Effects of engineering charged amino acids in the C_H3 domains on antibody heavy chain dimerization

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The effect on antibody heavy chain dimerization of complementary charged amino acids engineered on the C_H3 domains was investigated. The T366 and Y407 located on the C_H3 domain interface of two antitumor antibodies, CC49IL2 (CC49 with an interleukin-2 attached to its carboxyl end) and COL-1, were replaced with arginine and glutamic acid residues, respectively. The possible attraction of the complementary charges to form a heterodimer was detected by simultaneously expressing the engineered CC49IL2 and COL-1 constructs in *Spodoptera frugiperda* (Sf9) insect cells. Among the combinations studied, the Y407R / T366E combination yielded the highest amount of heterodimer which is about 68% of the total dimers produced compared to about 30% and 33% for the other two combinations, T366R / Y407E and T366R:Y407R / T366E:Y407E, respectively. Modeling of the engineered mutations showed that two H-bonds could form between R407 on CC49IL2 chain and E366 on COL-1 which could explain why the combination Y407R / T366E resulted in more of the heterodimer than the other two combinations studied. These results suggest that pairing of complementary charges engineered on C_H3 region can result in the attraction of different antibody chains favoring formation of a heterodimer or a bispecific antibody.

KEYWORDS

bispecific antibody, electrostatic interaction, C_H3 domain, heterodimer, homodimer, monomer

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INTRODUCTION

Antibodies are proteins produced by the immune system in response to substances recognized by the body as foreign. Typical antibodies share the same common structural unit – two identical heavy (H) and light (L) chains composed of approximately 440 or 550 and 220 amino acids, respectively. Each chain is composed of an N-terminal variable domain (V_L and V_H) followed by a single constant domain in the light chain and three (in the case of IgG, IgA and IgD) or four (in the case of IgM and IgE) constant domains, C_H, in the heavy chain. The variable domains of the antibody form the antigen-binding site while the constant domains of the heavy chains, C_H2 and C_H3 (plus the C_H4 in the case of IgE and IgM), constitute the Fc region which is responsible for the effector functions of the antibody. There is close association of the two domains of the

light chain and the variable domain, V_H, and first constant domain, C_H1, of the heavy chain. There is also close association of the heavy chain domains in the Fc region of the molecule.

X-ray crystallographic studies of the Fc region of IgG antibodies have demonstrated that the most extensive site of interaction occurs between the C_H3 domains (Deisenhofer 1981). The C_H3 domain interface (Figure 1A) is the core antiparallel β-strands of the Fc region wherein, each sheet is made up of 11 amino acid residues (Ridgway et al. 1996). Studies have shown that contact residues on the C_H3 dimer interface, hidden between the monomeric subunits, are critical for the dimerization process and that hydrophobic and charged interactions play a major role in stabilizing the interface (Brinda et al. 2002, Lassila et al. 2002). This information has been utilized to form bispecific antibodies (bsAbs), protein heterooligomers which can bind to two different epitopes (on a target cell, for example). Maximal formation of heterodimers, with minimal formation of homodimers, was achieved by introducing sterically complementary mutations (big and small amino acids) in the C_H3 region, i.e., by 'knobs-into-holes' engineering (Ridgway et al. 1996).

We had previously suggested using positively and negatively charged amino acids to enhance the complementarity of chosen heavy chain pairs (Santos and Padlan 1998). Here, we present the results of a study wherein we explored the effect of introducing charged amino acids on antibody C_H3 domains that we expect would favor electrostatic attraction between two different single-chain antibody constructs, CC49 and COL-1 (Figure 1B). These antibodies have different but complementary specificities. The combination of these two antibodies has shown an increase in antibody binding and distribution in colon cancer tumor specimens by immunoperoxidase staining as compared to either antibody alone (Meredith et al. 1996). Combining these two antibodies in generating a bispecific antibody (heterodimer) may be useful in antibody localization as well as in the delivery of a larger amount of a toxin into tumor cells.

MATERIALS AND METHODS

Antibody format and nomenclature

Two antibody heavy-chain genes, CC49IL2 and COL-1, designed as single-chain gene constructs, were used in this study. CC49IL2 is a single-chain construct of the heavy chain of CC49, a murine antitumor antibody that binds the glycoprotein, TAG-72 (Muraro et al. 1988), and of interleukin-2 (Bei et al. 1995). The conjugation of interleukin-2 to the CC49 heavy chain allows for the easy determination, by molecular weight analysis, of which monomers or dimers had formed. COL-1 is a murine antitumor antibody that binds carcinoembryonic antigen (de Pascalis et al. 2002). Mutations are denoted by the original amino acid residue and position (based on the numbering scheme of Kabat), followed by the replacement amino acid. For example, T366R means that threonine at position 366 is replaced with arginine. The representation for a double mutation on the same antibody chain, for example threonine at position 366 and tyrosine at position 407, is denoted as T366R:Y407R. Mutations separated by a slash, for example T366R / Y407E, means that T366R is on CC49IL2 while Y407E is in COL-1. The single-chain CC49IL2 was engineered to contain arginine, either at position 366 or 407, or at both positions. The same was done for the single-chain COL-1 heavy chain, in which glutamic acid was introduced instead of arginine (Figure 2).

Plasmids and synthetic oligonucleotides

All recombinant plasmids were extracted using the modified alkaline lysis method by Sambrook et al (2000). The plasmids pGem-3Zf+ (Promega) and Topo 4.1 (Invitrogen) were used as intermediate cloning vectors for introduction of mutations, restriction sites, and sequencing of the antibody gene constructs. The pAcUW51 baculovirus transfer vector (Pharmingen) was used to clone the CC49IL2 and COL-1 genes downstream of the p10 and polH promoters, respectively. The oligonucleotide primers used to introduce the desired mutations via site-directed mutagenesis and to screen for inserts ligated into the pAcUW51 vector are listed in Table 1.

Introduction of mutations

The modified protocol of site-directed mutagenesis of

Table 1. Oligonucleotides used to introduce mutations on the CC49IL2 and COL-1 antibody genes labelled as ASOG followed by the amino acid substitution and position. The underlined nucleotides cause the desired amino acid substitutions. ASOG 11, ASOG 12, BV, p10 and AFcγ are the primers used to verify the orientation of the cloned CC49IL2 and COL-1 in the pAcUW51 vector.

Primer Name	Primer Sequence
5' ASOG T366R	5' GTCAGCCTGAGATGCCTGGTCAAAG3'
3' ASOG T366R	5' CTTTGACCAGGCATCTCAGGCTGAC 3'
5' ASOG T366E	5' GTCAGCCTGAAGTGCCTGGTCAAAG3'
3' ASOG T366E	5' CTTTGACCAGGCATTCCAGGCTGAC 3'
5' ASOG Y407R	5' TCCTTCTTCCTCCGCAGCAAGCTCAC3'
3' ASOGY407R	5' GTGAGCTTGCTGCGGAGGAAGAAGGA3'
5' ASOG Y407E	5' TCCTTCTTCCTCGAAAGCAAGCTCAC3'
3' ASOG Y407E	5' GTGAGCTTGCTTTCGAGGAAGAAGGA3'
ASOG 11 (F)	5' CTTTAATTCAACCCAACAC 3'
ASOG 12 (R)	5' AGGCTCTTCTGCGTGTAG 3'
p10 (F)	5' CTTTAATTCAACCCAACAC 3'
BV (R)	5' AACGCACAGAATCTACGCC 3'
AFcγ (R)	5' CAGGAGTTCAGGTGCTGGGC 3'

Stratagene™ was done to introduce the mutations on the C_H3 core domains of CC49IL2 and COL-1 gene constructs. Briefly, using Pfx polymerase (Invitrogen), a high fidelity proofreading thermostable DNA polymerase, and primers that were to introduce the mutations, PCR was done on the non-engineered (NE) antibody (CC49IL2 and COL-1) or a previously mutated antibody (CC49IL2 Y407R and COL-1 Y407E) as templates, to generate the single and double mutants, respectively. This was followed by digestion with *Dpn1* (to destroy the template DNA) and subsequent transformation of competent DH5α cells via heat shock. All mutations were verified by DNA sequencing using the ABI Prism™ Big Dye Terminator sequencing protocol (PE Applied Biosystems, Foster City, CA, USA).

Cloning of engineered antibody genes in the baculovirus transfer vector

Standard cloning procedures were done to position the mutated CC49IL2 and COL-1 gene constructs downstream of the p10 and polH promoters, respectively, of a single pAcUW51 vector (Figure 2). All ligations were performed at 4^0C followed by transformation of competent DH5α cells. PCR colony screening using primers specific for the vector and insert identified the clones carrying the inserts in the desired orientation. Recombinant plasmids were extracted and presence of the inserts was further verified by restriction enzyme digestion.

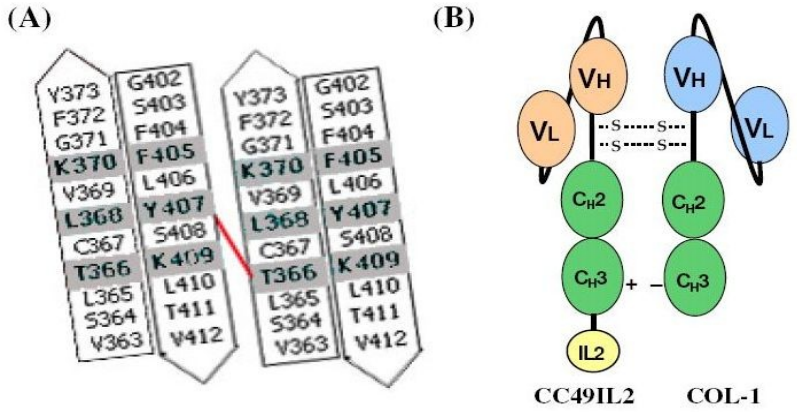

Figure 1. (A) Sequence of the amino acids found on the C_H3 dimer interface with the contact residues highlighted in gray and the red line indicating the H-bond which forms between the T366 and Y407 residues. Adapted from Ridgway et al. 1996. **(B)** Schematic representation of the CC49IL2 / COL-1 heterodimer showing the possible attractions that may occur between complementary charges engineered at the C_H3 domains.

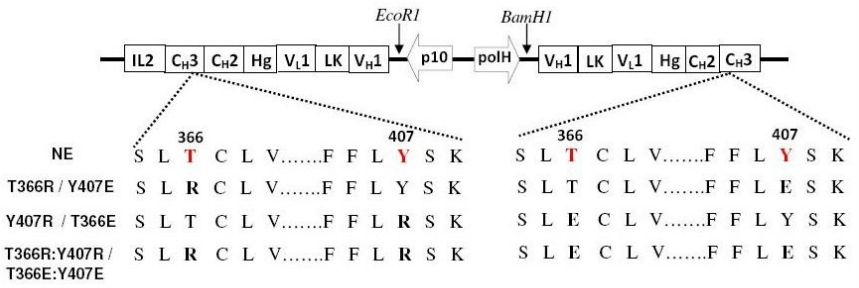

Figure 2. Diagram of the antibody gene constructs used for the heterodimerization assay. The engineered CC49IL2 and COL-1 were cloned in a single pAcUW51 vector under the control of p10 and polH, respectively. Linker (LK) is 15 amino acids made up of three Gly_4-Ser residues. Hg is hinge region. The non-engineered (NE) sequence shows threonine (T) at position 366 and Tyrosine (Y) at position 407 (shown in red). These are the amino acids which were replaced to introduce the complementary charges at the C_H3 position.

Expression in insect cells

Spodoptera *frugipderda* (Sf9) insect cells were cotransfected with the recombinant pAcUW51 transfer vector containing the mutated antibody gene inserts and the linearized baculovirus BaculogoldTM (Pharmingen). Approximately 5 µg of the recombinant engineered construct was combined with 0.5 µg BaculoGold™ linearised baculovirus DNA (Pharmingen) with 20 mM HEPES buffer in polystyrene tube (Falcon 2057) to make a final volume of 50 µL. In a separate polypropylene tube (Falcon 2063), 30 µg DOTAP (N-[1-92,3-Dioleoxyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate, Roche) was mixed with 20 mM HEPES buffer to make a 100 µL mixture volume. The DOTAP-HEPES mixture was added to the nucleic acid solution and was used to infect 1 x 10^6 insect cells adhered in a culture dish. Culture was made for six days at 28^0C, after which, the culture supernatant was harvested by centrifugation at 1600 g for 5 minutes. The transfection supernatant was collected and stored in 15 mL-conical tube at 4 ^{0}C until the plaque purification assay. Aliquots of the transfection supernatants were made for ELISA. For the plaque purification step, 10-fold (up to10^{-7}) dilutions of the transfection supernatants were prepared with the highest dilution used to infect 3 x 10^6 adhered insect cells. Infected cells were overlayed with 1% baculoagar, incubated for 5 days and then stained with neutral red solution. On the 6th day of culture, several isolated plaques were visible. Five plaques for each construct were extracted and stored in Sf9 media. For the viral titer amplification step, harvested plaques were used to infect new batch of 1x

10^6 insect cells. After 3 days of culture, the supernatant was harvested and used for immunoblotting.

Enzyme-linked Immunosorbent Assay (ELISA)

This was done to check whether the transfection procedure was successful by detecting the presence of BSM-reactive antibody in the transfection supernatant. A 96-well flat-bottom PVC plates coated with 1 µg/well bovine submaxilliary mucin or BSM (Sigma) was used for ELISA. Fifty microliters of diluted controls and 150 µL virus stock from the transfection supernatant were tested in duplicates. Negative controls were 50 µL PBS and 50 ng human IgG (Jackson Immunolabs). Positive controls were 100 ng of previously purified CC49 antibody expressed in insect. Samples were incubated at 37 °C for an hour. Wells were washed 3 times with 1% BSA in PBS. Fifty microliters of a 1:1000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-human Fcγ specific antibody (Jackson Immunolabs) in 1% BSA in PBS was added to each well. The plates were incubated at 37 °C for 1 hour and then washed 3 times with 1% BSA in PBS. The reaction was developed using O-phenylenediamine dihydrocholoride (OPD), a substrate of HRP, wherein an enzymatic reaction is indicated by a bright orange color reaction. OPD substrate (Sigma) was prepared in buffer containing 0.02 M citric acid and 0.05 M Na_2HPO_4 and 25 µL 3% H_2O_2. One hundred microliters of this complete substrate buffer was added to each well. Color development was allowed to proceed in the dark for about 15 minutes at room temperature. The enzyme reaction was stopped by adding 50 µL 1M H_2SO_4 to each well. Plates were photographed using a DigiDocIt photoimaging system.

SDS-PAGE and Immunoblotting

Twenty seven microliters of the culture supernatants, referred to as amplified supernatants, were added to three microliters of 10X treatment buffer, run in a 7.5% SDS-PAGE (denaturing, non-reducing conditions) and blotted in a polyvinylidine (PVDF) membrane. For reducing conditions, 5 µL β-mercaptoethanol was added per 30 µL reaction volume prior to boiling. The entire volume was loaded onto the SDS polyacrylamide gel and run at constant 100V. One gel was stained with silver while the other is blotted in a PVDF membrane. Membranes with the blotted proteins were blocked with 5% skim milk and incubated with goat anti-human IgG Fcγ specific antibody-conjugated to HRP. Detection was done using the Opti-4CN kit (Biorad) substrate of HRP. The dried PVDF membranes were scanned and the intensities of the signals were quantified using Scion Image 4.03 image analysis software.

RESULTS AND DISCUSSION

In this study, two antitumor antibody genes were used: CC49 and COL-1. These antibody genes were designed as single chain gene constructs for ease of protein assembly. This consisted of V_H and V_L joined by a 15 amino acid linker $(Gly_4\text{-}Ser)_3$ resulting in a single chain variable fragment (sFv) covalently joined to the human Fcγ (C_H2 and C_H3 domains) through the hinge region. A leader sequence was added to facilitate secretion of the expressed protein into the culture supernatant. To check whether the transfection of insect cells is successful and the antibodies are secreted outside the cell, an ELISA was performed (Figure 3, Lane 6) detecting the presence of the CC49 arm of the antibody in the harvested transfection supernatants. Moreover, for the CC49 gene construct, an interleukin-2 (IL2) was included primarily to have a more obvious difference in the molecular weight of the expressed protein product of CC49 as compared to COL-1. Approximate molecular weights of CC49IL2 and COL-1 homodimers were previously reported to be 165 and 115 kDa, respectively (Bei et al. 1995, Shu et al. 1993). With these weights, CC49IL2 and

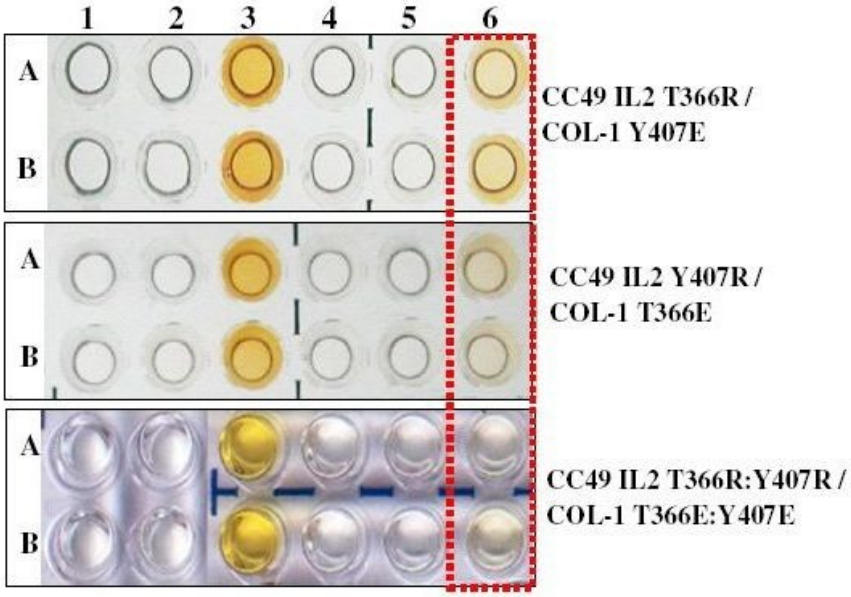

Figure 3. ELISA of the transfection supernatants of the three recombinant constructs. BSM-coated plates were incubated with the transfection supernatants. HRP-conjugated goat anti-human Fcγ specific antibody was used as the detecting antibody. O-phenylenediamine dihydrochloride (OPD) substrate reacts with HRP in the presence of H_2O_2 resulting in a bright orange to yellow color. **Top:** CC49IL2 T366R / COL-1 Y407E; **Middle:** CC49IL2 Y407R / COL-1 T366E ; **Bottom:** CC49IL2 T366R:Y407R / COL-1 T366E:Y407E. Columns 1-2: negative controls, PBS and human IgG, respectively; Lane 3: positive control purified CC49 antibody; Columns 4 and 5: transfection negative controls (untransfected insect cells supernatant); 6: insect cells supernatant transfected with the recombinant constructs. A and B are duplicates.

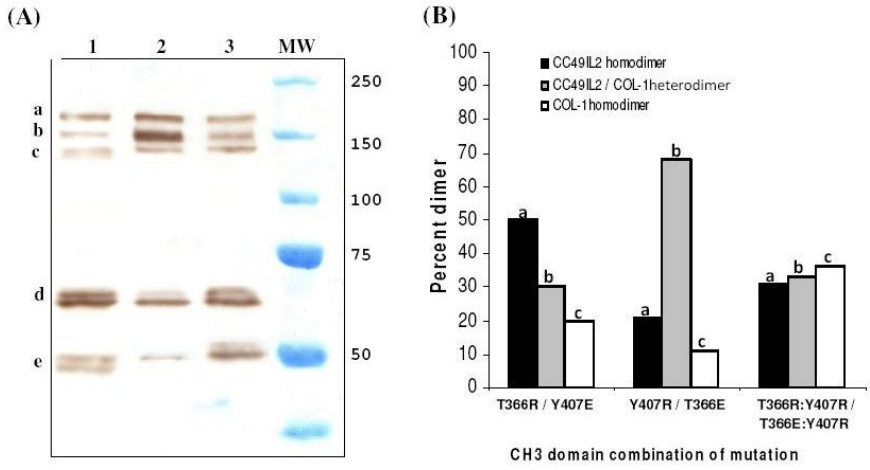

Figure 4. Quantification of the amounts of dimers formed for the three combinations of mutations on the C_H3 domain. (A) Western blot of the amplified supernatants of the engineered constructs under denaturing non-reducing conditions. Amplified supernatants were run in 7.5% SDS-PAGE and blotted on a PVDF membrane. MW: All blue prestained marker (BioRad™); Lane 1: T366R / Y407E; Lane 2: Y407R / T366E; Lane 3: T366R:Y407R / T366E:Y407E. CC49IL2 homodimers, CC49IL2 / COL-1 heterodimers, COL-1 homodimers, CC49IL2 monomers and COL-1 monomers are labeled a, b, c, d and e, respectively. Molecular weights are in kilodaltons. **(B)** The amount of dimers which formed determined by scanning the Western blot image and quantifying the signals from each band using the ScionImage analysis software. Each of the bands corresponding to the CC49IL2 homodimer, CC49IL2 / COL-1 heterodimer and COL-1 homodimer was selected and compared to the total signal contributed by all three bands. Note that in the analysis, signals corresponding to the monomers were not included.

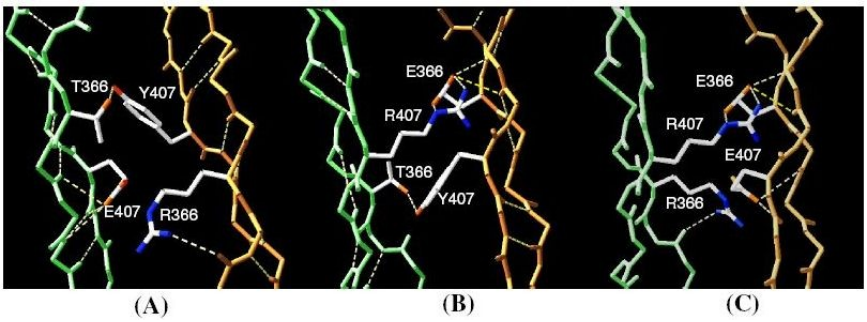

Figure 5. Modeling of the C_H3 domain interface with the introduced complementary mutations at 366 and 407 positions. H-bonds that may form between residues are indicated by white dashed lines. **A:** T366R / Y407E; **B:** Y407R / T366E; **C:** T366R:Y407R / T366E:Y407E. Note that all arginine mutations are in CC49IL2 while glutamic acid mutations are in COL-1.

COL-1 monomers should have molecular weights corresponding to half of the dimers, which are 82.5 and 57.5 kDa, respectively, while the CC49 IL2 / COL-1 heterodimer should have an approximate molecular weight equivalent to 140 kDa. These theoretical molecular weights allowed the identification of the respective dimers and monomers based on the migration of the bands after running in SDS-PAGE followed by Western blot using an anti-human Fcγ as the detecting antibody. The Western blot of the recombinant constructs was scanned and analyzed using ScionImage analysis software to obtain a quantitative measure of the amount of dimers expressed for each of the three engineered constructs being studied.

Before exploring the possibility of attraction between complementary charges on different antibody chains, we did a separate study to determine whether same charges on the C_H3 domain will repel, thus preventing formation of homodimers. We did this by expressing CC49IL2 with arginine mutations and COL-1 with glutamic acid mutations in separate pAcUW51 vectors and quantifying the amounts of CC49IL2 or COL-1 homodimers and their respective monomers formed. We found that the introduced positively and negatively charged amino acids were shown to impair homodimer formation since the non-engineered constructs formed dimers exclusively, while the engineered constructs with either arginine or glutamic acid, in single or double copy at positions 366 and/or 407, caused the formation of a significant amount of monomers (data not shown). Formation of monomers for the engineered constructs is expected in view of the removal of the two hydrogen bonds which occur between residues T366 and Y407 which are crucial for the correct folding of the C_H3 domain (Atwell et al. 1997). We were also able to observe that a single arginine or glutamic acid is more disruptive to homodimerization when introduced in place of the tyrosine at 407 than when it is used to replace the threonine at 366. This supports a previous study that claims that a hydrophobic amino acid at position 407 is crucial to the stabilization of the antibody dimer interface (Espinosa et al. 2006). Increase in monomer formation was not observed with the double mutations compared with single mutations. Since charged residues can have a long-range

interaction with one another, non-additivity of the effects is expected, additivity being generally applicable only to spatially well-separated uncharged residues.

Simultaneous expression of CC49IL2 and COL-1 in insect cells produced dimers exclusively for the non-engineered construct (Ponce 2002), while in this report, the engineered constructs formed dimers and significant amount of monomers. The CC49IL2 Y407R / COL-1 T366E mutations yielded the greatest amount of the heterodimer which is approximately 68% of the total dimers expressed as compared to only about 30% for the T366R / Y407E and 33% for the T366R:Y407R / T366E:Y407E combination (Figures 4A and 4B). This result was consistent with all the harvested plaques of the engineered constructs which showed the expected five bands (three bands correspond to the dimers and two bands to the monomers) in the Western blot (see Supplementary Material A).

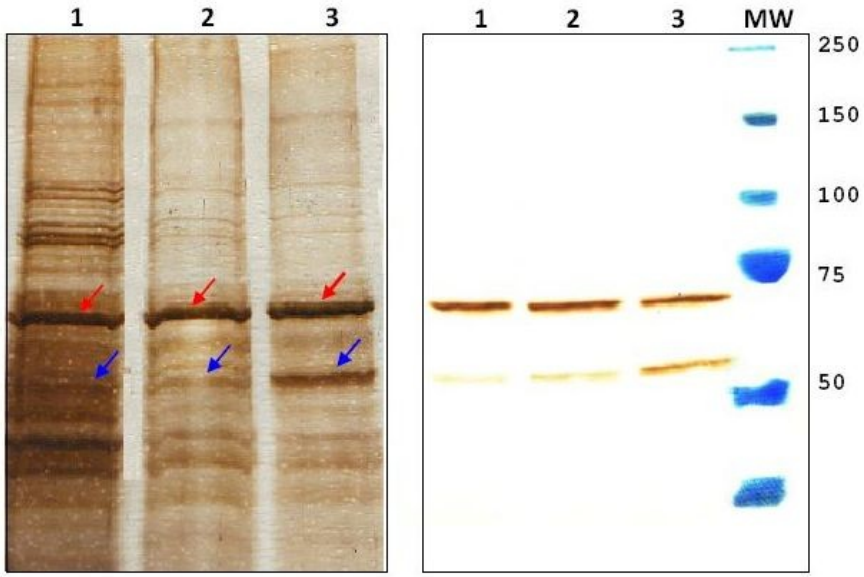

Figure 6. Silver-stained SDS-PAGE (Left) and Western blot (Right) of the amplified supernatants under reducing condition. Amplified supernatants of the engineered constructs were reduced using beta-mercaptoethanol and run in 7.5% polyacrylamide gel. MW: All blue prestained marker. 1: CC49IL2 T366R / COL-1 Y407E; 2: CC49IL2 Y407R / COL-1 T366E; 3: CC49IL2 T366R:Y407R / COL-1 T366E:Y407E. Molecular weights in kilodaltons. The CC49IL2 and COL-1 monomers are indicated by red and blue arrows, respectively.

The combination of a positive charge on the C_H3 domain of CC49IL2 and a negative charge on the other domain of COL-1 is hypothesized to form a favorable electrostatic interaction that enhances the formation of the heterodimer. Electrostatic interaction between oppositely charged amino acids to form a salt bridge is expected to occur when (a) the side-chain charged group centroids are within a 4 Å distance and (b) at least one pair of Asp/Glu side-chain carbonyl–oxygen and Arg/Lys/His side-chain nitrogen atoms are within 4 Å distance (Kumar and Nussinov 2002). Modelling of the introduced mutation using the most probable geometry with the least steric clashes with the neighbouring residues showed that two possible H-bonds could form between the R407 on CC49IL2 chain and E366 on the COL-1 chain but not with the R366 and E407 (Figure 5A and 5B). The very close distance between R407 and E366 can cause a possible salt bridge to be formed, with the resulting formation of more of the heterodimer for the Y407R / T366E combination. However, although the CC49IL2 T366R:Y407R / COL-1 T366E:Y407E combination contained one R407 and E366 interaction, the loss of the T366 and Y407 H-bond at the other position may have caused the decrease in the amount of heterodimer produced compared to the simpler Y407R / T366E combination (Figure 5C).

In theory, in the non-engineered CC49IL2 / COL-1 pairing, the probability that the heterodimer will form should be 50% while the two corresponding homodimers should form with equal probability of 25%, when the monomers are at a 1:1 molar ratio. With the introduction of charges in the contact region of the dimerization interface, homodimer association should be minimized by the repulsion of like charges on the same chain.

Nevertheless, homodimers could still be formed by the formation of disulfide bonds involving the cysteines in the hinge regions, without the association of the constant domains to form a proper Fc. This is demonstrated by the presence of CC49IL2 and COL-1 homodimers despite the repulsion of the same charges on their C_H3 domain interfaces. This result parallels that of our previous experiment which showed that CC49IL2 with arginine mutations and COL-1 with glutamic acid mutations may still result in the association of identical chains even though they harbor the same charge which should repel. Perhaps not unexpectedly, the formation of significant amounts of CC49IL2 and COL-1 monomers (not quantified) was also observed for all the engineered recombinant constructs (Figure 4A). This finding indicates that although repulsion of the same charge is a prerequisite to minimize homodimer association, it does not guarantee association of complementary charges to favor heterodimer formation. Moreover, differential strengths of the p10 and polH promoters of the baculovirus may have led to the unequal expression of the CC49IL2 and COL-1 as shown by the Western blot result under reducing condition (Figure 6) making the pairing to form the heterodimer less efficient.

CONCLUSION

Taken together, the results obtained in our study suggest that significant change in interdomain organization can arise from modification of amino acid residues on the C_H3 domain interface. Moreover, complementary charges introduced in this region can alter the association pattern of single-chain antibodies to form dimers. Additionally, although the Y407R / T366E formed the highest percentage of the heterodimer as compared to the other combinations of positive and negative mutations, the introduced complementary charges was not strong enough to bring the two antibody chains together to form exclusively the desired CC49IL2 / COL-1 heterodimer. A more accurate gauge of the amount of heterodimer produced from each of the engineered constructs should be possible with large scale expression and antibody purification prior to separation and quantification of the dimers by analytical ultracentrifugation or gel filtration.

CONFLICTS OF INTEREST

None

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

The idea of introducing charges on antibody C_H3 domains to enhance heterodimer formation was conceptualized by Dr. Eduardo Padlan and Dr. Ameurfina Santos. All the experiments were performed by Maria Aiko Diaz as part of her MS thesis. All authors read and approved the final manuscript.

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Supplementary Material A

Dilutions of the transfection supernatant were used for plaque purification assay. Five well-isolated plaques were obtained for each of the constructs and these were used for the first round of amplification. The amplified supernatants from the selected plaques were run in 12.5% or 7.5% SDS-polyacrylamide gel (non-reducing condition) and blotted on PVDF membrane.

Five bands were expected to be detected in the Western blot (based on the study of Ponce 2002) which corresponds to the dimers and monomers of CC49IL2 and COL-1 using a goat anti-human Fcγ specific as the detecting antibody. For the CC49IL2 T366R / COL-1 Y407E (upper left blot), only plaques 1 and 2 expressed the five expected bands, plaques 3 and 4 expressed only two bands which corresponds to the COL-1 homodimer and monomer, while no antibody was detected for plaque 5. For the CC49IL2 T366R:Y407R / COL-1 T366E:Y407E, all plaques showed the expected five bands except for plaque 1 (upper right blot) where no signal was detected. The presence of an "empty virus" or viral plaque which does not express the recombinant antibody or express only one of the recombinant protein controlled by one of the promoters could be due to failure or incomplete homologous recombination of the baculogold viral DNA and the pAcUW51 transfer vector with the engineered gene constructs. For the CC49IL2 T366R / COL-1 Y407E, plaques 1 and 2 were used for the succeeding amplification steps while plaques 3 and 4 were used for the CC49IL2 T366R:Y407R / COL-1 T366E:Y407E. Not very good separation resulted because PAGE was run using 12.5% polyacrylamide gel in a short period of time. For the CC49IL2 Y407R / COL-1 T366E (lower left blot), all five plaques were able to express the dimers and monomers of CC49IL2 and COL-1. Clear separation of the bands was more obvious for this construct since PAGE was run using 7.5% gel. For this construct, plaques 1 and 3 were used for the succeeding amplification steps.

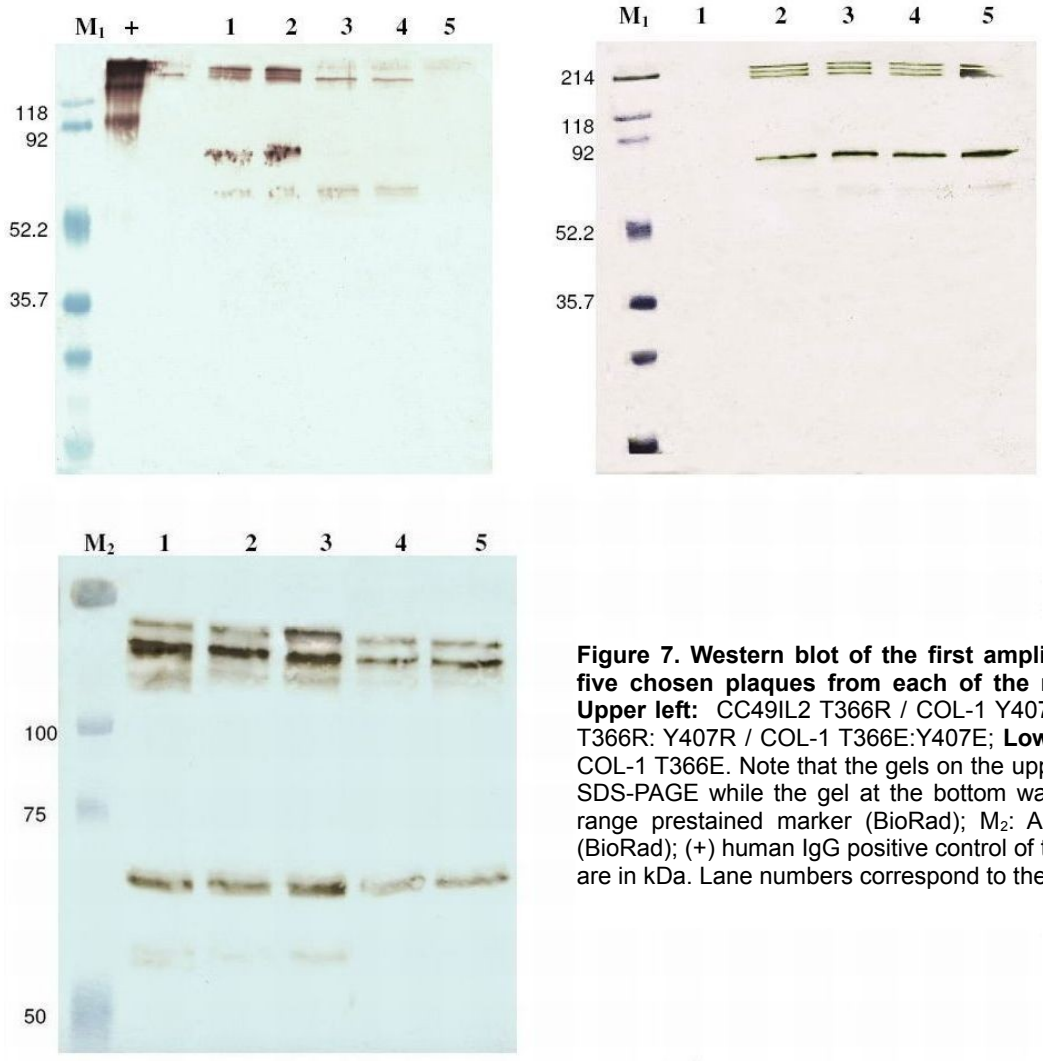

Figure 7. Western blot of the first amplification supernatants of five chosen plaques from each of the recombinant constructs. Upper left: CC49IL2 T366R / COL-1 Y407E; **Upper right:** CC49IL2 T366R: Y407R / COL-1 T366E:Y407E; **Lower left:** CC49IL2 Y407R / COL-1 T366E. Note that the gels on the upper part were run in 12.5% SDS-PAGE while the gel at the bottom was run at 7.5%. M_1: Broad range prestained marker (BioRad); M_2: All blue prestained marker (BioRad); (+) human IgG positive control of the blot. Molecular weights are in kDa. Lane numbers correspond to the extracted plaques.