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'Knobs-into-holes' engineering of antibody C_H3 domains for heavy chain heterodimerization

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'Knobs-into-holes' was originally proposed by Crick in 1952 as a model for the packing of amino acid side chains between adjacent α-helices. 'Knobs-into-holes' is demonstrated here as a novel and effective design strategy for engineering antibody heavy chain homodimers for heterodimerization. In this approach a 'knob' variant was first obtained by replacement of a small amino acid with a larger one in the C_H3 domain of a CD4-IgG immunoadhesin: T366Y. The knob was designed to insert into a 'hole' in the C_H3 domain of a humanized anti-CD3 antibody created by judicious replacement of a large residue with a smaller one: Y407T. The anti-CD3/CD4-IgG hybrid represents up to 92% of the protein A purified protein pool following co-expression of these two different heavy chains together with the anti-CD3 light chain. In contrast, only up to 57% of the anti-CD3/CD4-IgG hybrid is recovered following co-expression in which heavy chains contained wild-type C_H3 domains. Thus knobs-into-holes engineering facilitates the construction of an antibody/immunoadhesin hybrid and likely other Fc-containing bifunctional therapeutics including bispecific immunoadhesins and bispecific antibodies.

Keywords: bispecific antibody/heterodimerization/immunoadhesin

Introduction

Bispecific antibodies (BsAb) have significant potential for human therapy (reviewed by Nolan and O'Kennedy, 1990; Songsivilai and Lachmann, 1990; Fanger *et al.*, 1992) which has been effectively stymied by the difficulty of obtaining BsAb in sufficient quantity and purity using either hybrid-hybridoma technology (Milstein and Cuello, 1983) or by directed-chemical coupling of Fab' fragments obtained from intact Ab (Brennan *et al.*, 1985; Glennie *et al.*, 1987). Recent progress in directed chemical coupling of *Escherichia coli*-derived Fab' fragments (Rodrigues *et al.*, 1992; Shalaby *et al.*, 1992) together with direct recombinant methods (reviewed by Holliger and Winter, 1993; Carter *et al.*, 1996) offer a variety of ways of efficiently preparing BsAb lacking an Fc region. However, BsAb designed for many clinical applications preferably include an Fc region to extend serum permanence time and permit effector functions. Here Ab heavy (H) chains have been engineered for heterodimerization to facilitate the construction of Fc-containing bifunctional molecules for human therapy.

X-ray crystallography has demonstrated that the intermolecular association between human IgG_1 H chains in the Fc region includes extensive protein–protein interaction between C_H3 domains whereas the glycosylated C_H2 domains interact via their carbohydrate (Deisenhofer, 1981). In addition, there are two inter H chain disulfide bonds which are efficiently formed during Ab expression in mammalian cells unless the H chain is truncated to remove C_H2 and C_H3 domains (King *et al.*, 1992). Thus H chain assembly appears to promote disulfide bond formation rather than vice versa. Taken together, these structural and functional data led us to hypothesize that Ab H chain association is directed by the C_H3 domains. We further speculated that the interface between C_H3 domains might be engineered to promote formation of heterodimers of different H chains and hinder assembly of corresponding homodimers.

A 'knobs-into-holes' strategy was adopted to engineer C_H3 for heterodimerization. Knobs were created by replacing small amino side chains at the interface between C_H3 domains with larger ones, whereas holes were constructed by replacing large side chains with smaller ones. This approach was inspired by Crick's model (Crick, 1952, 1953) for the packing of amino acid side chains between adjacent α-helices which was subsequently demonstrated by the X-ray crystallographic structure of a leucine zipper (O'Shea *et al.*, 1991). In Crick's model, the side chains of residues in an α-helix are represented as spaced knobs on the surface of a cylinder alternating with holes in which knobs of an adjacent α-helix might fit.

Knob-into-hole C_H3 variants were designed and compared with wild-type C_H3 in their ability to direct the formation of the Ab immunoadhesin hybrid (Ab/IA), anti-CD3/CD4-IgG (Chamow *et al.*, 1994). This was accomplished by co-expression of humanized anti-CD3 light (L) and H chains (Rodrigues *et al.*, 1992; Shalaby *et al.*, 1992) together with CD4-IgG (Byrn *et al.*, 1990). Formation of heterodimers and homodimers was assessed by protein A purification followed by SDS–PAGE and scanning laser densitometry.

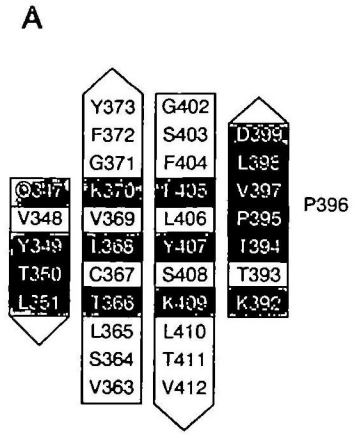
Materials and methods

Construction of C_H3 variants

Mutations were constructed in the C_H3 domain of the humanized anti-CD3 Ab H chain (T366Y, F405A, Y407T, and T366Y:F405A) and in CD4-IgG (T366Y, T394W, Y407T and T394W:Y407T) by site-directed mutagenesis (Kunkel *et al.*, 1987; Carter, 1991) using previously described phagemid vectors (Chamow *et al.*, 1994) and the following oligonucleotides:

T366Y, 5′ TTGACCAGGCAGTACAGGCTGACCTG 3′;
F405A, 5′ CTTGCTGTAGAGGGCGAAGGAGCCGTC 3′;
Y407T, 5′ CACGGTGAGCTTCGAAGTGAGGAAGAAG-GAG 3′; and
T394W, 5′ GCACGGGAGGCCAGGTCTTGTAGTTG 3′.

Mutations are denoted by the amino acid residue and number [Eu numbering scheme of Kabat *et al.* (1991)], followed by the replacement amino acid. Multiple mutations are represented



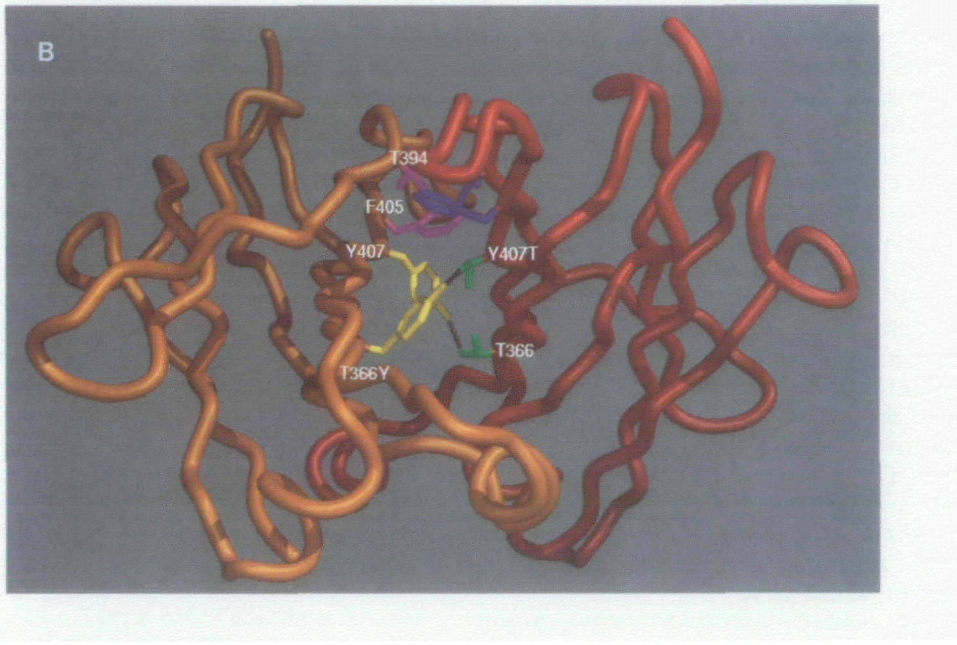


Fig. 1. C_H3 dimer interface. (**A**) Anti-parallel β-strands at C_H3 dimer interface showing non-contact and contact residues in black and white lettering, respectively (Miller, 1990). (**B**) C_H3 dimer based upon a 2.9 Å structure of human IgG_1 Fc (Deisenhofer, 1981) highlighting T366Y and Y407T mutations on opposite sides of the interface together with residues F405 and T394.

by the single mutation separated by a colon. Mutants were verified by dideoxynucleotide sequencing (Sanger *et al.*, 1977) using Sequenase version 2.0 (United States Biochemicals, Cleveland, OH).

Expression and purification of Ab/IA variants

Phagemids encoding anti-CD3 H and L chains (Rodrigues *et al.*, 1992; Shalaby *et al.*, 1992) and a CD4-IgG IA (Byrn *et al.*, 1990) were co-transfected into human embryonic kidney cells, 293S, for transient expression as described (Chamow *et al.*, 1994). The total amount of transfected DNAs was fixed at 15 μg whereas the ratio of input DNAs was varied. Excess L over H chain DNA was used in attempt to avoid the L chain from being limiting.

Products were affinity purified using Staphylococcal protein A (ProSep A, BioProcessing Ltd, UK). The efficiency of recovery of IA homodimer and IgG from conditioned culture media following protein A purification was 41.5% and 40.3%, respectively, as judged by antigen-binding ELISA. Additional purification of Ab/IA hybrid was achieved by ion-exchange chromatography using a gradient of 0–300 mM NaCl in 20 mM Tris–HCl, pH 8.0, on a mono S HR 5/5 column (Pharmacia, Piscataway, NJ). Products were analyzed by SDS–PAGE, (8% acrylamide, Novex, San Diego, CA) followed by staining with Coomassie Brilliant Blue R-250 and scanning laser densitometry (model GS-670, Bio-Rad, Hercules, CA). The densitometric signal response was found to be linear (r = 0.9993) over the experimental range used (0.02–10 μg) as judged by a control experiment using a closely related humanized antibody, huMAb4D5–8 (Carter *et al.*, 1992).

Results

Design of knob-into-hole C_H3 variants

A 'knobs-into-holes' strategy was adopted to engineer C_H3 for heterodimerization. In this approach, knob and hole variants were anticipated to heterodimerize by virtue of the knob inserting into an appropriately designed hole on the partner C_H3 domain. Knobs were constructed by replacing small side chains with the largest side chains, tyrosine or tryptophan. Holes of identical or similar size to the knobs were created by replacing large side chains with the smaller ones, in this case alanine or threonine.

The C_H3/C_H3 interface involves 16 residues on each domain located on four anti-parallel β-strands which buries 1090 Å^2 from each surface (Deisenhofer, 1981; Miller, 1990). Mutations were targeted to residues located on the two central anti-parallel β-strands (Figure 1A). This strategy was adopted to

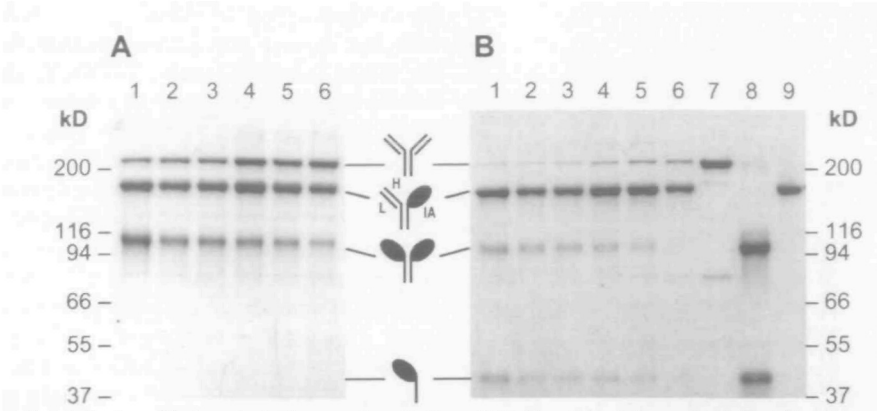


Fig. 2. SDS–PAGE analysis of protein A purified products from co-transfection of Ab H and L chains with IA for **(A)** wild-type chains and **(B)** Ab Y407T, IA T366Y variants. The ratio (by mass) of IA:H chain:L chain input DNAs (15 μg total) was varied as follows: lane 1, 8:1:3; lane 2, 7:1:3; lane 3, 6:1:3; lane 4, 5:1:3; lane 5, 4.1:3; lane 6, 3:1:3; lane 7, 1:0:0; lane 8, 0:1:3; and lane 9, sample from lane 5 after ion-exchange chromatography (mono S,

minimize the risk that a knob variant could homodimerize by rotating into solvent to accommodate the additional side chain volume.

Residue T366 is within hydrogen-bonding distance of residue Y407 on the partner C_H3 domain. Indeed, the principal intermolecular contact to residue T366 is to residue Y407 and vice versa. A knob-into-hole pair was created with the reciprocal mutations of T366Y in one C_H3 domain and Y407T in the partner domain, thus maintaining the volume of side chains at the interface (Figure 1B). A second knob-into-hole pair, F405A and T394W, was evaluated both alone and in combination with the first mutant pair to install both a knob and a hole on each C_H3 domain: T366Y:F405A and T394W:Y407T.

Analysis of products from expression of C_H3 variants

Knob-into-hole and wild-type C_H3 variants were compared in their ability to direct the formation of the Ab immunoadhesin hybrid (Ab/IA), anti-CD3/CD4-IgG (Chamow *et al.*, 1994). Phagemids encoding anti-CD3 L and H chain variants (Rodrigues *et al.*, 1992; Shalaby *et al.*, 1992) were co-transfected into human embryonic kidney cells, 293S, together with a CD4-IgG variant encoding phagemid (Byrn *et al.*, 1990). The total amount of transfected phagemid DNAs was fixed whereas the ratio of different DNAs was varied in an attempt to optimize the yield of Ab/IA hybrid. The products were affinity purified using Staphylococcal protein A prior to analysis by SDS–PAGE and scanning laser densitometry. The identity of products was verified by electroblotting on to a poly(vinylidene difluoride) membrane (Matsudaira, 1987) followed by amino terminal sequencing (data not shown).

Products from wild-type C_H3 variants

Co-transfection of phagemids for the anti-CD3 L chain together with those for the corresponding H chain and anti-CD3/CD4-IgG IA incorporating wild-type C_H3 resulted in a mixture of Ab/IA hybrid, IgG and IA homodimer products (Figure 2A), as shown previously (Chamow *et al.*, 1994). The larger the fraction of input DNA encoding Ab H plus L chains or IA, the higher was the fraction of corresponding homodimers recovered. An input DNA ratio of 6:1:3 of IA:H:L yielded 54.5% Ab/IA hybrid with similar fractions of IA homodimer (22.5%) and IgG (23.0%). These ratios are in good agreement with those expected from equimolar expression of each chain followed by random assortment of H chains with no bias being introduced by the method of analysis: 50% Ab/IA hybrid, 25% IA homodimer and 25% IgG.

Products from knob-into-hole C_H3 variant

In contrast to chains containing wild-type C_H3, Ab/IA hybrid was recovered in yields of up to 92% from co-transfections in which the anti-CD3 H chain and CD4-IgG IA contained the Y407T hole and T366Y knob mutations, respectively (Figures 2B and 3B). Similar yields of Ab/IA hybrid were obtained if these reciprocal mutations were installed with the knob on the H chain and the hole in the IA (Figure 3C). In both cases monomer was observed for the chain containing the knob but not the hole. Thus the T366Y knob is apparently more disruptive to homodimer formation than the Y407T hole. The fraction of Ab/IA hybrid obtained with T366Y and Y407T mutant pair was virtually independent of the ratio of input DNAs over the wide range tested (Figure 3B). Furthermore, the contaminating species were readily removed from the Ab/IA hybrid by ion-exchange chromatography (Figure 2B, lane 9). This augurs well for the preparation of larger quantities of Ab/IA hybrids using stable cell lines where the relative expression levels of Ab and IA are less readily manipulated than in the transient expression system used here.

The fraction of Ab/IA hybrid was not significantly changed by increasing the size of both knob and hole (Ab T366W, IA Y407A) (not shown). A second knob-into-hole pair (Ab F405A, IA T394W) yielded up to 71% Ab/IA hybrid using a small fraction of IA input DNA to offset the unanticipated proclivity of the IA T394W knob variant to homodimerize (Figure 3D). Combining the two independent knob-into-hole mutant pairs (Ab T366Y:F405A, IA T394W:Y407T) did not improve the yield of Ab/IA hybrid over the Ab T366Y, IA Y407T pair (compare Figures 3C and 3E). As anticipated, installation of the T394W mutation into the IA Y407T variant reduced the amount of IA homodimer, albeit with a concomitant increase in the expression of IA monomer.

Discussion

We have used a 'knobs-into-holes' strategy to engineer human IgG_1 C_H3 domain homodimers for efficient heterodimerization in the construction of an Ab/IA hybrid. In particular, we demonstrate that the T366Y and Y407T mutant pair at the C_H3 domain interface is sufficient to promote efficient formation of an anti-CD3/CD4-IgG hybrid. In contrast, the mutant pair, F405A and T394W, gave only a small improvement over wild-type domains in the formation of the Ab/IA hybrid. Furthermore, the T394W knob yielded significant amounts of

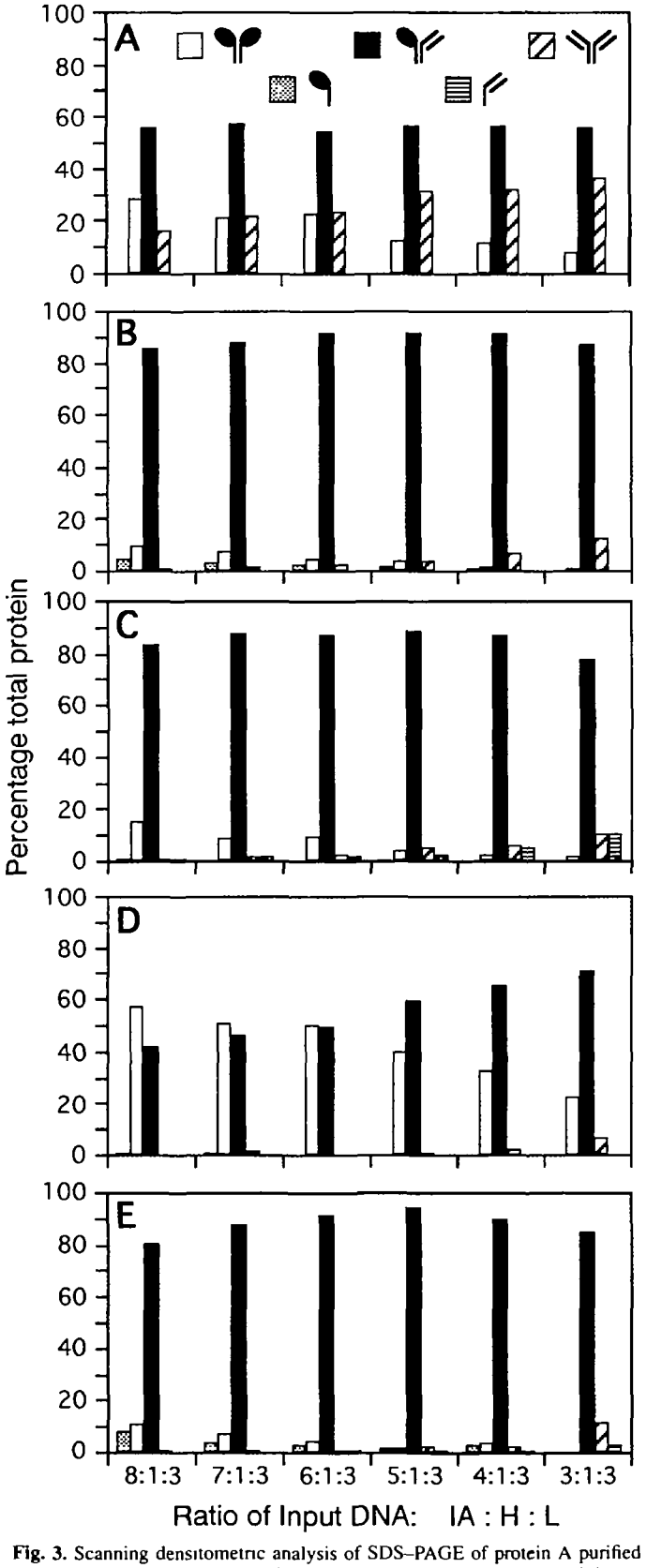


Fig. 3. Scanning densitometric analysis of SDS–PAGE of protein A purified products from co-transfection of Ab H and L chains with IA. (**A**) Wild-type; (**B**) Ab Y407T, IA T366Y; (**C**) Ab T366Y, IA Y407T; (**D**) Ab F405A, IA T394W; (**E**) Ab T366Y:F405A, IA T394W:Y407T. Data presented are

the corresponding IA homodimer. These data are consistent with the notion that knobs created towards the center of the dimer interface, such as T366Y, are more disruptive to homodimer formation than those located near the edge of the dimer interface, such as T394W (Figure 1A).

Tyrosine and tryptophan replacements were chosen for constructing knobs whereas threonine and alanine replacements were utilized for creating holes. Glycine and cysteine replacements were avoided as potential holes to minimize the effect of mutations upon main chain conformation, and prevent possible oxidation or mispairing of disulfide bonds, respectively. Other large and small residues are currently being evaluated in the design of knobs and holes.

A priori, knobs-into-holes mutations may affect the relative association rates of Ab H chains with IA chains and/or the thermodynamic stability of corresponding homodimers and heterodimers. The yield of Ab/IA hybrid likely reflects primarily the relative rates of association of chains into homodimers and heterodimers rather than the thermodynamic stability of Ab/IA hybrids. This is a consequence of hinge disulfide bond formation which restricts free exchange of partners. Knob mutations were found to disfavor strongly homodimer formation as anticipated, since extra side chain volume at the interface must perturb the packing of interface residues. Hole mutations were also found to disfavor homodimer formation, which may reflect the loss of some favorable intermolecular contacts with the partner chain as compared to the wild-type homodimer.

In contrast to the co-expression experiments, the knob variant, T366Y, when expressed alone forms significant amounts of IA monomer as well as IA homodimer (Figure 2B, lane 8). This is in spite of an increase in side chain volume of 155 $Å^3$ at the C_H3 domain interface. *A priori* this additional side chain volume might be accommodated by local structural perturbation and/or by small displacement of one C_H3 domain with respect to the partner.

The T366Y and Y407T mutations are directly applicable to the construction of bispecific IA (Dietsch *et al.*, 1993), which further expand IA as a class of novel therapeutic (Ashkenazi *et al.*, 1993). In addition, the mutations identified are anticipated to increase the clinical potential of Fc-containing BsAb by reducing the complexity of the mixture of products obtained from a possible 10 major species (Suresh *et al.*, 1986) down to four or less. The T366Y and Y407T mutant pair will likely be useful for heterodimerization of other human IgG isotypes since T366 and Y407 are fully conserved and other residues at the C_H3 domain interface of IgG_1 (Deisenhofer, 1981; Miller, 1990) are highly conserved (Kabat *et al.*, 1991).

The construction of heteroligomers from homoligomers where different subunits contain different mutations provides a powerful tool for studying the relationship between protein structure and function (Robey and Schachman, 1985; Carter *et al.*, 1986; Ward *et al.*, 1987). Aside from Ab Fc domains, knobs-into-holes engineering may have additional utility in the generation of heterooligomers of other proteins which are normally homooligomers. For example, this strategy has recently been used to enhance successfully the production of bispecific diabodies (Z.Zhu, P.Carter and L.G.Presta, unpublished data).

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