HuCC49ΔCH2 is a heavy chain constant domain 2 domain-deleted antibody under development as a radioimmunotherapeutic for treating carcinomas overexpressing the TAG-72 tumor antigen. Mammalian cell culture biosynthesis of HuCC49ΔCH2 produces two isoforms (form A and form B) in an approximate 1:1 ratio, and consequently separation and purification of the desired form A isoform adversely impact process and yield. A protein engineering strategy was used to develop a panel of hinge-engineered HuCC49ΔCH2 antibodies to identify hinge sequences to optimize production of the form A isoform. We found that adding a single proline residue at Kabat position 243, immediately adjacent to the carboxyl end of the core middle hinge CPPC domain, resulted in an increase from 39 to 51% form A isoform relative to the parent HuCC49ΔCH2 antibody. Insertion of the amino acids proline-alanine-proline (PAP) at positions 243–245 enhanced production of the form A isoform to 72%. Insertion of a cysteine-rich 15-amino acid IgG3 hinge motif (CPEPKSCDTPPPCPR) in both of these mutant antibodies resulted in secretion of predominantly form A isoform with little or no detectable form B. Yields exceeding 98% of the form A isoform have been realized. Preliminary peptide mapping and mass spectrometry analysis suggest that at least two, and as many as five, inter-heavy chain disulfide linkages may be present. Radioimmunotherapy has been shown to be efficacious in treating hematological diseases, such as relapsed or refractory B cell non-Hodgkin lymphoma, with two radioimmunotherapeutic drugs currently approved for use in humans, (1, 2). However, the treatment of solid tumors with radiolabeled monoclonal antibodies (mAbs) has recently approved for use in humans (1, 2). However, the treatment of solid tumors with radiolabeled monoclonal antibodies (mAbs) has also been shown previously with protein G-purified chimeric B72.3 and HuCC49ΔCH2 can be described as containing an atypical hinge region. Native human antibody hinge regions can be structurally defined as consisting of an upper hinge region (UH) extending from the last residue of Cμ1 up to but not including the first inter-heavy chain cysteine, a middle hinge region (MH) extending from the first inter-heavy chain cysteine to a proline residue adjacent to the carboxyl-end of the last MH cysteine, and a highly conserved 7–8-amino acid lower hinge (LH) (14). The atypical hinge region in HuCC49ΔCH2 is similar to that described in the antircarcinoembryonic antigen minibody (15), whereas the MH proline at position 243 (Kabat numbering system) (16) and the entire Cμ2 domain, including the LH residues, APELLGGP (the first eight amino-terminal residues of the IgG1 Cμ2 domain), are deleted and replaced by the 10-amino acid peptide Gly/Ser spacer.

Biosynthesis of HuCC49ΔCH2 in mammalian Chinese hamster ovary cells has been observed to produce two homodimeric isoforms present in approximately a 50:50 mixture. The presence of two species has also been shown previously with protein G-purified chimeric B72.3 or chimeric CC49 Cμ2 domain-deleted antibodies (7, 8). For HuCC49ΔCH2, one isoform, referred to as form A, contains covalent interchain disulfide bonds at heavy chain MH positions 239 and 242, Kabat numbering system. The second isoform, form B, is held together by noncovalent interactions through the Cμ3 domains, and it fails to develop an interchain hinge disulfide bond as evidenced by the formation of a 60-kDa product following nonreducing, denaturing gel electrophoresis (Fig. 1). The form B isoform is thought to contain heavy chain intrachain disulfide bonds covalently linking the cysteine residue at position 239 to that at position 242. Compound stability studies support

**The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.**

1. To whom correspondence should be addressed: Biogen Idec, Inc., 2500 Research Place San Diego, CA 92122. Tel.: 858-401-8645; Fax: 858-401-8714; E-mail: scott.glaser@biogenidec.com.

2. The abbreviations used are: mAb, monoclonal antibody; Cμ2, heavy chain constant domain 2; ΔCH2, Cμ2 domain-deleted; C, constant; UH, upper hinge; MH, middle hinge; LH, lower hinge; TAG-72, tumor associated glycoprotein-72; CHO, Chinese hamster ovary; PAP, proline-alanine-proline; HPLC, high pressure liquid chromatography; DTT, dithiothreitol; CPPC, core middle hinge domain (Cys-Pro-Pro-Cys); SOE, splicing by overlap extension; MS, mass spectrometry; endo, endoproteinase; Hu, human.

3. P. Chinn, personal communication.
form A HuCC49 ΔCH2 as the preferred molecule for therapeutic development, and methods for the separation and purification of form A from form B using hydrophobic interaction chromatography have been described (17). However, we projected that development of a cell line that expressed only form A would avoid synthesis of unwanted antibody by-product and, in turn, eliminate the requirement for physical separation of form A from form B, resulting in a more efficient recombinant protein production process. We hypothesized that generation of the two HuCC49 ΔCH2 antibody isoforms is a consequence of hinge heterogeneity because of variation in disulfide bond formation. It follows, therefore, that stabilization of the hinge region should favor production of the desired form A isoform. By using a protein engineering strategy, we describe here a series of variant hinge-connecting peptides that were found to improve significantly the homogeneity and yield of CH2 domain-deleted antibodies.

MATERIALS AND METHODS

Construction of HuCC49 ΔCH2 Hinge Variant Vectors

Synthetic oligonucleotides (Sigma Genosys) encoding the various hinge region connecting peptides were introduced into the HuCC49 ΔCH2 vector using PCR and splicing by overlap extension (SOE) methods. The HuCC49 ΔCH2 vector contains a translation-impaired modified (intron-containing) neomycin phosphotransferase gene to select for transcriptionally active integration events and a murine dihydrofolate reductase gene to permit amplification with methotrexate (18).

Middle and lower hinge mutations were introduced into the parental HuCC49 ΔCH2 heavy chain gene by PCR-based site-directed mutagenesis (19). PCR primer sets were designed with 5’ Agel and 3’ Xhol restriction sites for cloning the hinge region PCR products into HuCC49 ΔCH2 as Agel/Xhol fragments. PCRs consisted of HuCC49 ΔCH2 DNA template, the 5’ primer, MB-04F 5’-TTTCCCC-GAACCGGTGACGGTG-3’ and the following 3’ primers: Pro-243 (MB-06R, 5’-GATCCGCTCACCCATCGACGCCACCCCCGAGCC- GTGGGCATGTG-3’); PAP (MB-010R, 5’-GATCCGCCCTCA- CTCGACCCACCTCCGGGTGACGGTG-3’); and C242S + Pro-243 (MB-08R, 5’-GATCCGCCCTCCTCGAAGCCACCCCCGAGCC- GTGGGCATGTG-3’); C242S + PAP (MB-024R, 5’-GATCCGCCCTCCTCGAAGCCACCCCCGAGCC- GTGGGCATGTG-3’); C239S + Pro-243 (MB-11R, 5’-GATCCGCCCTCCTCGAAGCCACCCCCGAGCC- GTGGGCATGTG-3’); and C239S + PAP (MB-012R, 5’-GATCCGCCCTCCTCGAAGCCACCCCCGAGCC- GTGGGCATGTG-3’). PCRs were initiated with HotStarTaq™ DNA polymerase (Qiagen, Inc., Valencia, CA) according to the manufacturer’s instructions. Cycle conditions were of 15 min denaturation at 95 °C followed by 30 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 54 °C, and 1 min of synthesis at 72 °C, followed by a final strand extension for 10 min at 72 °C.

Construction of hinge regions containing a cysteine-rich 15-amino acid IgG3 hinge insertion (CPEKPSCTDPPCPR) in addition to middle and lower hinge mutations were performed using SOE (20). PCR primer sets were also designed with 5’ Agel and 3’ Xhol restriction sites and consisted of HuCC49 ΔCH2 DNA template, the 5’ primer MB-04F, and the following 3’ primers: G1/G3 “SOE overlap” (MB-13R, 5’-GACC- GTGGGCACCCCAAGGAGGTGTGCTCAAAGATTTGGCTTG- GCACGCTGGGCGACGTTG-3’); G1/G3:MB-14R, 5’-GATTCC- GCTTACGAGCCACCCCAAGGAGGTGTGCTCAAAGATTTGGCTTG- GCACGCTGGGCGACGTTG-3’; and G1/G3:MB-15R, 5’-GATCCGCTGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG-3’). Briefly, the first PCR contained DNA template, 0.8 μM of the forward primer MB-04F, and a limiting concentration (0.2 μM) of the reverse SOE primer MB-13R. The reaction mixture was denatured for 15 min at 95 °C followed by 20 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 54 °C, and 1 min of synthesis at 72 °C. The second PCR was performed by adding either 0.8 μM MB-14R or MB-15R reverse primer for an additional 20 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 54 °C, 1 min of synthesis at 72 °C followed by 10 min extension at 72 °C. PCR products were purified, digested with restriction endonucleases, and ligated into Agel/Xhol-digested HuCC49 ΔCH2 vector. Escherichia coli strain XL-1 Blue (Strategene, La Jolla, CA) was used for plasmid propagation. Correct modifications to the hinge region were confirmed by DNA sequence analysis.

Protein Expression and Western Blot Analysis

Plasmid DNA was used to transform CHO DG44 cells for stable production of antibody protein. The CHO cell line, DG44, was grown in CHO-SSFMI medium supplemented with hypoxanthine and thymidine (Invitrogen). Approximately 1 μg of PacI-linearized plasmid DNA was transfected into 4 × 10⁶ CHO cells by electroporation using a Bio-Rad Gene Pulser II electroporation device (Bio-Rad). Conditions for electroporation were 350 V, 600 microfarads, with high capacitance setting. Each electroporation was plated into a 96-well dish (about 400,000 cells/well). Dishes were fed with media containing 400 μg/ml G418 (Geneticin; Invitrogen) beginning 2 days after electroporation and thereafter every 2nd or 3rd day until colonies arose. Supernatants from colonies were assayed for the presence of antibody by an enzyme-linked immunosorbent assay specific for human antibody.

Colonies producing various amounts of antibody were further tested by Western blot analysis to evaluate the secreted isoforms. Briefly, 3 ng of total antibody protein was analyzed by nonreducing 4–20% Tris-glycine SDS-PAGE (Invitrogen) followed by Western blot probed with an anti-human κ antibody (Roche Applied Science), and then an anti-rabbit horseradish peroxidase antibody (Amersham Biosciences) to detect form A and form B isoforms. Membranes were processed with the ECL Western blotting analysis system according to the manufacturer (Amersham Biosciences). Colonies producing the highest amount of antibody were expanded, and supernatants dilutions ranging from 30 to 0.23 ng were tested by Western blot analysis as described above. Band intensities corresponding to the A and B isoforms were semi-quantified by imaging the developed film with a CCD camera (Alpha Innotech Corp., San Leandro, CA). Band intensities from four lanes that fell within the linear range of the exposed film were measured using the spot densitometry function. The relative ratios of the form A and B isoforms were calculated (mean ± S.E.).

Protein Purification and Characterization

Select G418-resistant CHO cell lines producing HuCC49 ΔCH2 PAP and HuCC49 ΔCH2 G1/G3:PAP antibodies were adapted to CD-CHO media (21). Cell line cultures were scaled up in 25-liter cell bags in a WAVE BIOREACTOR (wave BIOTECH, LLC; Bridgewater, NJ), and supernatants were harvested for affinity purification by protein G-Sepharose (Amersham Biosciences). In some cases protein G-enriched immunoglobulin isoforms were further purified by hydrophobic interaction chromatography. The elution peaks representing form A and form B isoforms were separately pooled, dialyzed, and concentrated, and the amount of protein determined by modified Lowry (Bio-Rad) proteins were analyzed for purity by scanning densitometry (SI 375 personal densitometer; Amersham Biosciences) of reduced and nonreduced SDS-PAGE stained with Coomassie Blue. Size exclusion HPLC was used to analyze the percentage of monomer antibody products.
Competitive Binding Assay

A 96-well assay plate was coated with 8 μg/ml bovine submaxillary mucin, a source of the TAG-72 antigen, and blocked with 1% phosphate-buffered saline:bovine serum albumin. HuCC49/H9004 CH2, HuCC49/H9004 CH2 PAP, and HuCC49/H9004 CH2 G1/G3:PAP antibodies were incubated for 2 h at room temperature starting at 100 μg/ml in 3.5-fold serial dilution with 0.2 μg/ml of Eu³⁺-labeled HuCC49/H9004 CH2 tracer, followed by incubation with 200 μl/well DELFIA enhancement solution (PerkinElmer Life Sciences). Time-resolved fluorescence was read using the Europium protocol. IC₅₀ values were calculated using GraphPad Prism 4.0 (San Diego, CA).

Characterization of Hinge Region Disulfides by Peptide Mapping

Trypsin Digestion of HuCC49/H9004 CH2 and Hinge Variant Antibodies—Samples of the HuCC49/H9004 CH2, HuCC49/H9004 CH2 PAP, and HuCC49/H9004 CH2 G1/G3:PAP antibodies were denatured, reduced, and digested with trypsin as follows. Aliquots of 150 μg were diluted to 100 μl in HPLC water and denatured in 6 M guanidine hydrochloride, 50 mM Tris, pH 8.0. The samples were reduced by the addition of 20 μM DTT and incubated for 30 min at 37 °C. The reduced samples were alkylated with 50 mM iodoacetic acid for 30 min at 37 °C. The alkylation reaction was quenched by the addition of excess DTT. The reduced and alkylated samples were buffer-exchanged into 25 mM isoacetic acid for 30 min at 37 °C. The alkylation reaction was quenched by the addition of excess DTT. The reduced and alkylated samples were buffer-exchanged into 25 mM Tris, 20 mM CaCl₂, pH 7.5, using PD-10 columns. Trypsin was added to each sample in a 1:15 (w/w) ratio and incubated for 4 h at 37 °C. The digestion was stopped by the addition of trifluoroacetic acid to a final concentration of 0.1%. Trypsindigested samples (15 μg) were then analyzed according to the chromatographic procedure described below under "HPLC/Mass Spectrometry Analysis."

Endoproteinase Lys-C Digestion of HuCC49/H9004 CH2 and Hinge Variant Antibodies (Determination of Disulfide Linkages)—Denatured and reduced samples were prepared by adding a final concentration of 4 M guanidine HCl and 25 mM DTT to 1.5 mg/ml sample. Nonreduced and reduced endoproteinase Lys-C digested samples (12 μg) were then analyzed according to the procedure described below.

HPLC/Mass Spectrometry Analysis—Samples were analyzed on an Agilent 1100 HPLC system connected to an Agilent MSD single qua-
A reverse phase C18 column (Vydac catalog number 218TP52) was used with an eluant system of water, 0.1% trifluoroacetic acid (v/v) (Buffer A) and acetonitrile, 0.1% trifluoroacetic acid (v/v) (Buffer B), at a flow rate of 0.2 ml/min. A post-column “trifluoroacetic acid fixative” solution of acetonitrile and acetic acid (1:1 v/v) at 0.1 ml/min was added to enhance ionization. The column temperature was controlled at 45 °C, and the elution profile was monitored by absorbance at 215 and 280 nm. The total ion chromatogram was

**FIGURE 2. Western blot analysis of form A and B isoforms of HuCC49ΔCH2 and hinge-engineered HuCC49ΔCH2 antibodies.** Supernatants from representative transfected cell lines were chosen for analysis. Samples are 2-fold serial dilutions ranging from 30 to 0.23 ng antibody. A, HuCC49ΔCH2 parent antibody and the Pro-243 and PAP hinge variants. B, C239S:Pro-243, C239S:PAP, C242S:Pro-243, and C242S:PAP hinge variants. C, G1/G3:Pro-243 and G1/G3:PAP hinge variants. Markers indicate expected location of form A and B isoforms.
Hinge Engineering of a $\mathrm{CH}_2$ Domain-deleted Antibody

monitorable in positive ion mode. Samples were injected onto the column, and the gradient was held at 0% Buffer B for 5 min. Elution was accomplished with a linear gradient of 0–50% Buffer B over 125 min, followed by a 75% Buffer B wash over 10 min and a 0% Buffer B re-equilibration over 30 min.

RESULTS

Hinge-engineered HuCC49ΔCH2 Antibodies, Expression, and Western Blot Analysis—To examine the effect that hinge region modifications have on the secreted fraction of HuCC49ΔCH2 form A and form B isoforms, a series of genetically engineered HuCC49ΔCH2 hinge variants were constructed and the proteins expressed in CHO cells. TABLE ONE shows the UH and MH amino acid sequences for the parent HuCC49ΔCH2 antibody and the engineered hinge variants. Supernatants from ~10 individually isolated transfected cell lines representing each hinge variant were collected, and the concentration of antibody in the culture supernatants was determined by immunoassay. Expression levels among the entire set of hinge variants ranged from 300 to 1200 ng/ml with no set of variants showing preferentially high or low expression (data not shown). This would imply that the hinge modifications do not have a major effect on protein expression. 3 ng of total antibody protein from each isolate was analyzed by nonreducing SDS-PAGE followed by Western blot with an anti-human $\kappa$ chain antibody to detect HuCC49ΔCH2 form A and form B isoforms using methods shown in Fig. 1 (data not shown). This preliminary screen showed that differences in the observed ratios of the A:B isoforms among the different engineered antibodies were independent of expression levels and also indicated that the A:B isoform ratios were consistent within the set of approximate 10 clones for each of the hinge-engineered variants. We then performed a more comprehensive Western blot analysis examining a series of supernatant dilutions from representative cell lines for each of the hinge-engineered antibodies (Fig. 2). Under these denaturing conditions, form A antibodies migrate as single 120-kDa disulfide-linked homodimers, and form B antibodies, which fail to form inter-heavy chain disulfide bonds, migrate as two 60-kDa half-molecules. Also visible are disulfide-linked $\kappa$ dimers and $\kappa$ chain monomers. A number of lesser intense bands are visible between the A and B antibody isoforms and most likely represent degradation products. The HuCC49ΔCH2 Pro-243 antibody contains an insertion of a single middle hinge proline residue at Kabat position 243, resulting in a modest increase in the form A isoform fraction relative to parent HuCC49ΔCH2 antibody as evidenced by the presence of a 120-kDa band relative to the 60-kDa band at dilute sample concentrations (Fig. 2A). The HuCC49ΔCH2 PAP antibody containing the MH and LH PAP insertion (Kabat positions 243–245) further enhances production of the A isoform (Fig. 2A). Substituting the cysteine at position 239 with serine in both molecules HuCC49ΔCH2 C239S:Pro-243 and HuCC49ΔCH2 C239S:PAP conversely favors production of the B isoform (Fig. 2B). Substituting the cysteine at position 242 with serine in HuCC49ΔCH2 C242S:Pro-243 had a marginal effect on the relative quantity of secreted A:B isoforms, yet in contrast it dramatically increased production of the B isoform in HuCC49ΔCH2 C242S:PAP (Fig. 2B). Both antibodies containing the cysteine-rich 15-amino acid IgG3 hinge peptide CPEPKSCDTPPPCPFR, HuCC49ΔCH2 G1/G3:Pro-243 and HuCC49ΔCH2 G1/G3:PAP, produced predominantly form A isoform with little or no detectable form B (Fig. 2C). In a similar trend as seen with the HuCC49ΔCH2 PAP antibody, the presence of the PAP addition in the HuCC49ΔCH2 G1/G3:PAP antibody resulted in a greater enhancement of secreted form A isoform compared with HuCC49ΔCH2 G1/G3:Pro-243 containing only the Pro-243 insertion.

| Construct | Fraction form A | Fraction form B |
|-----------|----------------|----------------|
| HuCC49ΔCH2 (parent) | 39 ± 7.5 | 61 ± 7.5 |
| Pro-243 | 51 ± 7.2 | 49 ± 7.2 |
| PAP | 72 ± 3.1 | 28 ± 3.1 |
| C239S:Pro-243 | 23 ± 1.8 | 77 ± 1.8 |
| C239S:PAP | 34 ± 0.8 | 66 ± 0.8 |
| C242S:Pro-243 | 36 ± 2.3 | 64 ± 2.3 |
| C242S:PAP | 22 ± 4.5 | 78 ± 4.5 |
| G1/G3:Pro-243 | 91 ± 1.7 | 9 ± 1.7 |
| G1/G3:PAP | 98 ± 1.2 | 2 ± 1.2 |

Spot densitometry was used to semi-quantify form A and form B band intensities from exposed films shown in Fig. 2 (TABLE TWO). Addition of a single proline residue at position 243 showed modest, albeit statistically insignificant, enhanced production levels of form A relative to the parent HuCC49ΔCH2 molecule (51 ± 7.2% versus 39 ± 7.5%, respectively). Addition of PAP at positions 243–245 increased the fraction of form A to 72 ± 3.1%, whereas the addition of the cysteine-rich 15-amino acid IgG3 hinge peptide sequence plus PAP resulted in up to 98 ± 1.2% secreted form A.

Protein Purification and Characterization—HuCC49ΔCH2 PAP and HuCC49ΔCH2 G1/G3:PAP antibodies were selected for scale-up bio-

FIGURE 3. SDS-PAGE analysis of purified HuCC49ΔCH2, HuCC49ΔCH2 PAP, and HuCC49ΔCH2 G1/G3:PAP antibodies. G418-resistant CHO cell lines expressing HuCC49ΔCH2 and hinge variant antibodies were scaled up in 25-liter cell bags. Antibodies were purified by protein G chromatography, and in the case of HuCC49ΔCH2 and HuCC49ΔCH2 PAP, the form A and form B isoforms were further purified by hydrophobic interaction chromatography. The peaks representing form A and B isoforms were separately pooled, dialyzed, and concentrated. 5-μg samples were run under nonreducing denaturing conditions and visualized with Coomassie Blue stain. Lane M, mark 12 marker; lanes 1–3, HuCC49ΔCH2; lanes 4–6, HuCC49ΔCH2 PAP; lane 7, HuCC49ΔCH2 G1/G3:PAP. Lanes 1, 4, and 7, antibodies following protein G chromatography; lanes 2 and 5, purified form A following hydrophobic interaction chromatography; lanes 3 and 6, purified form B following hydrophobic interaction chromatography. HuCC49ΔCH2 G1/G3:PAP (lane 7) was essentially >98% pure following a single protein G purification step.
synthesis by mammalian expression in CHO cells. Antibodies from precleared culture supernatants harvested from 10- to 25-liter capacity cell bags were purified by protein G affinity chromatography. HuCC49ΔCH2 G1/G3:PAP form A antibody was efficiently purified using protein G. 41.3 mg of HuCC49ΔCH2 G1/G3:PAP form A antibody was recovered by protein G chromatography from 7.5 liters of supernatant for a yield of 5.5 mg/liter and purity of 98.2% as assessed by scanning densitometry of Coomassie Blue-stained polyacrylamide gels. 213 mg of HuCC49ΔCH2 PAP form A:form B mixture was recovered by protein G chromatography with the form A isoform being further purified by hydrophobic interaction chromatography for a final yield of 138 mg from 25 liters or 5.52 mg/liter. Final purity of HuCC49ΔCH2 PAP A isoform was 98.4%.

Nonreducing SDS-PAGE analysis of purified HuCC49ΔCH2, HuCC49ΔCH2 PAP, and HuCC49ΔCH2 G1/G3:PAP antibodies showed that the protein G affinity chromatography step resulted in form A protein yields consistent with the results seen by Western blot analysis (Fig. 3, lanes 1, 4, and 7). HuCC49ΔCH2 yielded ~45% form A antibody, HuCC49ΔCH2 PAP yielded 83% form A, and HuCC49ΔCH2 G1/G3: PAP yielded 98.2% form A antibody.

Form A HuCC49ΔCH2 PAP and HuCC49ΔCH2 G1/G3:PAP antibody elution profiles were analyzed by gel filtration HPLC (Fig. 4). Both antibodies eluted predominantly as single peaks with similar retention times. No aggregates or breakdown products were detected.

**Competitive Binding Activity**—The binding activities of purified HuCC49ΔCH2, HuCC49ΔCH2 PAP, and HuCC49ΔCH2 G1/G3:PAP form A antibodies were compared in a DELFIA time-resolved fluorescence competitive binding assay to bovine submaxillary mucin, a source of the TAG-72 antigen (Fig. 5). In this assay increasing concentrations of unlabeled hinge-engineered antibodies are used to compete with a Eu³⁺-labeled form A HuCC49ΔCH2 antibody for binding to antigen. The data were analyzed using a one-site competition equilibrium binding model. For both of the hinge-engineered antibodies, HuCC49ΔCH2 PAP and HuCC49ΔCH2 G1/G3:PAP, the binding activities were indistinguishable from that of the parent form A HuCC49ΔCH2 antibody.

**Characterization of the Engineered Hinge Regions by Peptide Mapping**—Peptide mapping was used to determine the integrity of disulfide bond formation in the heavy chain hinge regions of HuCC49ΔCH2, HuCC49ΔCH2 PAP, and HuCC49ΔCH2 G1/G3: PAP antibodies. Fig. 6 shows the A₁₁₁ nonreduced (Fig. 6A), endo Lys-C reduced (Fig. 6B), and trypsin (Fig. 6C). Endo Lys-C mapping was performed under both nonreducing and reducing conditions to compare the expected molecular masses of the unlinked, monomeric hinge region peptides to the corresponding disulfide-linked hinge peptides (TABLE THREE). Finally, trypsin mapping was performed under reducing conditions to confirm the identities of the hinge region peptides for each construct (TABLE THREE). The masses of tryptic fragments differ from the reduced endo Lys-C fragments because of digestion procedure and specificity of enzymatic cleavage.

Approximately 99% amino acid coverage was obtained by endo Lys-C mapping under nonreducing conditions, confirming the identity of each construct. Disulfide-linked fragments were detected in the variable light, Cᵥ, variable heavy, hinge, Cᵥ1, and Cᵥ3 regions, as expected, for all constructs (data not shown). The G1/G3:PAP sample showed an additional inter-heavy chain disulfide linkage in fragment (H232–275), below the original (H224–227) CPPC hinge region.

Theoretical and observed masses for the nonreduced hinge region peptides digested with endo Lys-C are summarized in TABLE THREE. The HuCC49ΔCH2 hinge peptide (residues H221–257) had an observed Mᵣ of 7419.1, in good agreement with the calculated mass of 7419.4 g/mol for a linked hinge containing two inter-chain disulfide bridges. The HuCC49ΔCH2 PAP hinge peptide (residues H221–260)
had an observed $M_r$ of 7949.7, also in good agreement with the calculated mass of 7949.8 g/mol for a linked hinge containing two inter-chain disulfide bridges. Two hinge peptide fragments resulted from digestion of HuCC49ΔCH2 G1/G3: PAP by endo Lys-C, because of an internal lysine residue at Kabat position 241II in the 15-amino acid IgG3 hinge peptide (TABLE ONE). Peptide fragment THTCPPCPEPK (residues H221–231) had an observed $M_r$ of 2414.3, in good agreement with the calculated mass of 2413.0 for a linked hinge containing at least one and possibly two inter-chain disulfide bridges. Peptide fragment SCDTPP-PCPRCPAPGGSSGGSGGQPREPQVYLPPSDELTK (residues H232–275) had an observed $M_r$ of 8782.6, in good agreement with the calculated mass of 8782.0 g/mol for a linked fragment containing at least two and possibly three disulfide linkages with at least one of these linkages as an inter-chain disulfide bridge. Although our preliminary peptide mapping/MS analyses do not permit us to discriminate mass differences in the H221–275 hinge region to definitively account for all possible disulfide linkage arrangements, it is apparent from these studies that the engineered hinge region of the HuCC49ΔCH2 G1/G3: PAP antibody possesses a minimum of two, and perhaps as many as five, inter-chain hinge disulfide bonds.

FIGURE 6. Detection of interchain hinge region disulfide bonds in hinge-engineered HuCC49ΔCH2 antibodies. Enzymatically digested and denatured samples were fractionated by reverse phase-HPLC and the elution profiles monitored at 215 and 280 nm. A, endo Lys-C digest, nonreduced. B, endo Lys-C digest, reduced. C, tryptic digest, reduced. Both fragments 29 (H221–231) and 30 (H232–275) in the nonreduced endo Lys-C HuCC49ΔCH2 G1/G3: PAP sample contain interchain disulfide bonds. The chromatograms revealed a conserved point mutation (Leu → Met) in HuCC49ΔCH2 G1/G3: PAP Cγ1 domain that was subsequently corrected.
DISCUSSION

We sought to improve the production of form A HuCC49ΔCH2 by using a protein engineering strategy focusing on the hinge region sequences. We approached this problem from the following three perspectives: 1) adding back middle and lower hinge residues may alter hinge conformation and favor stable disulfide bond formation; 2) deleting one of the two core middle hinge cysteine (CPPC) residues might favor interchain bond formation if only one pair of cysteine residues were available for disulfide formation; and 3) engineering a hinge containing additional hinge disulfide bonds beyond those contributed by the core middle hinge cysteines may facilitate stable interchain formation. In HuCC49ΔCH2, a flexible Gly/Ser spacer immediately follows the core CPPC hinge region. This Gly/Ser spacer is required for proper assembly of the HuCC49ΔCH2 homodimer antibody, and deletion of the Gly/Ser spacer in HuCC49ΔCH2 results in formation of a HuCC49ΔCH2 tetrameric species (homotetramer antibody) presumably through tail-to-tail interactions mediated by the CH3 domains.4

Pioneering work by Moroder and co-workers (22, 23) using synthetic peptides of human IgG1 hinge core region showed that antibody hinge dimers could be assembled into a disulfide-bonded, parallel configuration that were conformationally similar to the hinge seen in native IgG structures. It is thought that the conserved middle and lower hinge amino acid residues (Pro-243, Ala-244, and Pro-245) immediately adjacent to the core CPPC hinge region could influence the stability of the hinge disulfides. Based on this information, we speculated that re-introducing these residues into the HuCC49ΔCH2 molecule might augment the formation of hinge interchain disulfide bonds favoring secretion of the form A isoform. Indeed, we found that adding back the single middle hinge proline residue at position 243 gave a modest improvement in the yield of secreted HuCC49ΔCH2 form A. Addition of lower hinge residues proline, alanine, and proline at positions 243, 244, and 245, respectively, further increased the yield of the domain-deleted HuCC49 form A isoform up to 72%. The two lower hinge residues Ala-244 and Pro-245 are completely conserved across all human IgG subclasses and have not been found to account for differences in FcR binding or effector function among these immunoglobulin subclasses; therefore, we would not expect the addition of these residues to HuCC49ΔCH2 to restore effector function to any significant degree (24).

Cμ2 domain-deleted antibodies share a similar characteristic with IgG4 antibodies with respect to possessing hinge heterogeneity. IgG4 mAbs exist as mixtures of disulfide-bonded heterotetramers and as two half-molecules held together through noncovalent associations (25). These two forms can be easily distinguished by nonreducing, denaturing SDS-PAGE. Peptide mapping studies of recombinant IgG4 have provided chemical evidence that noncovalently assembled half-molecules contain intrachain hinge disulfide bonds (26). It has been proposed that the two forms reflect equilibrium products, but little is known about what mechanisms might lead to the interchange, although disulfide exchange oxidation/reduction reactions may play a role perhaps through enzymatic activity of protein-disulfide isomerases (27).

---

4 G. Brawslasky, personal communication.
Hinge Engineering of a C\textsubscript{H}2 Domain-deleted Antibody

Preferential synthesis of the interchain disulfide-bonded form of IgG4 has been achieved using site-directed mutagenesis to mutate the core CPSC middle hinge region, substituting the serine at position 241 with a proline resulting in the “IgG1-like” sequence CPPC (28). This strategy would not apply to the HuCC49\textsubscript{ΔCH2} molecule because the IgG1 core hinge already contains proline at position 241. However, in a series of experiments investigating the assembly of disulfide bonds in an IgG4 molecule, Schuurman et al. (29) mutated the cysteine at Kabat position 239 within the core CPSC hinge region to a serine with the thought that if one member of the pair of core cysteines were absent, then formation of an intrachain disulfide bond would be impossible, favoring assembly of interchain disulfide form (29). This construct was shown to reduce dramatically the proportion of half-molecules compared with that seen with the native hinge. By extending this concept to HuCC49\textsubscript{ΔCH2}, we designed and constructed a series of hinge-connecting peptides substituting the core CPPC hinge region cysteines with serine at either Kabat positions 239 or 241. The MS data also showed two alternative models where the Cys-241KK, Cys-241QQ, and Cys-242 hinge residues may participate in forming a single interchain disulfide bridge. In summary, we have shown that a protein engineering strategy has been used to design and produce a novel chimeric antibody hinge region for the efficient production of form A HuCC49\textsubscript{ΔCH2}. Our studies highlight the importance of hinge region disulfides as well as residues located outside of the core hinge region in producing a homogeneous preparation of the HuCC49\textsubscript{ΔCH2} antibody. Incorporation of the G1/G3:PAP hinge into C\textsubscript{H}2 domain-deleted antibodies is expected to result in significant improvements in manufacturing and cost savings. In addition, production of minibodies, another class of C\textsubscript{H}2 domain-deleted engineered antibodies, may also benefit from our G1/G3:PAP hinge design and is worth investigating (15). We are currently further studying the activity of \textsuperscript{111}I-labeled HuCC49\textsubscript{ΔCH2} G1/G3:PAP by examining pharmacokinetic and biodistribution properties in a murine xenograft model.

Acknowledgments—We gratefully acknowledge the expertise of Mike Favis for cell culture scale-up and Dr. Alexey Lugovskoy with the molecular modeling. We also thank Drs. Michael LaBarre, Paul Chinn, Brian Miller, and Stephen Demarest for valuable discussions and Kathleen Murphy for assistance with manuscript preparation.
REFERENCES

1. Witzig, T. E., Gordon, L. I., Cahanillas, F., Cruzcuzman, M. S., Emmanouilides, C., Joyce, R., Pohlman, B. L., Bartlett, N. L., Wiseman, G. A., Padre, N., Grillo-Lopez, A. J., Multani, P., and White, C. A. (2002) J. Clin. Oncol. 20, 2453–2463
2. Kaminski, M. S., Estes, J., Zasadny, K. R., Francis, I. R., Ross, C. W., Tuck, M., Regan, D., Fisher, S., Gutierrez, J., Kroll, S., Stagg, R., Tidmarsh, G., and Wahl, R. L. (2000) Blood 96, 1259–1266
3. Liu, T., Meredith, R. F., Saleh, M. N., Wheeler, R. H., Khazaeli, M. B., Ploj, W. E., Schlom, J., and LoBuglio, A. F. (1997) Cancer Biother. Radiopharm. 12, 79–87
4. Divgi, C. R., Scott, A. M., Danis, L., Capitelli, P., Siler, K., Hilton, S., Finn, R. D., Kemeny, N., Kelsen, D., and Kostakoglu, L. (1995) J. Nucl. Med. 36, 586–592
5. Meredith, R. F., LoBuglio, A. F., Ploj, W. E., Orr, R. A., Brezovich, I. A., Russell, C. D., Harvey, E. B., Yester, M. V., Wagner, A. J., and Spencer, S. A. (1991) J. Nucl. Med. 32, 1162–1168
6. Mueller, B. M., Reisfeld, R. A., and Gillies, S. D. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5702–5705
7. Calvo, B., Kashmiri, S. V., Hutzell, P., Hand, P. H., Slavin-Chiorini, D. C., Schlom, J., and Zaremba, S. (1993) Cancer Biother. 8, 95–109
8. Slavin-Chiorini, D. C., Kashmiri, S. V., Schlom, J., Calvo, B., Shu, L. M., Schott, M. E., Milenic, D. E., Snoy, P., Carraquillo, J., and Anderson, K. (1995) Cancer Res. 55, 55957–55967
9. Slavin-Chiorini, D. C., Kashmiri, S. V., Lee, H. S., Milenic, D. E., Poole, D. J., Bermon, E., Schlom, J., and Hand, P. H. (1997) Cancer Biother. Radiopharm. 12, 305–316
10. Muraro, R., Kuroki, M., Wunderlich, D., Poole, D. J., Colcher, D., Thor, A., Greiner, J. W., Simpson, J. F., Molinolo, A., and Noguchi, P. (1988) Cancer Res. 48, 4588–4596
11. Forero, A., Meredith, R. F., Khazaeli, M. B., Carpenter, D. M., Shen, S., Thornton, J., Schlom, J., and LoBuglio, A. F. (2003) Cancer Biother. Radiopharm. 18, 751–759
12. Agnese, D. M., Abdessalam, S. F., Burak, W. E., Jr., Arnold, M. W., Sobel, D., Hinkel, G. H., Young, D., Khazaeli, M. B., and Martin, E. W., Jr. (2004) Ann. Surg. Oncol. 11, 197–202
13. Sheikh, N. (2003) Curr. Opin. Mol. Ther. 5, 428–432
14. Roux, K. H., Strellets, L., Brekke, O. H., Sandle, I., and Michaelaen, T. E. (1998) J. Immunol. 161, 4083–4090
15. Hu, S., Shively, L., Raubitschek, A., Sherman, M., Williams, L. E., Wong, J. Y., Shively, J. E., and Wu, A. M. (1996) Cancer Res. 56, 3055–3061
16. Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S., and Foeller, C. (1991) Sequences of Proteins of Immunological Interest, 5 Ed., pp. iv–lvii, U. S. Department of Health and Human Services, Washington, D. C.
17. Brawlskaya, G., Glaser, S., Yang, T.-H., Hopp, J., and Chinn, P. (July 28, 2005) U. S. Patent PCT/US2004/020944
18. Barnett, R. S., Limoli, K. L., Huynh, T. B., Ople, E. A., and Reff, M. E. (1995) in Antibody Expression and Engineering (Wang, H. Y., and Imanaka, T., eds) pp. 27–40, Oxford University Press, New York
19. Kadowaki, H., Kadowaki, T., Wondisford, F. E., and Taylor, S. I. (1989) Gene (Amst.) 76, 161–166
20. Horton, R. M. (1993) in PCR Protocols: Current Methods and Applications (White, B. A., ed) Vol. 15, pp. 251–261, Humana Press Inc., Totowa, NJ
21. Nakamura, T., Kloepper, W. S., Brans, P., Hariharan, K., Chatam, S., Cao, X., LaBarre, M. J., Chinn, P. C., Morena, R. A., Shetowsky, W. S., Li, Y. P., Chen, A., and Reff, M. E. (2000) Int. J. Immunopharmacol. 22, 131–141
22. Wunsch, E., Moroder, L., Gohring-Romani, S., Musiol, H. J., Gohring, W., and Bovermann, G. (1988) Int. J. Pept. Protein Res. 32, 368–383
23. Kessler, H., Mronga, S., Muller, G., Moroder, L., and Huber, R. (1991) Biopolymers 31, 1189–1204
24. Clark, M. R. (1997) Chem. Immunol. 65, 88–110
25. King, D. J., Adair, J. R., Angal, S., Low, D. C., Proudfoot, K. A., Lloyd, J. C., Bodmer, M. W., and Yarranton, G. T. (1992) Biochem. J. 281, 317–323
26. Bloom, J. W., Madanat, M. S., Marriott, D., Wong, T., and Chan, S. Y. (1997) Protein Sci. 6, 407–415
27. Aalberse, R. C., and Schuurman, J. (2002) Immunology 105, 9–19
28. Angal, S., King, D. J., Bodmer, M. W., Turner, A., Lawson, A. D., Roberts, G., Pedley, B., and Adair, J. R. (1993) Mol. Immunol. 30, 105–108
29. Schuurman, J., Perdomo, G. J., Gorter, A. D., and Aalberse, R. C. (2001) Mol. Immunol. 38, 1–8
30. Aherr, K., Buchacher, A., Beerer, G., Josic, D., and Jungbauer, A. (2003) J. Chromatogr. A 1009, 89–96
31. Sali, A., and Blandell, T. L. (1993) J. Mol. Biol. 234, 779–815