Orientation of antigen binding sites in dimeric and trimeric single chain Fv antibody fragments

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Received 9 December 1997; revised version received 4 March 1998

Abstract Electron microscopy of dimeric and trimeric single chain antibody Fv fragments (scFvs) complexed with anti-idiotype Fab fragments was used to reveal the orientation of antigen binding sites. This is the first structural analysis that discloses the multivalent binding orientation of scFv trimers (triabodies). Three different scFv molecules were used for the imaging analysis; NC10 scFv-5 and scFv-0, with five- and zero-residue linkers respectively between the V_H and V_L domains, were complexed with 3-2G12 anti-idiotype Fab fragments and 11-1G10 scFv-0 was complexed with NC41 anti-idiotype Fab fragments. The scFv-5 molecules formed bivalent dimers (diabodies) and the zero-linker scFv-0 molecules formed trivalent trimers (triabodies). The images of the NC10 diabody-Fab complex appear as boomerangs, not as a linear molecule, with a variable angle between the two Fab arms and the triabody-Fab complexes appear as tripods.

Key words: Antibody; Dimer; Trimer; Diabody; Triabody; Single chain Fv; Antigen complex

1. Introduction

Recombinant single chain Fv fragments (scFvs), in which the two variable domains are covalently joined via a flexible peptide linker, have been shown to fold in the same conformation as the Fv domain of the parent antibody [1,2]. ScFvs with linkers greater than 12 residues in length can form stable monomers which usually exhibit a similar antigen binding affinity to the parent antibody [1,3]. Reduction of the linker length (to 2–12 residues) prevents pairing of V_H and V_L domains on the same chain to form an Fv monomer and forces pairing with complementary domains of another chain to form bivalent dimers, termed diabodies [4–8]. The five-residue linker sequence usually chosen for diabodies, Gly_4Ser, was originally designed as a flexible and hydrophilic hinge [4]. Further reduction of the linker length to fewer than two residues, including direct ligation of the C-terminal residue of V_H to the N-terminal residue of V_L, results in the formation of trivalent trimers, termed triabodies [8–10]. Models of diabodies with five-residue linkers (scFv-5, [5]) and triabodies (scFv-0, [8–10]) have confirmed that significant flexibility between Fv domains is possible, resulting in different relative orientations of the antigen binding sites. A model of scFv-0 molecules as a diabody revealed the structure was severely constrained [4] and incompatible with the crystal structure of an scFv-5 diabody [6]. We have now demonstrated, in two cases [8,9], that trivalent trimers (triabodies) are the preferred association of scFv-0 molecules with directly linked V_H-V_L domains. We have proposed that the propensity to trimerise is a general property of scFv-0 molecules constructed by direct ligation of V_H and V_L domains [8,9]. Our understanding of flexibility and binding site orientation in triabodies is based to date on only one modelling study of a trivalent trimer [8] and one crystal structure of an inactive V_H-V_L trimer [10]. Flexibility between the antigen binding sites of scFv dimers and trimers is an important aspect in the design of cross-linking molecules for either cell recruitment immunotherapy [5] or whole blood diagnostic applications [7]. Furthermore, the increase in functional affinity (avidity) for multivalent scFvs [8] is dependent on the ability to simultaneously bind adjacent antigens. Flexibility is obviously an important factor in the ability to bind simultaneously to surface receptors on the same cell. To characterise the flexibility and orientation of the antigen binding sites in diabodies and triabodies, we pre-formed stable molecular complexes with the corresponding anti-idiotype Fab fragment and revealed their low-resolution structure by single-molecule imaging. Fab fragments were expected to appear as relatively rigid, linear arms extending outwards from the antigen binding sites [11,12].

2. Materials and methods

2.1. Construction of NC10 and 11-1G10 scFv genes

The V_H and V_L genes were amplified by PCR from the parent NC10 [13] and 11-1G10 [14] hybridomas and scFvs were constructed by PCR overlap extension. NC10 scFv-5 was constructed using a Gly_4Ser five-residue linker [8] and the scFv-0 genes were constructed by ligation between codons for C-terminal V_H-Ser^{112} and N-terminal V_L-Asp^1 for NC10 and between C-terminal V_H-Ser^{113} and N-terminal V_L-Gln^1 for 11-1G10. Residues are numbered according to the Kabat nomenclature as described [1]. The NC10 scFv genes were cloned into the expression vector pPOW as described previously [8], whereas the 11-1G10 scFv gene was expressed in vector pGC as described [9]. Both pPOW and pGC vectors provide an N-terminal pelB leader sequence and C-terminal FLAG octapeptide tag tail [8,9]. The entire DNA sequences of the cloned scFv inserts were determined using DNA purified by alkaline lysis and sequencing reactions performed using the PRISM Cycle Sequencing Kit (ABI).

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Abbreviations: FPLC, fast protein liquid chromatography; IPTG, isopropyl-β-thiogalactoside; Fab, antibody fragment produced by proteolysis; Fv, complex of V_H and V_L domains; NA, influenza neuraminidase; M_r, molecular mass; PCR, polymerase chain reaction; scFv, single chain Fv molecule; SDS-PAGE, electrophoresis in a 15% polyacrylamide gel containing 1% SDS; V_H, variable region from antibody heavy chain; V_L, variable region from antibody light chain; YT, yeast tryptone medium

PII S0014-5793(98)00292-0

2.2. ScFv synthesis, purification and formation of complexes with Fab fragments

The NC10 scFv-5 and scFv-0 genes in pPOW were expressed [13] and the proteins recovered from insoluble periplasmic aggregates by denaturation, refolding in vitro and purified by affinity and size exclusion chromatography as described previously [8]. Purified scFvs were concentrated to ~1–2 mg/ml, dialysed against phosphate-buffered saline (PBS), pH 7.4 which contained 0.02% (w/v) sodium azide and stored frozen.

The 11-1G10 scFv-0 gene in pGC was expressed as a soluble protein and purified from the culture supernatant by affinity chromatography on an NC41 Fab Sepharose 4B affinity column as described [9]. 11-1G10 scFv-0 was concentrated to ~1 mg/ml, dialysed against TBS, pH 7.4 and stored at 4°C.

The affinity purified scFv molecules were fractionated by size exclusion FPLC on either a Superdex 75 column (HR10/30) or a Superose 12 column (HR 10/30, Pharmacia) in PBS to determine their molecular size and to remove higher molecular mass aggregates and any Fv monomers produced by proteolysis in the scFv-0 preparations [8]. The concentrations of the scFv fragments were determined spectrophotometrically using the values for the extinction coefficient ($\varepsilon^{0.1\%}$) at 280 nm of 1.73 and 1.75 for NC10 scFv-5 and scFv-0, respectively, and of 2.03 for 11-1G10 scFv-0 calculated from the protein sequences [15].

The monoclonal antibodies NC41 (an IgG2a, anti-idiotype to 11-1G10) and 3-2G12 (an IgG1, anti-idiotype to NC10) were isolated from ascites fluid by protein A-Sepharose chromatography (Pharmacia). NC41 and 3-2G12 Fab fragments were prepared by proteolysis of Ig and purified by gel filtration on a Superdex 75 column (HR 10/30) in PBS, pH 7.4, as described previously [8,16]. Complexes were formed between scFv and anti-idiotype Fab as described [8,9] and purified by size exclusion FPLC on a Superose 6 column (HR 10/30) in PBS (flow rate 0.5 ml/min) to remove unbound scFv or Fab.

2.3. Electron microscopy

Three complexes (NC10 scFv-5 diabody/Fab, NC10 scFv-0 triabody/Fab and 11-1G10 scFv-0 triabody/Fab) and also a mixture of NC10 scFv-0 triabody/Fab with free 3-G12 anti-idiotype Fab were examined by electron microscopy. In each case, proteins were diluted in PBS to concentrations of the order of 0.01–0.03 mg/ml. Prior to dilution, 10% glutaraldehyde (Fluka) was added to the PBS to achieve a final concentration of 1% glutaraldehyde. Droplets of ~3 μl of this solution were applied to thin carbon film on 700-mesh gold grids which had been glow-discharged in nitrogen for 30 s. After 1 min the excess protein solution was drawn off, followed by application and withdrawal of 4–5 droplets of negative stain (2% potassium phosphotungstate adjusted to pH 6.0 with KOH). The grids were air-dried and then examined at 60 kV in a JEOL 100B transmission electron microscope at a magnification of 100 000×. Electron micrographs were recorded on Kodak SO-163 film and developed in undiluted Kodak D19 developer. The electron optical magnification was calibrated under identical imaging conditions by recording single molecule images of the NC10 antibody (Fab) complex with its antigen, influenza virus neuraminidase heads [17].

Measurements of particle dimensions were made on digitised micrographs using the interactive facilities of the SPIDER image processing suite [18] to record the coordinates of particle vertices. Particle arm lengths and inter-arm angles were calculated from the coordinates for 229 diabodies and 114 triabodies.

3. Results

Three different scFv molecules were used for this study. Two scFvs (scFv-5 and scFv-0) were derived from a parent murine antibody NC10 with specificity to avian influenza virus N9 neuraminidase (NA). The molecular structure of this neuraminidase and those of its complexes with NC10 Fab and scFv have been determined to 2.5 Å resolution by X-ray crystallographic analysis [1,19]. The third scFv was derived from a parent murine antibody 11-1G10, an anti-idiotype that competes with the antigen, avian influenza virus N9 NA, for binding to murine NC41 antibody [20]. Protein chemical characterisation has shown that the NC10 scFv-5 was a dimer (diabody) [8] and that both scFv-0 molecules derived from antibodies NC10 and 11-1G10 were trimers (triabodies) with three active antigen combining sites [8,9]. The NC10 scFv-5 dimer and scFv-0 trimer used in this study were produced by refolding from bacterial periplasmic inclusion bodies solubilised in 6 M guanidine hydrochloride, while the 11-1G10 scFv-0 trimer was recovered as an active, soluble molecule from the culture supernatant by affinity chromatography [9]. The molecular masses of isolated complexes between 3-2G12 anti-idiotype Fab and either NC10 scFv dimer or trimer confirmed the stoichiometry of two 3-2G12 Fabs binding to the NC10 scFv-5 dimer and three 3-2G12 Fabs binding per NC10 scFv-0 trimer (Fig. 1) [8]. These complexes were isolated by gel filtration for imaging and were stable to dilution and freezing (data not shown).

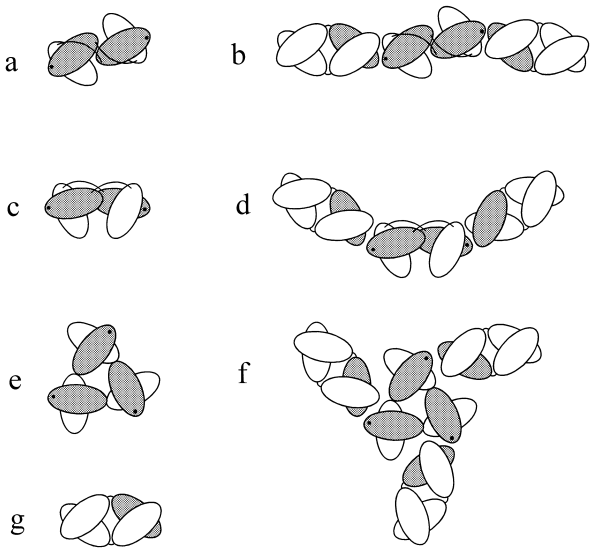
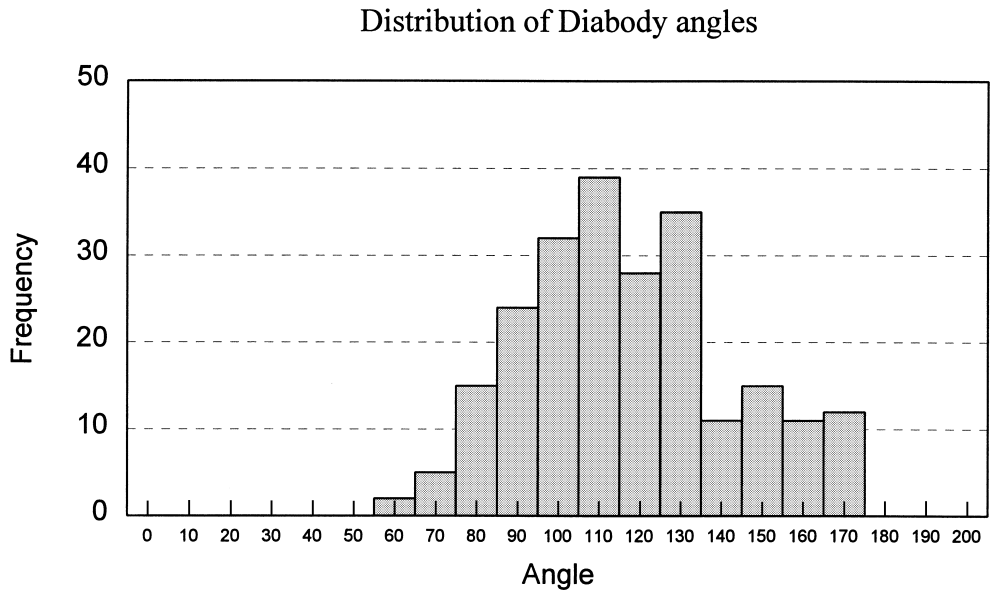


Fig. 1. Schematic 'static' interpretation of scFv diabodies, triabodies and their complexes with anti-idiotype Fab fragments. Ovals represent Ig V or C domains; the V_H domains are shown shaded and the N-terminal end of the scFv V_H domain is marked with a dot. (a) Rigid 'back-to-back' diabody [1] and (b) its linear Fab complex with two-fold symmetry axis perpendicular to page. (c) Flexible 'twisted' diabody [6,10] and (d) its boomerang-shaped Fab complex with vertical two-fold symmetry axis. (e) Triabody [8,9] and (f) its Y-shaped Fab complex with three-fold symmetry axis perpendicular to page. Fab molecules (g) are shown extending outwards from the scFv antigen binding site and, obviously, other orientations of V-domain pairing and linker geometry in both diabodies and triabodies are possible.

Electron micrographs of the NC10 scFv-5 diabodies complexed with two anti-idiotype 3-2G12 Fab molecules ($M_r \approx 156$ kDa) showed boomerang-shaped projections with the angle between the two arms ranging from 60 to 180° (Fig. 2a, Fig. 3a). The mean angle was 122°, with an approximately normal distribution of angles about the mean (Table 1). Each arm corresponds to a Fab molecule (Fig. 1) and, despite the potential 'elbow' flexibility between Fv and C modules, appears as a relatively rigid, linear molecular rod which extends outwards from the antigen binding sites [11,12]. Linearity of the Fab arms under the current imaging conditions was confirmed by the appearance of free 3-2G12 anti-idiotype Fabs imaged in conjunction with triabodies (data not shown). The variation in the angle between the arms indicates that there

Table 1
Histogram of inter-arm angles and lengths taken from 229 images of diabody-Fab complexes (NC10 scFv-5 complexed with 3-2G12 Fab)



Diabody Measurements

	Mean length (arbitrary units)	Std Dev
end-to-end	47.0	4.8
shorter arm	21.6	2.9
longer arm	25.4	2.6
Mean angle	122.4°	
Min angle	60.5°	
Max angle	178.8°	

appears to be considerable flexibility in the linker region joining the two scFvs in the diabody.

In micrographs of NC10 scFv-0 triabodies complexed with three 3-2G12 Fab molecules ($M_r \approx 212$ kDa), most fields showed a mixture of predominantly Y-shaped and V-shaped projections (Fig. 2b). There was some variation in particle appearance depending on the thickness of the stain on the carbon film. The Y-shaped projections (Fig. 3b) were interpreted as tripods (viewed from above), which had adopted an orientation in which all three legs (i.e. the distal ends of the three Fab molecules) were in contact with the carbon film. The three Fab legs were separated by two angles of mean 136° and one of mean 80°. However, the range of angles was such that for approximately 10% of particles the arms were evenly spaced with angles all 120° (±5°). The Y-shaped projections were unlikely to be planar as invariably one of the Fab legs appeared foreshortened. The V-shaped projections (Fig. 3c) were interpreted as tripods (triabody complexes) lying on their sides on the carbon film, with two Fab legs forming the V and the third Fab leg extending upward and out of the stain which would account for the increase in density sometimes observed at the junction of the V. The V-shaped structures (Fig. 3c) were clearly different to the boomerang-shaped diabody complexes (Fig. 3a), both in the angle between Fab arms and in the projected density in the centre of the molecules, consistent with the expected models (Fig. 1). The interpretation of tripods lying on their side is consistent with the appearance of a few projections with all three Fab legs pointing in the same direction.

Images of the 11-1G10 scFv-0 triabody/NC41 Fab complex were similar to the NC10 scFv-0 triabody/3-2G12 complex with numerous Y-shaped tripods in which all three Fab arms extended towards the same quadrant/direction (data not shown). As before, many V-shaped projections showed an increase in density at the junction of the V indicating a

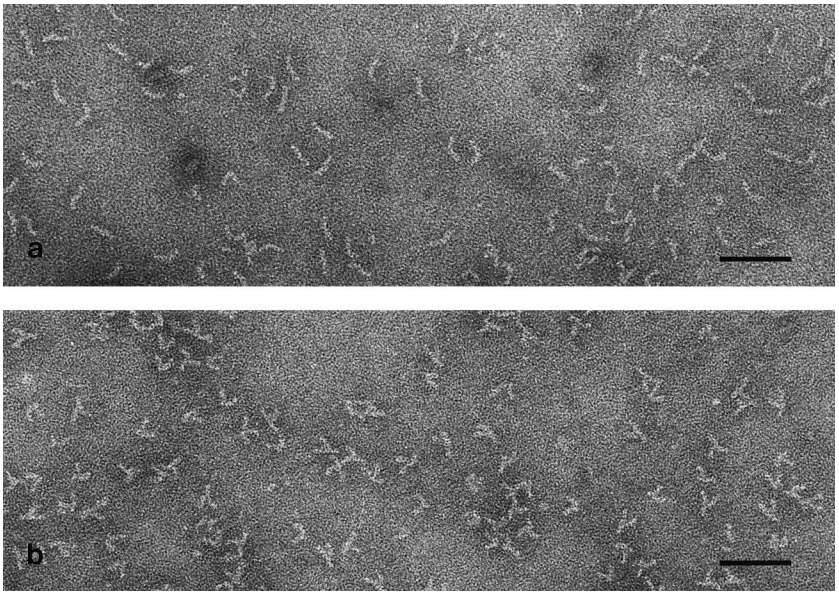


Fig. 2. Electron micrographs of complexes stained with potassium phosphotungstate (2%). Magnification bar is 50 nm. a: NC10 scFv-5 diabodies with two anti-idiotype 3-2G12 Fab molecules. The angle between the arms of the boomerang-shaped particles can be seen to vary from about 60° to 180°. b: NC10 scFv-0 triabodies with three 3-2G12 Fab molecules. Particles are predominantly either Y-shaped or V-shaped molecular complexes.

similar extension of the third leg of the tripod upward and out of the stain.

4. Discussion

The imaging of the NC10 scFv-5 dimer complexed with two anti-idiotype 3-2G12 Fabs revealed a planar boomerang-shaped structure with two Fab arms extending from the diabody and with a variable angle of about 60–180° between Fab arms with a mean of 122° (Fig. 2a). Few linear dimers (rods) were observed (fewer than 5% of particles had angles greater than 170°), indicating that NC10 scFv-5 diabodies are unlikely to form the rigid 'back-to-back' structure revealed in the X-ray structure of crystals of the NC10 scFv-15 (monomer) complexed to NA (Fig. 1b) [1]. Instead, the variable angle observed between Fab arms in the boomerang-shaped projections (Table 1) indicates there is considerable flexibility in the linker region joining the two Fv structures. This orientation is consistent with a 'twisted' or bent diabody structure (Fig. 1d), similar to that modelled by Holliger et al. [4,5] and confirmed in a crystal structure of a five-residue linker diabody [6]. However, in modelling studies of the L5MK16 diabody [5] the predicted angle between the Fv domains was larger than 122° and ranged from 138° to 166°.

The observed angle (Table 1) was interpreted as the true angle between Fab arms lying flat on the support film and not the result of variable orientations of Fab arms tilted out of the plane of the film for the following reasons. Firstly, if one Fab arm of the boomerang was flat on the support film, while the other projected out of the plane of the film, the upwardly projecting arm would appear foreshortened in projection compared to the other arm. It would be expected that if the distribution of measurements of the longer arm of each particle were compared with the distribution of measurements of the

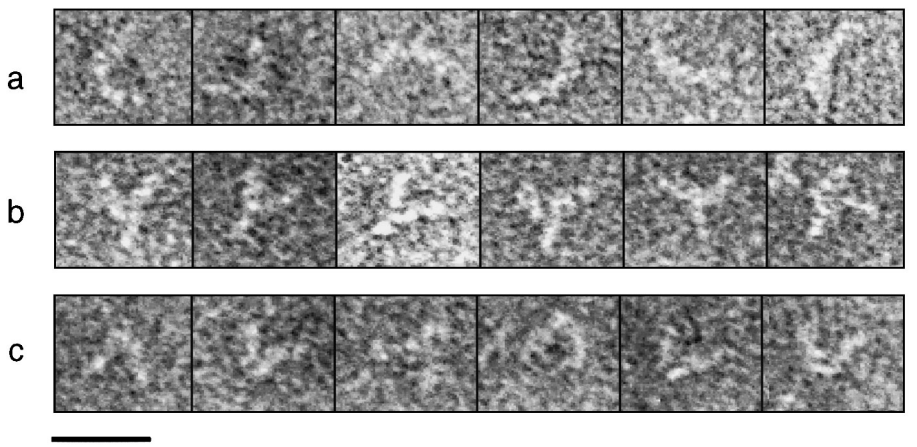


Fig. 3. Gallery of selected particles from electron micrographs of)a) boomerangs; NC10 scFv-5 diabody/3-2G12 Fab complex, (b) Y-shaped tripods; NC10 scFv-0 triabody/3-2G12 Fab complex, (c) V-shaped projections; NC10 scFv-0 triabody/3-2G12 Fab complex. Staining and photography as for Fig. 2; magnification bar 20 nm.

shorter arm, the shorter arm measurements would show a significantly greater standard deviation from the mean, due to the effect of varying angle of projection. However, it was found that the standard deviation for the shorter arm lengths was in fact smaller than that for the longer arm lengths (Table 1). Secondly, if the boomerangs were rigid, with a fixed Fab arm angle but tilted out of the plane of the support film with either the ends of the boomerang or the central point in contact with the film, those molecules lying almost in the plane of the film would appear to have the most acute inter-arm angle, and the end-to-end length of the molecule would be greatest. Also, those molecules oriented at right angles to the plane would have more linear projections and the projected end-to-end lengths would be shortest. However, a significant proportion of the straightest particles were also among the longest measured, and there was only a weak negative correlation between end-to-end length and angle (correlation coefficient = −0.33). Some variation in Fab arm length, even when lying flat on the support grid, is expected due to staining artifacts, radiation damage, and the effects of negative stain and dehydration on the protein.

The electron micrograph images (Figs. 2 and 3) of NC10 scFv-0 triabodies complexed with three 3-2G12 Fab molecules showed that the three Fab arms of the trimer-Fab complex are not coplanar, but are angled together in one direction and appear as the legs of a tripod (Fig. 3b) consistent with the triabody model of NC10 scFv-0 [8]. Similar Y-shaped tripod structures were also clearly observed for the 11-1G10 scFv-0 triabody complexed with three NC41 Fab molecules (data not shown). Triabodies are obviously flexible molecules, with observed angles between Fab arms in the NC10 triabody-Fab complexes distributed around two angles of mean 136° and one of mean 80°, and are not rigid molecules as shown schematically in Fig. 1f. Numerous V-shaped images with an increased density at the apex of the V were interpreted as the third arm of the tripod pointing upwards (Fig. 3c). Since the triabody-Fab complexes are stable in solution and all three antigen combining sites have equivalent affinity [8,9], it is unlikely that one Fab leg has dissociated from the complex to form the V-shaped projections. When free Fab arms were imaged in a mixture with triabodies, they typically appeared as distinctly linear rods of length comparable to that of triabody arms (data not shown). It is highly unlikely that one Fab arm had dissociated during preparation since imaging of purified triabody preparations did not reveal free Fab molecules in the proportion that would be anticipated: in fact few, if any, such Fabs were observed. However, we cannot unequivocally rule out the possibility of some Fab dissociation since the images of free Fab arms are at the limit of micrograph resolution.

In the construction of the NC10 scFv-0 molecule the V_H C-terminal residue Ser^{112} and the V_L N-terminal residue Asp^1 were ligated by direct fusion. While the absence of a flexible linker in this construct presumably prevents the formation of a dimer, the V_H and V_L domains paired to form a trimer. Precise structural data, obtained by crystallographic analysis [8,19], has shown that the C-terminal residue Ser^{112} is the last constrained residue in the V_H domain framework before the start of the flexible hinge region and similarly, Asp^1 of V_L is known to be hydrogen bonded to the V-domain framework and is close to, but not involved in, the antigen binding region. Therefore, this zero-residue construct has no sites of flexibility between the two domains. A computer graphic model for the NC10 scFv-0 triabody, constructed using circular three-fold symmetry [8] and with V_H and V_L domains rotated around the peptide bond to minimise steric clashes between domains, showed that the Fv conformation and CDR positions were consistent with a pyramid shaped molecule. The three antigen binding sites are at the apices of the pyramid; this would therefore produce a tripod structure when complexed with three anti-idiotype Fab molecules (Fig. 1), compatible with the Y-shaped projections observed in the EM images (Fig. 3).

In the design of 11-1G10 scFv-0, an additional residue (V_H-Ser^{113}) was present in the linker between V_H and V_L compared to NC10 scFv-0. The zero-linker scFv-0 'diabody' described by Holliger et al. [4,5] also contained this additional residue. The 11-1G10 scFv-0 exclusively formed trimers which were shown to be fully active and trivalent for Fab binding [9]. It is tempting to speculate that the trimeric conformation (triabodies) will be preferred over dimers (diabodies) for other scFv-0 molecules, although this property may be dependent on the particular antibody chosen and the precise choice of residues for the V_H-V_L linkage [8,9].

The gain in functional affinity through multivalent binding (often termed avidity [21]) makes trimeric scFvs attractive for in vivo tumour imaging as an alternative reagent to diabodies [22,23] and multivalent chemical conjugates [24–27]. The gain in functional affinity (avidity) for scFv trimers compared to scFv monomers is due to apparently reduced off-rates which result from both multiple binding and rebinding to the target antigens [8,21]. Multiple binding to surface-bound antigens is dependent on correct alignment and orientation in the Fv modules of diabodies and triabodies. If multiple binding is not sterically possible, particularly for surface-bound antigens, then apparent gains in functional affinity are likely to be small and due only to the effect of increased rebinding, which is dependent on diffusion rates and surface antigen concentration. Antigen orientation also affects the ability of diabodies and triabodies to simultaneously bind to multiple antigens on a cell surface, and this factor is particularly important in the design of any therapeutic reagent required to cross-link surface receptors on either the same or adjacent cells [21,28]. Indeed, receptor cross-linking and enhanced cell activation have recently been demonstrated using intact Ig molecules conjugated together into flexible dimers [29]. Flexibility in both scFv diabodies and triabodies is evident in our single molecule imaging studies. The effect of manipulating linker length, sequence and structure on diabody and triabody stability and flexibility can now be analysed using single molecule imaging of anti-idiotype complexes. The ability of triabodies to cross-link surface receptors is unknown, and will obviously depend on flexibility between the Fv modules and the orientation of the antigen binding sites, as well as the structure of the receptor. Furthermore, the construction of tricistronic expression vectors should enable the production of trispecific scFv-0 trimers capable of cross-linking different target antigens, with obvious applications in specific cell recruitment and activation.

Acknowledgements: The authors thank Professors D. Metzger and R. Webster for providing the 11-1G10, NC10, 3-2G12 and NC41 hybridoma cell lines, Dr D. Hewish for preparing the 3-2G12 and NC41 antibodies, J. Burns and G. Oddie for assistance with protein purification, and Drs N. McKern, J. Atwell and M. Lawrence for their

critical review of the manuscript. P. Iliades was supported by a CSIRO scholarship.

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