Protein Science (1997), 6:781–788. Cambridge University Press. Printed in the USA.

Remodeling domain interfaces to enhance heterodimer formation

ZHENPING ZHU,[1] LEONARD G. PRESTA,[2] GERARDO ZAPATA,[3] AND PAUL CARTER[1]

[1]Department of Molecular Oncology, Genentech Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080
[2]Department of Immunology, Genentech Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080
[3]Department of Process Sciences, Genentech Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080

(RECEIVED November 25, 1996; ACCEPTED January 22, 1997)

Abstract

An anti-p185^{HER2}/anti-CD3 humanized bispecific diabody was previously constructed from two cross-over single-chain Fv in which V_H and V_L domains of the parent antibodies are present on different polypeptides. Here this diabody is used to evaluate domain interface engineering strategies for enhancing the formation of functional heterodimers over inactive homodimers. A disulfide-stabilized diabody was obtained by introducing two cysteine mutations, V_L L46C and V_H D101C, at the anti-p185^{HER2} V_L/V_H interface. The fraction of recovered diabody that was functional following expression in *Escherichia coli* was improved for the disulfide-stabilized compared to the parent diabody (>96% versus 72%), whereas the overall yield was >60-fold lower. Eleven "knob-into-hole" diabodies were designed by molecular modeling of sterically complementary mutations at the two V_L/V_H interfaces. Replacements at either interface are sufficient to improve the fraction of functional heterodimer, while maintaining overall recoverable yields and affinity for both antigens close to that of the parent diabody. For example, diabody variant v5 containing the mutations V_L Y87A:F98M and V_H V37F:L45W at the anti-p185^{HER2} V_L/V_H interface was recovered as 92% functional heterodimer while maintaining overall recovered yield within twofold of the parent diabody. The binding affinity of v5 for p185^{HER2} extracellular domain and T cells is eightfold weaker and twofold stronger than for the parent diabody, respectively. Domain interface remodeling based upon either sterically complementary mutations or interchain disulfide bonds can facilitate the production of a functional diabody heterodimer. This study expands the scope of domain interface engineering by demonstrating the enhanced assembly of proteins interacting via two domain interfaces.

Keywords: bispecific diabody; engineered disulfide bond; knobs-into-holes

Designed protein heterooligomers containing different mutations on closely related subunits have proved to be highly useful in probing the relationship between protein structure and function (Robey & Schachman, 1985; Bedouelle & Winter, 1986; Carter et al., 1986; Wente & Schachman, 1987). In addition, bispecific antibodies and other heterooligomers have significant therapeutic potential (Carter et al., 1995). Unfortunately, the utility of designer hetero-oligomers has been greatly restricted by the lack of efficient methods to prepare such molecules.

Purification of heterodimers from stochastic mixture of dimerized monomers has a maximum possible yield of 50%. Remodeling domain interfaces to prevent homodimer formation and promote heterodimer formation offers a potentially more efficient approach. For example, sterically complementary "knobs-into-holes" mutations on either side of a domain interface (Ridgway et al., 1996) have been used successfully to engineer antibody heavy chains for heterodimerization.

Here we evaluate different domain interface engineering strategies for enhancing the preference of a pair of proteins to form heterodimers rather than homodimers. Our test system is an anti-p185^{HER2}/anti-CD3 humanized bi-specific diabody (Zhu et al., 1996). Bi-specific diabodies are heterodimers of two cross-over single-chain (sc) Fv fragments in which V_L and V_H domains of the two antibodies are present on different polypeptide chains (Holliger et al., 1993). Intra-chain pairing of variable domains is prevented by separating them with linkers of 10 residues or less. After secretion of these two chains from *E. coli* they can associate to form a non-covalent heterodimer that may reconstitute both antigen-binding specificities. Also possible are inactive homodimers (Fig. 1A). Separate expression of component scFv for the anti-p185^{HER2}/anti-CD3 diabody leads to inactive homodimers, whereas when coexpressed these crossover scFv fragments show some preference for heterodimer formation (Zhu et al., 1996).

Reprint requests to: Paul Carter, Department of Molecular Oncology, Genentech Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080; email: pjc@gene.com.

Abbreviations: CDR, complementarity determining region, ds, disulfide-stabilized; ECD, extracellular domain; FR, framework region; IRF, immunoreactive fraction; sc, single-chain.

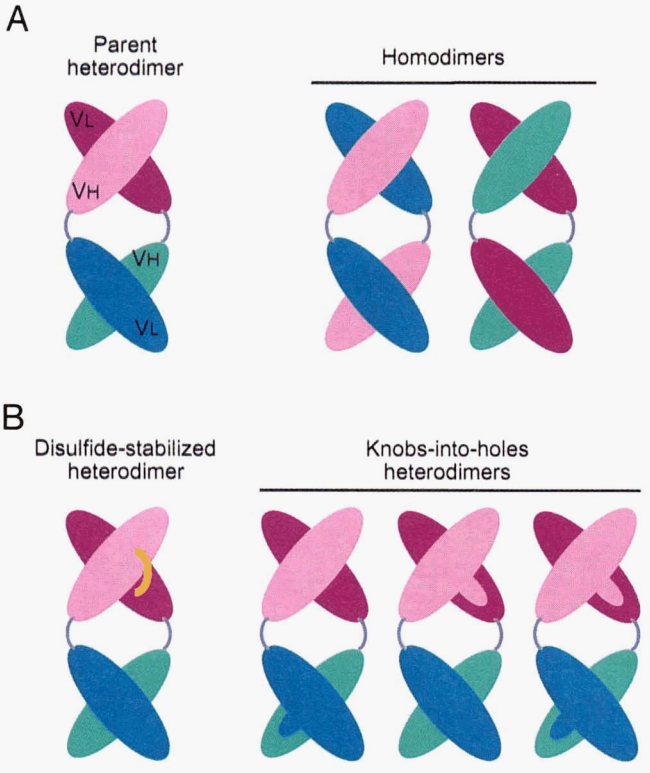

Fig. 1. Domain interface design strategies. **A:** Pairing of cross-over scFv to form heterodimers and homodimers in which cognate and non-cognate variable domains (labeled at N-termini) are paired, respectively. **B:** Disulfide and knobs-into-holes engineering approaches to promote heterodimerzation over homodimerization of cross-over scFv.

Two strategies are evaluated here for redesigning the anti-p185^{HER2}/anti-CD3 crossover scFv to promote heterodimerization over homodimerization (Fig. 1B). First, a disulfide-stabilized (ds) diabody was created by installing a disulfide bond across the anti-p185^{HER2} V_L/V_H interface. The second approach utilizes "knobs-into-holes" mutations at the anti-p185^{HER2} and anti-CD3 V_L/V_H interfaces, both separately and in combination. Diabody variants are compared in their recovery following secretion from *E. coli*, antigen binding affinities, and the fraction of preparations that are functional—the immunoreactive fraction (IRF).

Results

Design of diabody variants

An anti-p185^{HER2}/anti-CD3 humanized bispecific diabody (Zhu et al., 1996) was previously constructed that comprises two cross-over scFv fragments: anti-p185^{HER2} V_L followed by a GGGGS linker and then anti-CD3 V_H; anti-CD3 V_L followed by a GGGGS linker and then anti-p185^{HER2} V_H. This diabody is used here to evaluate domain interface engineering strategies for enhancing the formation of functional diabody heterodimers over inactive homodimers. The two design strategies tested are installing a disulfide bond or sterically complementary mutations across V_L/V_H interfaces (Fig. 1B).

A disulfide bond was introduced across the anti-p185^{HER2} V_L/V_H interface by means of the cysteine mutations, V_L L46C and V_H D101C.[4] These particular mutations were chosen because installing them into the anti-p185^{HER2} Fv resulted in efficient disulfide bond formation and retention of p185^{HER2} extracellular domain (ECD) binding affinity (Rodrigues et al., 1995). In contrast, two other pairs of cysteine replacements gave inefficient disulfide bond formation and reduction of p185^{HER2} ECD binding affinity (Rodrigues et al., 1995). We anticipated that the desired ds diabody heterodimer would be the only soluble disulfide-linked dimer. It seemed likely that it would be possible to purify the ds diabody away from homodimers and other inactive species lacking the inter-chain disulfide bond.

A second strategy tested is knobs-into-holes engineering using sterically complementary mutations (Ridgway et al., 1996). Knobs-into-holes diabody variants were designed by molecular modeling and created by mutagenesis of residues at the anti-p185^{HER2} and anti-CD3 V_L/V_H interfaces, both separately and also in combination. Knobs were constructed by replacing small side chains with larger ones, whereas holes of identical or similar size to the knobs were created by replacing large side chains with smaller ones. Knob and hole variants were anticipated to preferentially heterodimerize by virtue of the knob inserting into a judiciously placed hole on the partner variable domain.

The choice of residues to replace in the anti-p185^{HER2} Fv was guided by the corresponding X-ray crystallographic structure (Eigenbrot et al., 1993). The V_L/V_H interface involves seven complementarity-determining region (CDR) and eight framework-region (FR) residues from V_L plus 11 CDR and 7 FR residues from V_H. FR residues were mutated, whereas the antigen binding CDR loops were left unchanged to lessen the likelihood of impairing antigen binding. Knobs-into-holes mutations were targeted to four FR residues located at the center of the V_L/V_H interface: V_L Y87 and F98, V_H V37 and L45 (Fig. 2A). Other FR residues at the V_L/V_H interface were left unchanged for a variety of reasons: V_L Y36 and L46, plus V_H W47 contact CDR residues and may indirectly affect antigen binding, V_H Q39 and V_L Q38 play a structural role in hydrogen bonding to each other, whereas V_L Y49 and V_H G44 are significantly exposed to solvent.

In the parent anti-p185^{HER2} Fv, V_H residues V37 and L45 pack closely against V_L residues F98 and Y87 (Fig. 2B). A large knob was created in the anti-p185^{HER2} V_H by the mutations V37F and L45W. A complementary hole to accommodate this knob was created in the anti-p185^{HER2} V_L by the mutations F98M and Y87A. Additional diabody variants were designed using permutations of the following knob and hole mutations: V_L Y87A or W; V_L F98S, M or W; V_H V37A, F or Y and V_H L45A or W. Anti-CD3 and anti-p185^{HER2} Fv fragments have identical FR residues and likely closely related structures. This encouraged us to mutate the same set of FR residues in both of these diabody components.

Diabody expression and purification

The parent diabody was recovered in good yield (~6 mg/L) by protein A chromatography of periplasmic extracts of *E. coli* cultured in shake flasks. The two component scFv fragments of the parent diabody are not readily resolved by SDS-PAGE and give rise to a single major band with mobility close to that anticipated (Zhu et al., 1996). The molecular masses of the component cross-

[4] Variants are denoted by the original amino acid residue and number according to Kabat et al. (1991) followed by the replacement amino acid. Multiple mutations are separated by colons.

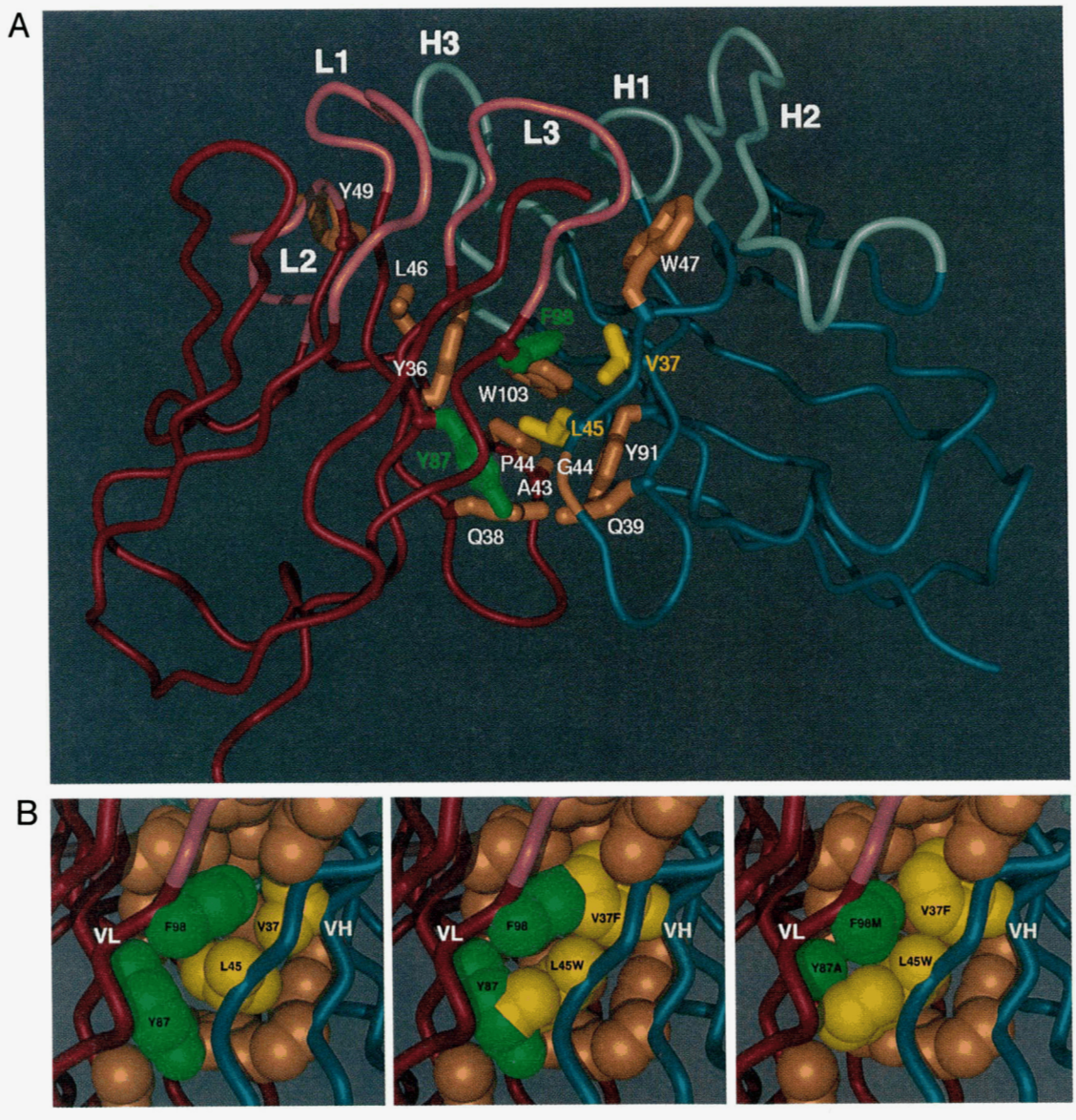

Fig. 2. Design of knobs-into-holes anti-p185^{HER2} Fv. **A:** Representation of the X-ray crystallographic structure of the anti-p185^{HER2} Fv (1FVC, Eigenbrot et al., 1993). The V_H FR (aquamarine) and CDR (H1-H3, pale green) residues and V_L FR (red) and CDR residues (L1–L3, pink) are shown together with the side chains of FR residues at the V_L/V_H interface targeted for mutagenesis (yellow and green) or left unchanged (brown). Interface residues are defined as those in which at least one side chain atom is within 6 Å of any atom in the other variable domain. **B:** Packing of side chains of V_L Y87 and F98 (green) with V_H L45 and V37 (yellow) for the parent Fv (left panel), showing the steric clash introduced by the knob, V_H L45W:V37F (middle panel), and relief of the steric clash by the complementary hole, V_L Y87A:F98M (right panel).

over scFv of the diabody determined by high-resolution electrospray MS are in excellent agreement with those anticipated (Zhu et al., 1996).

Knobs-into-holes diabodies were recovered in approximately similar yield (1.3–8.4 mg/L, Table 1) and purity (not shown) as the parent diabody. In contrast, only very small quantities of ds diabody (v1) were recovered from shake flasks (<0.1 mg/L) and useful amounts (5 mg/L) of the ds diabody could only be obtained after culturing the corresponding *E. coli* host to high cell density (124 OD_{550}) in a fermentor. In addition, a large amount of insoluble diabody (>300 mg/L) was found associated with the cells (not shown). About 65% of ds diabody preparations are disulfide-linked heterodimer as judged by SDS-PAGE and scanning laser densitometry (Fig. 3). The ds diabody was purified to near homogeneity by cation exchange chromatography and gave rise to bands of the expected electrophoretic mobility in the absence (M_r = 51,000) and presence of reducing agent (M_r = 25,500).

Characterization of parent and disulfide-stabilized diabodies

The binding affinity of diabodies for p185^{HER2} ECD was determined by surface plasmon resonance, whereas the stoichiometry was determined by diabody titration with p185^{HER2} ECD and size-exclusion chromatography. Diabody binding to CD3 was investigated by competition with ^{125}I-anti-CD3 v9 Fab (Rodrigues et al., 1992) for binding to the Jurkat T cell line (Zhu & Carter, 1995).

Table 1. *Comparison of diabody variants*[a]

Variant	Anti-p185^{HER2} mutations		Anti-CD3 mutations		p185^{HER2} ECD binding		Jurkat binding	Recovery
	V_L	V_H	V_L	V_H	IRF (%)[b]	K_d (nM)[c]	IC_{50} (nM)[d]	(mg/L)[e]
Parent[f]	—	—	—	—	72 ± 2.6	0.8 ± 0.3	6.0 ± 0.3	6.4 ± 2.4
v1 (ds)	L46C	D101C	—	—	96 ± 1.0	3.7 ± 0.3	2.9 ± 0.4	<0.1
v2	F98S	V37Y	—	—	81 ± 1.0	47 ± 20	3.0 ± 0.8	8.4 ± 0.3
v3	Y87A	L45W	—	—	58 ± 3.6	1.0 ± 0.2	5.4 ± 1.9	3.4 ± 0.4
v4	Y87W	L45A	—	—	70 ± 1.5	1.3 ± 0.2	5.1 ± 0.7	2.1 ± 0.3
v5	Y87A:F98M	V37F:L45W	—	—	92 ± 1.0	6.4 ± 0.8	3.5 ± 0.7	3.0 ± 0.2
v6	Y87A:F98S	V37Y:L45W	—	—	87 ± 2.0	200 ± 60	4.9 ± 1.6	3.7 ± 0.7
v7	—	—	F98M	V37F	60 ± 4.6	1.3 ± 0.1	6.3 ± 0.1	2.2 ± 0.2
v8	—	—	Y87A	L45W	83 ± 1.9	0.8 ± 0.2	6.4 ± 1.2	4.0 ± 0.2
v9	—	—	Y87W	L45A	84 ± 0.6	0.7 ± 0.2	2.1 ± 0.5	7.3 ± 0.2
v10	—	—	Y87A:F98M	V37F:L45W	71 ± 2.8	1.4 ± 0.4	5.3 ± 0.8	1.7 ± 0.2
v11	—	—	Y87W:F98W	V37A:L45A	<30	3.7 ± 1.9	12.9 ± 1.5	2.8 ± 0.8
v12	Y87A:F98M	V37F:L45W	Y87W	L45A	<30	20.4 ± 4.2	4.7 ± 0.6	1.3 ± 0.2

[a]Data shown are the mean ± SE of ≥ two independent experiments.
[b]IRF were determined by antigen titration followed by size exclusion chromatography (Zhu et al., 1996).
[c]K_d values for p185^{HER2} ECD binding were estimated by surface plasmon resonance (Kelley & O'Connell, 1993).
[d]IC_{50} values were obtained by competition with ^{125}I-anti-CD3 v9 Fab for binding to Jurkat cells (Zhu & Carter, 1995).
[e]Diabody recovery following protein A purification from shake flask cultures was estimated from A_{280} and $\epsilon^{0.1\%} = 2.03$ (Zhu et al., 1996).
[f]The parent diabody is functionally indistinguishable from the diabody described previously (Zhu et al., 1996) and differs only in that the GGPGS linker between V_L and V_H domains is replaced by GGGGS.

The purified parent diabody consists of ~87% dimer with the remainder comprising earlier eluting species that likely include higher order multimers and aggregates (Fig. 4). Approximately 83% of the parent diabody dimer is capable of binding p185^{HER2} ECD to give an IRF of 72% (83 × 0.87). In contrast, the ds diabody (v1) is recoverable almost entirely as functional heterodimer (IRF > 96%) (Fig. 4), which is free of detectable higher order multimers and aggregates. The ds diabody has comparable affinity for CD3 as the parent diabody but approximately fivefold weaker binding to p185^{HER2} ECD (Table 1).

Characterization of knob-into-hole diabodies

Knob-into-hole mutations were installed at the anti-p185^{HER2} (v2–v6), anti-CD3 (v7–v11), or both V_L/V_H interfaces (v12). The most successful diabody mutagenized at the anti-CD3 V_L/V_H interface (v9: V_L Y87W and V_H L45A) gave 84% functional heterodimer. Diabody variant v9 is unique among those constructed in that in addition to enhanced heterodimer formation, it compares favorably with the parent diabody in binding affinity for both antigens. In particular, v9 shows similar binding affinity for p185^{HER2} as the parent diabody, whereas the affinity for T cells is approximately threefold improved for v9. Almost as promising as v9 is v8 in which the knob-into-hole pair was inverted: V_L Y87A and V_H L45W. Neither of this pair of mutations increased the yield of functional heterodimer when installed at the anti-p185^{HER2} V_L/V_H interface (v3 and v4).

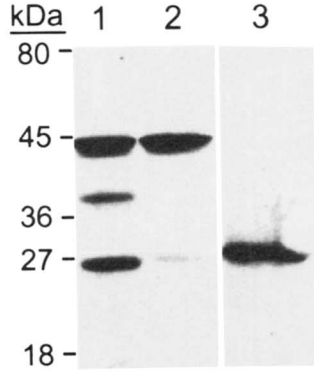

Fig. 3. Purification of the ds diabody following secretion from *E. coli*. Lane 1, protein A pool. Lane 2, Mono S pool. Lane 3, Mono S pool in the presence of DTT. Also shown are the positions of molecular weight standards.

Diabody variant v5 containing the mutations V_L Y87A:F98M and V_H V37F:L45W at the anti-p185^{HER2} V_L/V_H interface (Fig. 2B) gave the highest yield of functional heterodimer (IRF = 92%) (Table 1). This reflects in part that variant v5 is free of higher order multimers and aggregates, in striking contrast to the parent diabody (Fig. 4). The binding affinity of v5 for p185^{HER2} ECD is eightfold weaker than for the parent diabody, whereas T cell binding is approximately twofold tighter.

Installing the FR mutations present in v5 at the anti-CD3 V_L/V_H interface (v10) did not enhance the formation of functional heterodimer. Replacement of V_L residue 98 with a serine and V_H residue 37 with a tyrosine in v5 to create v6 gave a small decrease in the yield of functional heterodimer but reduced the binding affinity for p185^{HER2} ECD by 30-fold.

The most productive mutations at the anti-p185^{HER2} (V_L Y87A:F98M, V_H V37F:L45W) and anti-CD3 (V_L Y87W, V_H L45A) V_L/V_H interfaces were installed into the same diabody (v12) and gave very low yield (<30%) of functional heterodimer. This reflects that v12, in contrast to other diabodies, is primarily monomer rather than dimer as judged by size exclusion chromatography (not shown).

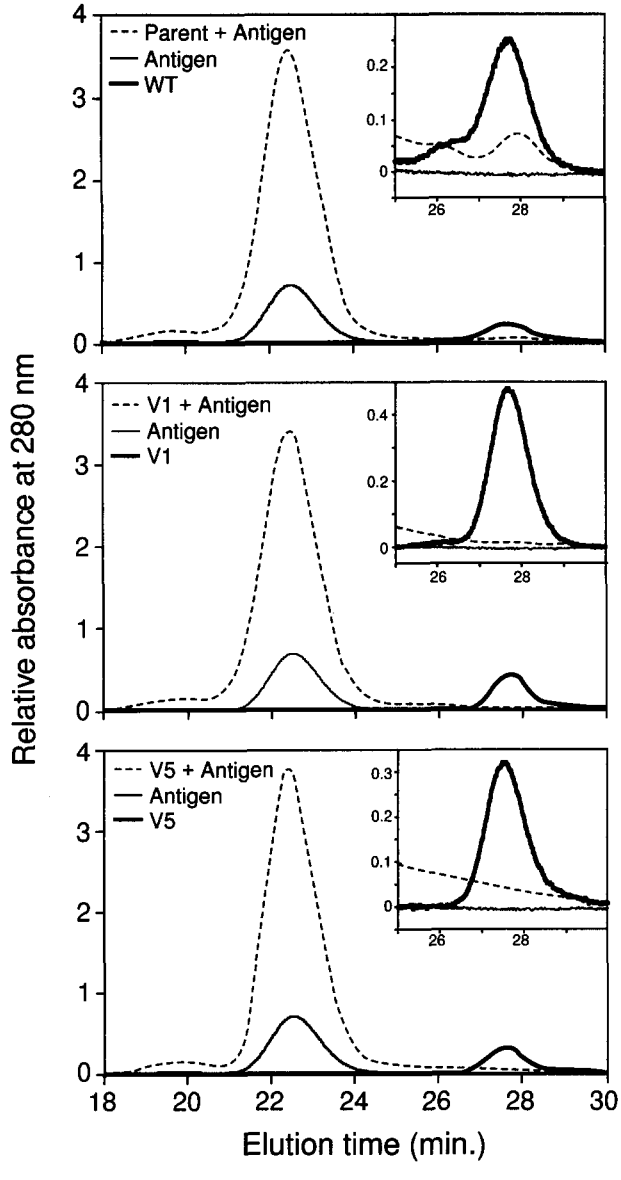

Fig. 4. FPLC size exclusion chromatography of parent, v1 (ds) and v5 diabodies. The diabodies (1 μM) were run in the presence or absence of p185^{HER2} ECD antigen (4 μM). Also shown are chromatographs with antigen alone (1 μM). Plot inserts highlight titration of the diabody variants with antigen by expanding the relative absorbance scale leaving the time scale unchanged. Under these conditions the retention times of other antibody fragments are as follows: dsFv (25 kDa) 29.5 min, Fab (50 kDa) 26.8 min, and F(ab')$_2$ (96 kDa) 23.5 min.

Discussion

The significant potential of designed heterooligomers for structure–function analysis and as human therapeutics has been broadly stymied by the dearth of efficient preparation methods for such molecules. Here, we have evaluated two alternative strategies for enhancing heterodimer formation: installing an interchain disulfide bond and knobs-into-holes engineering of domain interface residues. These approaches were chosen as they build upon our prior experience with dsFv fragments (Rodrigues et al., 1995) and in remodeling antibody heavy chains for heterodimerization (Ridgway et al., 1996). Domain interface engineering strategies were tested in the context of an anti-p185^{HER2}/anti-CD3 humanized bispecific diabody heterodimer (Zhu et al., 1996).

Strengths and limitations of disulfide-stabilized diabody

A ds diabody (v1) was obtained by introducing mutations, V_L L46C and V_H D101C (Rodrigues et al., 1995), at the anti-p185^{HER2} V_L/V_H interface. The ds diabody was much improved over the parent diabody in eliminating higher order aggregates and multimers (Fig. 4) and in higher IRF (96% versus 72%, Table 1). An additional attractive feature of the ds diabody is that the disulfide-linked heterodimer can be readily distinguished from other species by SDS-PAGE. The major drawback of the ds diabody is the very low yield of soluble protein compared to the parent diabody. In addition, the affinity of the diabody for p185^{HER2} EC D was reduced by approximately fivefold on installing the inter-chain disulfide bond.

The vast majority of the ds diabody was insoluble and found in inclusion bodies. It may be possible to oxidatively re-fold the ds diabody as shown by Pastan and colleagues for dsFv-linked immunotoxins (Brinkmann et al., 1993; Reiter et al., 1994a, 1994b). Engineering a disulfide bond across the C_H3 domain interface of an antibody facilitated heterodimerization without compromising expression titers from human embryonic kidney (293) cells (A. M. Merchant, L. Presta, & P. Carter, unpubl. obs.). This encourages us to express the ds diabody in 293 cells and other eukaryotic hosts.

Strengths and limitations of knobs-into-holes diabodies

A second strategy evaluated for enhancing diabody formation was installation of sterically complementary domain interface mutations. A priori, mutations at either one or both V_L/V_H interfaces might be required to enhance diabody formation. In practice, it was readily possible to enhance diabody formation with mutations at either anti-p185^{HER2} or anti-CD3 V_L/V_H interface alone (Table 1).

It has proved much more difficult, however, to enhance heterodimer formation while simultaneously maintaining the antigen binding affinity of the parent diabody. Indeed, only v9 and v8 of the five variants that showed improved IRF had antigen-binding affinities that compare favorably with parent diabody. This was is spite of the precaution of making replacements of FR residues away from the antigen-binding CDR loops. Small relative movements of variable domains may accompany antigen binding, as shown by structure determination of complexed and uncomplexed Fab and Fv fragments (Bhat et al., 1990). Replacements at the V_L/V_H interface may potentially interfere with the relative movement of these domains and thereby impair antigen binding.

Alternative routes to active diabodies

Small (microgram) quantities of fully functional anti-p185^{HER2}/anti-CD3 diabody can be readily isolated by affinity purification using immobilized p185^{HER2} antigen (Zhu et al., 1996). No evidence was found for conversion of the active anti-p185^{HER2}/anti-CD3 diabody back to inactive homodimers. Such low yields of active diabody are inconvenient for biophysical or X-ray crystallographic studies and unsuitable for human therapy where gram or even kilogram amounts of material are needed. In contrast, knobs-into-holes engineering *does* permit preparation of large (milligram to gram) quantities of predominantly functional anti-p185^{HER2}/anti-CD3 diabody. In addition, domain interface engineering has

potential utility for diabodies where the corresponding antigens are not available and also for other proteins.

Extending V_L/V_H interface mutations to other Fv fragments

We have identified 4 FR residues (V_L 87 and 98, V_H 37 and 45) whose replacement can enhance heterodimerization of a corresponding diabody. Replacement of these FR residues in additional Fv fragments is encouraged by the very high sequence conservation at V_L/V_H interfaces between different human antibodies (Kabat et al., 1991).

The effects of domain interface mutants are context dependent: FR replacements at the anti-p185^{HER2} V_L/V_H interface that enhance diabody formation did not do so when installed at the anti-CD3 V_L/V_H interface and vice versa. This context dependence may reflect differences in CDR residues at the V_L/V_H interface (FR interface residues are identical) and their relative contribution to heterodimer and homodimer formation. In addition, there may be differences between the anti-p185^{HER2} and anti-CD3 antibodies in their requirements for relative movement of V_L and V_H for optimal antigen binding. Thus, the replacements of the key FR residues identified (V_L Y87 and F98, V_H V37 and L45) will require optimization for individual Fv. This problem is readily amenable to a phage display solution. We recently used a phage display strategy to select for C_H3 domain interface variants that form stable heterodimers (S. Atwell, J. B. B. Ridgway, J. A. Wells, & P. Carter, unpubl. obs.). It will likely be possible to develop analogous phage display libraries to select for diabodies that are stable and that also bind tightly to their cognate antigens.

Comparison of interface remodeling for diabodies and C_H3 domains

Diabodies represent a much more demanding test for domain interface engineering than do previously targeted antibody heavy chains. First, maintenance of antigen binding affinity by the diabody provides a stringent functional constraint beyond efficient heterodimerization assayed for antibody heavy chains (Ridgway et al., 1996). For example, v6 is improved for heterodimer formation but is significantly impaired for p185^{HER2} ECD binding. Second, diabodies, unlike antibody heavy chains, lack inter-chain disulfide bonds so de-stabilizing interface mutations may lead to heterodimer dissociation; for example, v11 and v12 are predominantly monomers. Third, there are two inter-chain domain interfaces in the diabody, whereas significant protein–protein interaction is limited to the C_H3 interface for antibody heavy chains. This significantly increases the complexity of domain interface engineering with the diabody as mutations need to be evaluated at each interface separately and also in combination.

Alternative strategies to remodeling domain interfaces

Engineering single complementary charges at a hydrophobic interface in different mutants of the enzyme tyrosyl tRNA synthetase gave preferential formation of heterodimers over homodimers. Unfortunately this specificity was gained at the expense of greatly reduced stability of the heterodimer compared to the wild-type homodimer (Ward et al., 1987). More successful was the addition of six lysine or six glutamate residues at the domain interface of GCN4-derived leucine zippers. This pair of designed zippers forms a stable heterodimer (K_d = 30 nM) and have $>10^5$-fold preference for forming heterodimers over homodimers (O'Shea et al., 1993). This chain pairing specificity is due to electrostatic de-stabilization of homodimers rather than stabilization of heterodimers by salt bridges (O'Shea et al., 1993). It remains to be seen if charge replacements of FR residues at the V_L/V_H interface can enhance diabody formation without significantly destabilizing the diabody, impairing antigen binding, or compromising expression.

Sterically complementary mutations can be combined with charge mutations in remodeling a domain interface for heterodimerization. Such a design strategy was recently used to obtain inactive mutants of HIV protease that do not readily homodimerize but efficiently heterodimerize with the wild-type protease, thereby inactivating it (McPhee et al., 1996). Unfavorable steric interactions between bulky substitutions and repulsion between similarly charged residues were predicted to disfavor homodimerization of the mutant protease, whereas enhanced hydrophobic interaction and a potential salt bridge were anticipated to promote heterodimerization with the wild-type enzyme. The distribution of heterodimer and homodimers formed awaits biochemical investigation. Nevertheless, the success of this approach is clearly demonstrated by reduction in viral polyprotein processing and diminished titers of infectious virus.

Conclusions

We have demonstrated that installing an interchain disulfide bond or sterically complementary mutations can facilitate the production of a functional heterodimer, thereby enhancing the clinical potential of a diabody. This expands the scope of domain interface engineering by showing that it is possible to direct the assembly of proteins that interact via two domain–domain interfaces. Further optimization of V_L/V_H interfaces is required to identify combinations of residues that give fully functional diabody while maintaining antigen binding and expression titers comparable to that of the parent diabody.

Materials and methods

Design of domain interface variants

Knobs-into-holes diabodies were designed using Insight II release 95.0 (Biosym/MSI) starting from the X-ray crystallographic structure of the anti-p185^{HER2} Fv (huMAb4D5-8, Eigenbrot et al., 1993; 1FVC) and a molecular model for the anti-CD3 Fv (huMAbUCHT1 v9, Rodrigues et al., 1992). Four buried residues at the V_L/V_H domain interface were targeted for replacement: V_L Y87 and F98, V_H V37 and L45. Knobs were first designed by replacement of each of these residues with larger ones. Holes of similar size to the knobs were then created on the partner variable domain by replacing side chains with smaller ones. Potential packing solutions of replaced side chains and their immediate neighbors were evaluated by sampling permutations of commonly observed rotamers (Ponder & Richards, 1987). Replacements were considered to be sterically complementary if no non-bonded atoms were within ~3.0 Å of each other while maintaining side-chain torsion angles within 30° of the commonly observed rotamers.

Vector construction

Phagemids pZZ1 and pZZ2 encoding anti-p185^{HER2}/anti-CD3 crossover scFv (Zhu et al., 1996) were mutagenized (Kunkel et al.,

1987; Carter, 1991) to replace the GGPGS linker between V_L and V_H domains with GGGGS to create pZZ1.1 and pZZ2.1, respectively. Diabody expression plasmid, pZZ4, was constructed by ligation of the small *Mlu* I/*Nhe* I and *Nhe* I/*Sph* I fragments from pZZ1.1 and pZZ2.1, respectively, with the large *Mlu* I/*Sph* I fragment from pAK19 (Carter et al., 1992). Diabody variants were obtained by mutagenesis of pZZ1.1 and pZZ2.1 using the following oligonucleotides for the anti-p185^{HER2} Fv:

V_L L46C 5′-GCTCCGAAATGCCTGATTTAC-3′;
V_H D101C 5′-TATGCTATGTGTTACTGGGGT-3′;

V_L F98S 5′-CCTCCCACGTCCGGACAGGGT-3′;
V_H V37Y 5′-ATACACTGGTATCGTCAGGCC-3′;

V_L Y87A 5′-GCAACTTATGCCTGTCAGCAA-3′;
V_H L45W 5′-GGTAAGGGCTGGGAATGGGTT-3′;

V_L Y87W 5′-GCAACTTATTGGTGTCAGCAA-3′;
V_H L45A 5′-GGTAAGGGCGCCGAATGGGTT-3′;

V_L F98M 5′-CCTCCCACGATGGGACAGGGT-3′;
V_H V37F 5′-ATACACTGGTTCCGTCAGGCC-3′;

and for the anti-CD3 Fv:

V_L F98M 5′-CCGTGGACGATGGGACAGGGC-3′;
V_H V37F 5′-ATGAACTGGTTCCGTCAGGCC-3′;

V_L Y87A 5′-GCAACTTATGCCTGTCAGCAA-3′;
V_H L45W 5′-GGTAAGGGCTGGGAATGGGTT-3′;

V_L Y87W 5′-GCAACTTATTGGTGTCAGCAA-3′;
V_H L45A 5′-GGTAAGGGCGCCGAATGGGTT-3′;

V_L F98W 5′-CCGTGGACGTGGGGACAGGGC-3′;
V_H V37A 5′-ATGAACTGGGCCCGTCAGGCC-3′.

All mutations were verified by dideoxynucleotide sequencing using Sequenase version 2.0 (United States Biochemicals). Expression plasmids for diabody variants were constructed in a similar manner to pZZ4.

Diabody production

E. coli strain 16C9 (W3110 Δ*ton*A *pho*AΔE15 Δ*deo*C2 Δ(*arg*F-*lac*)169) was transformed with individual expression plasmids, cultured in shake flasks (Zhu & Carter, 1995), and corresponding diabodies isolated from periplasmic extracts of cells by protein A chromatography (Pro-Sep A, Bioprocessing Inc.) as described (Zhu et al., 1996). For larger scale preparation of the ds diabody, *E. coli* strain 33B6 (Rodrigues et al., 1995) transformed with the corresponding expression plasmid was cultured for ~40 h at 30 °C in an aerated 10 L fermentor as described (Zhu et al., 1996). Following purification by protein A chromatography (Zhu et al., 1996) the ds diabody was concentrated and buffer exchanged into 10 mM MES (pH 5.5) using Centricon 10 concentrators (Amicon) and loaded on to a Mono S 5/5 column (Pharmacia Biotech). The column was washed with 10 column volumes of 10 mM MES (pH 5.5) containing 50 mM NaCl, prior to elution with a linear gradient of 50–100 mM NaCl in 10 mM MES (pH 5.5) over 20 column volumes. Purified diabodies were buffer exchanged into phosphate-buffered saline and their concentration estimated from A_{280} ($\epsilon^{0.1\%}$ = 2.03; Zhu et al., 1996).

Antigen binding by diabodies

The affinity of diabodies for p185^{HER2} ECD was determined by surface plasmon resonance using the BIAcore system (Pharmacia Biotech) as previously described (Kelley & O'Connell, 1993). The efficiency of binding of diabodies to CD3 on Jurkat T cell line was evaluated by competition binding with ^{125}I-labeled anti-CD3 v9 Fab (Zhu & Carter, 1995).

The stoichiometry of diabody/antigen interaction was determined by titration with p185^{HER2} ECD followed by size-exclusion chromatography. Diabodies (50 pmol) in phosphate-buffered saline in the absence or presence of 200 pmol p185^{HER2} ECD (Fendly et al., 1990) in a total volume of 50 μL were subjected to size-exclusion FPLC analysis with a Superose 12 column (Pharmacia Biotech) using 100 mM NaH_2PO_4, pH 6.5 and a flow rate of 0.5 mL/min (Zapata et al., 1995). The IRF of diabody preparations was estimated by peak integration (Zhu et al., 1996).

Acknowledgments

We thank Brad Snedecor and Han Chen for *E. coli* fermentations, Mark Vasser, Parkash Jhurani, Peter Ng, and Kristina Azizian for synthetic DNA, and Wayne Anstine and David Wood for help in preparing the figures.

References

Bedouelle H, Winter G. 1986. A model of synthetase/transfer RNA interaction as deduced by protein engineering. *Nature 320*:371–373.

Bhat TN, Bentley GA, Fischmann TO, Boulot G, Poljak RJ. 1990. Small rearrangements in structures of Fv and Fab fragments of antibody D1.3 on antigen binding. *Nature 347*:483–485.

Brinkmann U, Reiter Y, Jung S-H, Lee B, Pastan I. 1993. A recombinant immunotoxin containing a disulfide-stabilized Fv fragment. *Proc Natl Acad Sci USA 90*:7538–7542.

Carter P. 1991. Mutagenesis facilitated by the removal or introduction of unique restriction sites. In: McPherson MJ, ed. *Mutagenesis: A practical approach*. Oxford, UK: IRL Press. pp 1–25.

Carter P, Bedouelle H, Winter G. 1986. Construction of heterodimer tyrosyl-tRNA synthetase shows tRNA^{Tyr} interacts with both subunits. *Proc Natl Acad Sci USA 83*:1189–1192.

Carter P, Kelley RF, Rodrigues ML, Snedecor B, Covarrubias M, Velligan MD, Wong WLT, Rowland AM, Kotts CE, Carver ME, Yang M, Bourell JH, Shepard HM, Henner D. 1992. High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. *Bio/Technology 10*:163–167.

Carter P, Ridgway J, Zhu Z. 1995. Toward the production of bispecific antibody fragments for clinical applications. *J Hematother 4*:463–470.

Eigenbrot C, Randal M, Presta L, Carter P, Kossiakoff AA. 1993. X-ray structures of the antigen-binding domains from three variants of humanized anti-p185^{HER2} antibody 4D5 and comparison with molecular modelling. *J Mol Biol 229*:969–995.

Fendly BM, Kotts, C, Vetterlein D, Lewis GD, Winget M, Carver ME, Watson SR, Sarup J, Saks S, Ullrich A, Shepard HM. 1990. The extracellular domain of HER2/neu is a potential immunogen for active specific immunotherapy of breast cancer. *J Biol Resp Mod 9*:449–455.

Holliger P, Prospero T, Winter G. 1993. "Diabodies": Small bivalent and bispecific antibody fragments. *Proc Natl Acad Sci USA 90*:6444–6448.

Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C. 1991. In: *Sequences of proteins of immunological interest*, 5th edition. Bethesda, MD: National Institutes of Health.

Kelley RF, O'Connell MP. 1993. Thermodynamics of an antibody functional epitope. *Biochemistry 32*:6828–6835.

Kunkel TA, Roberts JD, Zakour RA. 1987. Rapid and efficient site-directed mutagenesis without phenotypic selection. *Methods Enzymol 154*:367–382.

McPhee F, Good AC, Kuntz ID, Craik CS. 1996. Engineering human immunodeficiency virus 1 protease heterodimers as macromolecular inhibitors of viral maturation. *Proc Natl Acad Sci USA 93*:11477–11481.

O'Shea EK, Lumb KJ, Kim PS. 1993. Peptide "velcro": Design of a heterodimeric coiled coil. *Curr Biol 3*:658–667.

Ponder J, Richards F. 1987. Use of packing criteria in the enumeration of allowed sequences for different structural classes. *J Mol Biol 194*:775–791.

Reiter Y, Brinkmann U, Kreitman RJ, Jung S-H, Lee B, Pastan I. 1994a. Stabilization of the Fv fragments in recombinant immunotoxins by disulfide bonds engineered into conserved framework regions. *Biochemistry 33*:5451–5459.

Reiter Y, Brinkmann U, Webber KO, Jung SH, Lee B, Pastan I. 1994b. Engineering interchain disulfide bonds into conserved framework regions of Fv fragments: Improved biochemical characteristics of recombinant immunotoxins containing disulfide-linked Fv. *Protein Eng 7*:697–704.

Ridgway JBB, Presta LG, Carter P. 1996. "Knobs-into-holes" engineering of antibody C_H3 domains for heavy chain heterodimerization. *Protein Eng 9*:617–621.

Robey EA, Schachman HK. 1985. Regeneration of active enzyme by formation of hybrids from inactive derivatives: Implications for active sites shared between polypeptide chains of aspartate transcarbamoylase. *Proc Natl Acad Sci USA 82*:361–365.

Rodrigues ML, Shalaby MR, Werther W, Presta L, Carter P. 1992. Engineering a humanized bispecific $F(ab')_2$ fragment for improved binding to T cells. *Intl J Cancer (Suppl) 7*:45–50.

Rodrigues ML, Presta LG, Kotts CE, Wirth C, Mordenti J, Osaka G, Wong WLT, Nuijens A, Blackburn B, Carter P. 1995. Development of a humanized disulfide-stabilized anti-$p185^{HER2}$ Fv-β-lactamase fusion protein for activation of a cephalosporin doxorubicin prodrug. *Cancer Res 55*:63–70.

Ward WHJ, Jones DH, Fersht AR. 1987. Effects of engineering complementary charged residues into the hydrophobic subunit interface of tyrosyl-tRNA synthetase. *Biochemistry 26*:4131–4138.

Wente SR, Schachman HK. 1987. Shared active sites in oligomeric enzymes: Model studies with defective mutants of aspartate transcarboylase produced by site-directed mutagenesis. *Proc Natl Acad Sci USA 84*:31–35.

Zapata G, Ridgway JBB, Mordenti J, Osaka G, Wong WLT, Bennett GL, Carter P. 1995. Engineering linear $F(ab')_2$ fragments for efficient production in *Escherichia coli* and enhanced anti-proliferative activity. *Protein Eng 8*:1057–1062.

Zhu Z, Carter P. 1995. Identification of heavy chain residues in a humanized anti-CD3 antibody important for efficient antigen binding and T cell activation. *J Immunol 155*:1903–1910.

Zhu Z, Zapata G, Shalaby MR, Snedecor B, Chen H, Carter P. 1996. High level secretion of a humanized bispecific diabody from *Escherichia coli*. *Bio/Technology 14*:192–196.