Leucine Zipper Dimerized Bivalent and Bispecific scFv Antibodies from a Semi-synthetic Antibody Phage Display Library*

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This report describes the construction of leucine zipper-based dimerization cassettes for the conversion of recombinant monomeric scFv antibody fragments to bivalent and bispecific dimers. A truncated murine IgG3 hinge region and a Fos or Jun leucine zipper were cloned into four scFv fragments previously isolated from a synthetic antibody phage display library. Cysteine residues flanking the zipper region were introduced to covalently link dimerized scFv fragments. The secreted fusion proteins were shown to spontaneously and efficiently form stable Fos-Fos or Jun-Jun homodimers in the Escherichia coli periplasm at levels comparable to their monovalent counterparts. The bivalent (scFv)2 fragments performed well in enzyme-linked immunosorbent assay, flowcytometric, and immunohistochemical analysis. Fos and Jun homodimer (scFv)2 antibodies with different specificities could be reduced, reshuffled, and reoxidized to form preparations of functional bispecific (scFv)2 Fos-Jun heterodimers. These Fos and Jun fusion protein cassettes provide a universal basis for the construction of dimeric scFv antibodies with enhanced avidity or dual specificity.

Surface display of Fab or single chain Fv (scFv) antibody fragments on filamentous phage particles in combination with an array of versatile selection procedures has become a powerful approach to obtain recombinant molecules with desired specificities and binding properties from large libraries (reviewed by Winter et al. (1994) and Burton and Barbas (1994)). The Fab and scFv fragments thus obtained are monovalent, whereas in many in vitro and in vivo applications, multivalency of antibody molecules is a desirable property. In addition, linking two or more binding sites efficiently increases the functional avidity of antibody molecules or results in the construction of antibodies with dual specificities (Plückthun, 1992). Several approaches have been employed to generate genetically engineered, multimerized antibody fragments. Bivalent (and bispecific) (scFv)2 and (Fab)2 fragments have been successfully produced by association of two molecules through flexible linker polypeptides, chemical cross-linking, and dimerization domains (reviewed by Holliger and Winter (1993)). In the latter approach, introduction of amphipathic helices or leucine zippers was shown to mediate dimerization of scFv or Fab fragments in vivo (Pack and Plückthun, 1992; Pack et al., 1993, 1995; Kostelný et al., 1992). These efforts have resulted in the production of higher valency antibody fragments with widely varying physicochemical properties.

In designing strategies for dimerization of antibody fragments, several issues need to be addressed including stability and homogeneity of the dimers, resistance to proteolytic cleavage during in vivo assembly, efficient production of preferably soluble protein, simple engineering steps, and general applicability for the construction of both bivalent and bispecific recombinant antibodies. With these issues in mind, we designed dimerization cassettes that allow the conversion of scFv antibodies from a number of published phage display libraries to bivalent or bispecific reagents involving a single cloning step. In this procedure, the flexible and proteolysis-resistant truncated mouse IgG3 upper hinge region (Pack and Plückthun, 1992) and either Fos or Jun leucine zippers were fused to scFv proteins. Two cysteine residues were engineered in the Fos and Jun zipper domains to produce disulfide-stabilized homodimers. Using four scFv antibodies previously isolated from a synthetic phage display library, we show that this approach results in the efficient in vivo production of stable, secreted homodimers that retain their specificity as assessed in a number of assays. Furthermore, exploiting preferential Fos-Jun heterodimer over Fos-Fos or Jun-Jun homodimer formation, we show that in vitro reduction, mixing, and re-oxidation of Fos and Jun scFv antibodies with different specificities results in the production of bispecific (scFv)2 molecules.

MATERIALS AND METHODS

scFv Fragments—The scFv antibodies were selected from a semi-synthetic antibody phage display library constructed in the phagemid vector pHEN1. scFv clone 3 is specific for an IgG paraprotein, scFv clones 22 and 23 are specific for dinitrophenol coupled to bovine serum albumin (DNP-BSA).† scFv clone 35 is specific for the α chain of the CD8 molecule expressed on a subpopulation of human T lymphocytes, and scFv clone 40 is specific for the β chain of the CD2 molecule expressed on mature human B lymphocytes. All scFv molecules have been described in detail elsewhere under different names (clone 3, IgG2; clone 22, DNP2; clone 23, DNP5 (de Kruif et al., 1995a); clone 35, T1; clone 40, B28 (de Kruif et al. 1995b)).

Construction of scFv-Zipper Proteins—Oligonucleotide primers (Table I) were developed to append NotI restriction sites and a murine IgG3 upper hinge region (Pack and Plückthun, 1992) to modified Fos and Jun leucine zipper regeps. At the N and C termini, the modified zipper regions contain cysteine residues added via Gly-Gly spacers (Crameri and Suter, 1993). Template consisting of the plasmid pJLFoS (encoding both modified zipper genes; Crameri and Suter (1993)) was polymerase chain reaction amplified with the appropriate primer set (30 s at 94°C, 30 s at 58°C, and 45 s at 72°C for 25 cycles), digested with NotI, and cloned into phagemid vector pHEN1 (Hoogenboom et al., 1991) to yield pHEN1-hFo and pHEN1-hJu. Sequences were verified by the dideoxy chain termination procedure. NotI digestion fragments from pHEN1-hFo and pHEN1-hJu were ligated into NotI-digested pHEN1-scFv clones to produce clones 3F (anti-IgG-hinge-Fos), 23 (an-
ti-DNP-hinge-Jun), 35F (anti-CD8 monoclonal antibody (Becton Dickinson). In control staining experiments, incubations with scFv and (scFv)2 fragments were omitted from the procedure.

Formation of Anti-IgG × Anti-DNP Bispecific (scFv)2 Fragments—Periplasmic preparations of clones 3F and 23J containing approximately equal amounts of (scFv)2 homodimers were reduced in 32 mM -mercaptoethanol at 37 °C for 1 h, mixed, and dialyzed against redox buffer (50 mM Tris-HCL, pH 8.5, 1 mM EDTA, 500 μM reduced glutathione, and 500 μM oxidized glutathione) for 24 h at 4 °C. Subsequently, the buffer was changed back to PBS by dialysis.

### RESULTS

Construction of scFv-Leucine Zipper Fusion Proteins—A polymerase chain reaction approach was used to append restriction sites and the mouse IgG3 upper hinge region to modified Fos and Jun leucine zippers. These “dimerization cassettes” were subsequently cloned into a unique NotI restriction site present in the 3′-end of the genes encoding scFv antibody fragments isolated from a semi-synthetic human phage antibody display library (Fig. 1).

### SDS-PAGE and Western Blot Analysis—Equal volumes of periplasmic preparations containing expressed scFv fragments or their Fos and Jun fusion protein derivatives were run on an SDS-PAGE gel and analyzed by Western blotting using the anti-Myc tag antibody 9E10. Under reducing conditions, equal amounts of scFv fragments are detectable in the periplasm of scFv, scFv-hinge-Fos (3F), and scFv-hinge-Jun (23J) expressing bacteria, indicating that expression levels of the scFv molecules are not affected by addition of the hinge region and leucine zipper domains (Fig. 2). A shift in the gel mobility of the scFv-zygote proteins is observed, corresponding to the 56 amino acids introduced by the hinge region and zipper domains. When SDS-PAGE is performed under non-reducing conditions, a set of proteins, with closely spaced bands, corresponding to approximately twice the size of scFv-zygote proteins is detected solely in periplasmic preparations containing zipper constructs. No protein bands corresponding to the size of monomeric scFv are observed.

### Bivalent and Bispecific scFv Antibodies

| scFV | PKSTPQGS | COG | LTDSL_FOS_ILAAH | GGC | MYC-TAG |
|------|----------|-----|-----------------|-----|---------|
| HD16 | —RIARL_JUN_KVMNH |      |                  |     |         |

Fig. 1. Diagram of the Fos and Jun dimerization cassettes cloned into scFv-containing pHEN1 phagemids. Cysteine residues are underlined.
lymphocytes is caused by the second and third step antibodies to any of the control antigens was observed (Fig. 4). Note that some nonspecific staining of antibodies against the CD83F (scFv)2 periplasm; lane 4, 23J (scFv)2 selected on IgG; lane 5, 23J (scFv)2 selected on DNP; lane 6, 23J (scFv)2 selected on IgG.

bound to IgG and DNP, respectively, whereas no significant binding to any of the control antigens was observed (Fig. 4).

**Immunohistochemical Staining of CD22**

Cells with Bivalent (scFv)2 Antibodies—COS cells were transfected with the CD22β cDNA cloned into the CDM8 vector. A periplasmic preparation from E. coli SF110 transformed with a unlinked anti-CD22β (scFv)2 (40J) was used to stain transfected cells. Results show an intense staining of approximately 10% of the transfected cells (Fig. 6), corresponding to the transformation efficiency in this particular experiment. No staining was observed when non-relevant (scFv)2 molecules with anti-DNP specificity were used to stain CD22β-transfected COS cells (results not shown).

**Formation and Performance of Anti-IgG × Anti-DNP Bispecific (scFv)2 Molecules**—The formation of bispecific (scFv)2 molecules was examined using anti-DNP and anti-IgG scFv clones 3F and 23J. Periplasmic preparations from bacteria transformed with these constructs were reduced in 2-mercaptoethanol. Upon this treatment, all detected proteins are present as scFv-zipper monomers (Fig. 7). To allow formation of heterodimeric (scFv)2 molecules, the proteins were mixed and incubated in a redox buffer. After subsequent dialysis in PBS, a set of proteins corresponding to the size of (scFv)2 molecules was observed (Fig. 7). Binding properties of these reshuffled (scFv)2s was first examined in ELISA. The 3F × 23J protein preparation detects both the DNP and the IgG antigen (Fig. 4). No binding to other antigens is detected. To test the bispecific properties of the reshuffled proteins, a sandwich ELISA was performed. The proteins were allowed to bind to IgG-coated ELISA plates. After washing, DNP was added to the wells. Bound DNP was detected by an anti-DNP phage antibody followed by a horseradish peroxidase-conjugated anti-M13 antibody. A strong signal developed in the wells incubated with the 3F × 23J bispecific antibody (Fig. 8). No signal was observed in wells incubated with 3F or 23J homodimers or when DNP is omitted in the procedure.

**DISCUSSION**

We have constructed scFv antibody fragment dimerization cassettes that can be readily introduced in the NotI restriction sites of genes encoding scFvs isolated from a variety of phage display libraries described in the literature (Hoogenboom and Winter, 1992; Nissim et al., 1994; de Kruif et al., 1995a). These cassettes add a truncated, flexible murine IgG3 hinge region and either a Fos or Jun leucine zipper to the scFv proteins. To increase stability of the bivalent antibodies, cysteine residues were incorporated at the N and C termini of each of the leucine zippers, facilitating disulfide bridge formation in the periplasmic space (Crameri and Suter, 1993).

**Fig. 2.** SDS-PAGE Western blot analysis of expressed scFv and (scFv)2 proteins. The upper panel shows the migration of anti-IgG (lane 1) and anti-DNP (lane 2) scFv and their Fos and Jun fusion protein derivatives (lanes 3 and 4, respectively) run under reducing conditions. In the lower panel, reducing agents were omitted from the sample SDS buffer.

**Fig. 3.** SDS-PAGE Western blot analysis of (scFv)2, proteins selected for binding to their target antigen. (scFv)2, proteins were incubated with paramagnetic beads coated with either IgG or DNP. After washing, the beads were boiled in non-reducing SDS sample buffer, and the resulting protein mixture was applied to the gel. Lane 1, 3F (scFv)2 periplasm; lane 2, 3F (scFv)2 selected on DNP; lane 3, 3F (scFv)2 selected on IgG; lane 4, 23J periplasm; lane 5, 23J (scFv)2 selected on DNP; lane 6, 23J (scFv)2 selected on IgG.

**Fig. 4.** Antigen specificity of (scFv)2 antibody fragments. Microtiter plates were coated with IgG, DNP, or a panel of control antigens including lysozyme, thyroglobulin, ovalbumin, HMGB-box protein, bovine serum albumin, and milk powder. (scFv)2, molecules were allowed to bind and were detected using the 9E10 antibody.
leucine zippers.

Monomeric scFv molecules were detectable as single bands in non-reducing SDS-PAGE, consistent with the notion that proteins secreted into the periplasmic space of Gram-negative bacteria refold properly with formation of the correct disulfide bonds (Huston et al. (1993) and references therein). In contrast, Fos or Jun homodimers presented themselves as multiple closely spaced bands in non-reducing SDS-PAGE. Immunoaffinity purification of (scFv)\(_2\) containing periplasmic preparations on antigen-coated beads showed that each of the closely spaced bands corresponded to functional protein retaining the capacity to specifically bind antigen. Others have also observed multiple scFv bands under non-reducing SDS-PAGE conditions (Neuberger et al., 1984; Kostelny et al., 1992; Huston et al., 1993), and it has been suggested that this results from anomalies associated with SDS binding to unreduced proteins.

**FIG. 5.** Flow cytometric analysis of peripheral blood leukocytes double stained with anti-CD8α scFv or (scFv)\(_2\) antibody fragments and a conventional fluorescein isothiocyanate-conjugated anti-CD8 monoclonal antibody. Peripheral blood leukocytes were incubated with periplasmic preparations of scFv (middle panel) and (scFv)\(_2\) (right panel) secreting bacteria, and bound fragments were detected using the 9E10 monoclonal antibody followed by a goat-anti-mouse phycoerythrin-labeled polyclonal antibody. As a control, the incubation step with scFv fragments was omitted (left panel). Only cells with a forward scatter/side scatter profile corresponding to lymphocytes are shown. Boxed area, CD8\(^+\) T cells as detected with a conventional monoclonal antibody.

**FIG. 6.** Immunohistochemical staining of COS7 cells transfected with a cDNA encoding the CD22\(β\) chain. COS7 cells were stained with a periplasmic preparation of bacteria transformed with the 40J construct enclosing a J un dimerized anti-CD22\(β\) (scFv)\(_2\). Bound antibodies were detected using the 9E10 anti-Myc antibody followed by goat-anti-mouse antibodies coupled to horse-radish peroxidase.

**FIG. 7.** Formation of bispecific anti-IgG \(×\) anti-DNP (scFv)\(_2\) fragments in vitro, visualized by non-denaturing SDS-PAGE and Western blotting. Periplasmic preparations of scFv clones 3F and 23J were reduced in 2-mercaptoethanol. Reduced proteins were mixed in a redox buffer followed by dialysis against PBS, resulting in the generation of bispecific antibody 3F \(×\) 23J.

**FIG. 8.** Detection of bispecific anti-IgG \(×\) anti-DNP fragments in a sandwich ELISA. IgG-coated plates were incubated with (scFv)\(_2\) proteins. After washing, DNP was added to the wells. Bound DNP was detected using an anti-DNP phage antibody followed by a horseradish peroxidase-coupled anti-M13 antibody. 3F \(×\) 23J, anti-DNP \(×\) anti-IgG bispecific (scFv)\(_2\) fragment; 3F and 23J, anti-IgG and anti-DNP dimers. –DNP and –(scFv)\(_2\), no DNP or scFv protein added.
Previously, a tendency of GCN4 zipper-linked “mini-antibodies” to display nonspecific binding to antigens coated to microtiter wells has been noted (Pack et al., 1993). We examined the binding specificities of our bivalent and bispecific (scFv)2 fragments in a number of assays, including ELISA, flow cytometry and immunohistochemistry. In none of these assays, significant nonspecific binding was observed. A reason for this apparent discrepancy between GCN4 zippers and Fos/Jun zippers may be a better shielding of the hydrophobic regions in the latter and/or the more stable configuration caused by covalently cross-linking the zipper regions.

Employing the much greater tendency of Fos and Jun zipper peptides to form heterodimers over homodimers (O’Shea et al., 1989; Kostelny et al., 1992), bivalent Fos and Jun leucine-zippered (scFv)2 can be rapidly converted to bispecific (scFv)2 molecules by simple reduction, mixing, and reoxidation steps. Using this approach, the anti-IgG and anti-DNP binding activities of two (scFv)2 homodimers were shown to be combined in a single heterodimeric molecule. A major advantage of this strategy is that only a single straightforward cloning step is required to produce bispecific antibodies obviating the need for extensive polymerase chain reaction and cloning efforts (Holliger, 1993; 1994; Mallender and Voss, 1994; Kurucz et al., 1995; Mack et al., 1995).

The dimerization system described here may be used to construct phage display libraries of bispecific antibodies. Bispecific antibodies that simultaneously recognize adjacent and non-overlapping epitopes on a target protein have higher avidities than the single chain or Fab antibodies obtained from conventional libraries (Neri et al., 1995). Thus, a Fos-linked scFv with a desirable specificity may be cloned into a phage library of Jun-scFv antibodies, permitting the direct recovery of high avidity bispecific antibodies using stringent selection procedures. We are currently performing experiments to assess the feasibility of this approach.

We show that using cysteine-modified Fos and Jun leucine zipper peptides, scFv antibody fragments isolated from phage display libraries can be simply converted to functional bivalent and bispecific molecules involving only a single doning step. It is important to note that scFv molecules obtained from phage display libraries have been through a stringent selection for correct expression, transport, and folding in bacterial cells. This explains why these antibodies and the derivatives described in this paper do not appear to suffer from many of the problems associated with bacterially expressed scFvs derived from hybridomas.

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