single dimerizing domain at the C-terminal part of the single-chain Fv fragment. We found that the CH3 domain of the human immunoglobulin γ1 chain was sufficient to promote dimerization of the scFvs, which were efficiently assembled and secreted by mammalian cells. This could represent an advantage compared with other dimerizing domains. The CH3 should not be immunogenic in humans, an important aspect when dealing with molecules destined to clinical use. Furthermore, the CH3 domain can serve as a tag to facilitate the detection and the purification of SIPs using standard anti-Ig reagents available for normal antibody molecules.

The size of a complete SIP molecule is similar to a Fab, but with the advantage of being dimeric. Only 30% of the molecular weight of the complete SIP molecule is contributed by the CH3 domain.

The inclusion of a single cysteine residue at the end of the linker separating the V$_i$ from the V$_H$ domain induced covalent stabilization of the bivalent dimer.

The SIP-short construct could also favour the production of SIPs with dual specificities (bispecific-SIP) in eukaryotic cells. In this case, two single chain SIP constructs containing scrambled V$_i$ and V$_H$ domains, corresponding to specificities for antigens a and b (V$_i$a/V$_H$b/CH3 and V$_i$b/V$_H$a/CH3), and co-transfected into a mammalian cell should produce three different types of dimers, i.e. (V$_i$a/V$_H$b/CH3)$_2$, (V$_i$b/V$_H$a/CH3)$_2$ and (V$_i$a/V$_H$b/CH3-V$_i$b/V$_H$a/CH3). Since only the last type of dimer should have both antigenic specificities, it could easily be separated by antigen affinity purification from the other two dimers that will not bind either antigen.

SIPs are easy to construct and to transfect since they represent a single chain construct and therefore no co-transfection is required. This could be useful for the expression in mammalian cells of Fv selected from phage display antibody libraries. To this respect, eukaryotic cells provide a more appropriate environment for the correct folding of the V$_i$/V$_H$ domains, thus better preserving the original antibody specificity. This is particularly important when dealing with scFv that do not reproduce the original specificity when expressed in bacteria.

We would like to emphasize the advantage provided by the molecular modelling in designing new constructs. The location of the cysteine used to obtain a covalent dimeric scFv was suggested by the three-dimensional model which showed the spatial proximity of the last residues in the V$_i$/V$_H$ linker. In addition the modelling also indicated the absence of steric hindrance in the SIP-short molecule, designed to dimerize through three interchain domain interactions. In this case, the availability of the X-ray diffraction derived structure of the ‘diabody’ molecule was of great value (Perisic et al., 1994).

The determination of the dissociation constants of the three SIPs showed that they were not substantially different, although they differed from that of the original mAb 6G6 (0.4×10^{-9} M) by a factor of 4–8 (Table I). This is not surprising since it is expected that single chain versions of complete antibody molecules have a reduced binding affinity. The SIP-long was the one with the highest relative affinity ($K_D = 1.3×10^{-9}$ M) when compared with the SIP-short and SIP-cys, which showed $K_D$ values of $3.1×10^{-9}$ M and $3.3×10^{-9}$ M respectively. This result could be the consequence of the different relative proximity and orientation of the V$_i$/V$_H$ binding units; alternatively, it could reflect the differences in the flexibility of the three SIPs, since the formation of the antigen–antibody complex involves in many cases structural changes in the antibody molecule. In this context, the addition of a disulphide bond in the linker between V$_i$ and V$_H$ (SIP-cys), though increasing the stability, can impair the relative mobility of the two binding domains and may eventually reduce the affinity. The same could hold true for the SIP-short which shows a more packed overall structure.

References


Received July 12, 1996; revised February 7, 1997; accepted February 17, 1997.