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Processing of an Antigenic Sequence from IgG Constant Domains for Presentation by MHC Class II

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Targeting of T cell epitopes to APC enhances T cell responses. We used an APC-specific Ab (anti-IgD) and substituted either of 18 loops connecting B strands in human IgG constant H (C\text{H}1) domains with a characterized T cell peptide epitope. All Ab-epitope fusion molecules were secreted from producing cells except IgG-loop 2(BC)/C\text{H}1, and comparing levels, a hierarchy appeared with fusions involving C\text{H}2>C\text{H}1>C\text{H}3. Within each domain, fusion at loop 6(FG) showed best secretion, while low secretion correlated with the substitution of native loops that contain conserved amino acids buried within the folded molecule. Comparing the APC-specific rAb molecules for their ability to induce T cell activation in vitro, the six mutants with epitope in C\text{H}2 were the most effective, with loop 4C\text{H}2 ranking on top. The C\text{H}1 mutants were more resistant to processing, and the loop 6C\text{H}1 mutant only induced detectable activation. The efficiency of the C\text{H}3 mutants varied, with loop 6C\text{H}3 being the least effective and equal to loop 6C\text{H}1. Considering both rAb secretion level and T cell activation efficiency, a total of eight loops may carry T cell epitopes to APC for processing and presentation to T cells, namely, all in C\text{H}2 in addition to loop 6 in C\text{H}1 and C\text{H}3. Comparing loop 4C\text{H}2 with loop 6C\text{H}1 mutants after injection of Ab in BALB/c mice, the former was by far the most efficient and induced specific T cell activation at concentrations at least 100-fold lower than 6C\text{H}1.


Increased presentation of antigenic peptide in complex with MHC molecules enhances T cell responses, and Ab-mediated targeting of peptide or whole Ag to APC is a way to increase the number of peptide–MHC complexes on the surface of APC (1). This is important since major efforts are concentrated on the development of safe vaccines that generate strong, specific T cell responses. Abs with T cell epitopes within their V region CDR loops have been described previously (2). Although such Abs may enter APC by way of FcYRs, Abs with peptides or whole Ag added C-terminally to Fab (3) or to Ab (4, 5) may target a preferred and defined APC surface molecule by way of V region specificity. We have introduced peptide epitopes into loops connecting B strands in constant heavy (C\text{H})\text{4} domains, and denoted the epitope-loaded rAb “Troybodies.” The loop-grafting experiments have involved the amino acid sequence 91–101 of the MOPC315 plasacytoma A2 L chain (91–101 \(\lambda^{2315}\)), which represents a minimal stimulating T cell epitope (5) presented on I-E\text{d} MHC class II for CD\text{4}+ T cells (7). In initial experiments, this epitope was exchanged or inserted into either of three loops (BC, DE, or FG) in C\text{H}1 of human (h) IgG3 (8) and murine (m) IgG2b (9). For simplicity, these loops are hereby denoted loop 2C\text{H}1, 4C\text{H}1, and 6C\text{H}1, respectively (see Fig. 1A). The loop 2C\text{H}1 HlgG3 rAb mutant was retained, while all other single loop substitution mutants were secreted. We also showed that loop 6C\text{H}1 HlgG3 could be substituted with model epitopes that show great variation in amino acid sequence, length, and secondary structure, namely, aa 323–339 from OVA, aa 110–120 from hemagglutinin, and aa 46–61 from hen egg lysozyme (10). Initially, the epitope was grafted into hapten-specific rAbs (8) and, subsequently, the rAbs were equipped with V genes encoding APC specificity. Following in vitro targeting, the epitopes were excised from the rAbs, loaded on MHC class II molecules, and presented to specific T cells. Such Troybody targeting to murine IgD results in a 10\(^3\)-fold improvement in presentation efficiency compared with rAbs with irrelevant specificity. Importantly, T cell activation was improved up to 10\(^5\)-fold compared with that achieved using synthetic peptide or whole protein (10, 11). The same results were obtained in experiments with 91–101 \(\lambda^{2315}\) in loop 6C\text{H}1 where the target was MHC class II (I-E) (12). Furthermore, a 10\(^2\)-fold enhanced presentation was seen in vivo in mice (12).

Comparing the in vitro presentation efficiency of APC loaded with mouse and human rAbs with 91–101 \(\lambda^{2315}\)-epitopes grafted in various loops in C\text{H}1 (8, 9), differences were found which prompted us to initiate a comprehensive analysis that involved grafting in all loops in all three C\text{H}1 domains. Each domain has six loops connecting B strands, offering 18 possibilities for loop replacement. To identify the loops that are best suited for epitope insertion, we exchanged each loop in the three HlgG3 C\text{H}1 domains with the amino acid sequence 89–105 from \(\lambda^{2315}\) (89–105) \(\lambda^{2315}\) and show here that a total of 17 such fusion molecules were secreted from transiently transfected 293E cells, although in different amounts. Levels of secretion are compared with variability as well as hydrophobicity and solvent accessibility score for each amino acid within each domain.

For each domain, the loop 6 mutant was secreted at the highest level. Furthermore, the C\text{H}1 and C\text{H}2 mutants (except loop 2C\text{H}1)
were secreted better than the C_i3 domain mutants, of which the loop 6 mutant only was secreted well. In general, low secretion correlated with the removal of conserved amino acids that were buried either within a domain core or between two interacting domains.

All secreted rAbs were tested for the ability to stimulate CD4^+ T cells in vitro in T cell activation and growth inhibition assays. Importantly, T cell epitope loop grafting on all three C_i3 domains was compatible with efficient stimulation of T cells. Eight mutants were both secreted in good amounts and found to induce T cell activation, namely, loop 6CH1, all C_i2 mutants and loop 6CH3. The difference in induction potential was large, as the best activator (loop 4CH2) induced T cell activation at a concentration that was 10^2-fold lower than the weakest (loop 6CH1). These two Abs were selected for further studies in vivo and both were injected into the tail vein of normal mice at various concentrations. Following in vivo targeting, isolated spleen cells as APC stimulated specific T cells in vitro and again the loop 4CH2 mutant was at least 10^2-fold more efficient than the loop 6CH1 mutant.

### Materials and Methods

**Mice, cell lines, and Abs**

BALB/c mice were bred by Taconic Farms. The study was approved by the National Committee for Animal Experiments (Oslo, Norway). 293E (CRL-10852) cells were obtained from American Type Culture Collection. The T cell clone 7A10B2 recognizes as 91–101 of A_{155} in complex with I-E^d (7). Spleen cells from BALB/c mice were used as APC, as were A20 B lymphoma cells transfected with a 2,4,6-trinitrophenyl-specific IgG BCR (A20; gift from Dr. N. Horumi, Department of Medical Genetics, University of Toronto, Toronto, Ontario, Canada) (13).

The 4B2A1 and 7A10B2 T cell clones recognize the same A_{155} I-E^d complex (7). The T cell hybridoma BW4B2A1 was produced by fusing lymph node cells from 4B2A1 T cell transgenic SCID mice (14) with the TCR-negative T cell hybridoma line BW51.47 (clonal) (15) under standard conditions. Hypoxanthine/aminopterin/thymidine-resistant clones were selected for surface expression of the 4B2A1 TCR. Functionality was confirmed by coculturing hybridoma cells with irradiated BALB/c splenocytes (20 Gy) and synthetic A_{155} peptide (89 –107), with subsequent detection of IL-2 in supernatant by sandwich ELISA (data not shown).

All cells were cultured in DMEM (BioWhittaker) or RPMI 1640 supplemented with 10% heat-inactivated FCS (PAAS), 2 mM L-glutamine (DMEM only), 25 μg/ml streptomycin, and 25 U/ml penicillin (both from BioWhittaker) under standard conditions. Abs for IFN-γ detection were rat anti-mouse IFN-γ (clone JES6-5H4), both from BD Pharmingen. Abs for IFN-α, anti-mouse IL-2 (clone JES6-1A12) and biotin rat anti-mouse IL-2 (clone BioWhittaker) under standard conditions. Abs for IL-2 detection were rat anti-mouse IL-2 (clone JES6-1A12) and biotin rat anti-mouse IL-2 (clone BioWhittaker) under standard conditions.

**Secondary structure analysis**

Secondary structure analysis (β strand or loop) was conducted using data and evaluation programs given in the PDB database (http://www.rcsb.org/pdb/) (22) and as described previously (9). Briefly, crystal structures of 1FC1 (hlgG1), 1HGT (mlgG1), and 1HTG (mlgG2a) were studied regarding both loop length and limits as determined by three-dimensional visualization and the program Structure Explorer (http://www.pdb.org). Loops were numbered 1–6 in each domain starting from the N-terminal end of the polypeptide chain. Variability of each amino acid position in Ig H chains was analyzed using the sequence analysis program provided by S. M. J. Searle (The Sanger Institute, Cambridge, U.K.) as described previously (9).

The relative total side chain accessibility of each amino acid in the C_i1, C_i2, and C_i3 domains was investigated using Naccess (http://swift. bms.umist.ac.uk/naccess/) and PDB ID: 1HZH (human IgG1) (23). Hydropathicity analysis of hlgG3 domains C_i1, C_i2, and C_i3 was performed using the program ProtScale (window size = 9; http://www.expasy.org/cgi-bin/protscale.pl) (24) and the Kyte-Doolittle scale (25), as provided by the Swiss Institute of Bioinformatics.

### Production of mutant hlgG3: construction of loop exchange mutants

The C_i chain gene encoding the G3m (b0) allotype (26) was a gift from Dr. M. P. LeFranc (International ImMunoGeneTics Information System, Montpellier, France) and cloned as a 2.8-kb fragment into HindIII-SphI sites in the polyclinker of pUC19 (Sigma-Aldrich). The resulting plasmid, pUC19y3wt, was template in all mutagenesis reactions. All predicted loop sequences in C_y3 C_i1, C_i2, and C_i3, were exchanged with aa 89–105 from A_{155} (FAALWFRNHFVPGGKT) in individual H chains. The cysteine in position 90 of the endogenous sequence was exchanged with alanine. Mutagenesis was performed by QuikChange mutagenesis (Stratagene). Table I shows loop sequences and Table II shows mutagenic primer sequences. Colonies were screened for the presence of the mutation by the simultaneous introduction of a silent DraIII restriction site and DraIII digestion of plasmid DNA. All 18 mutations were subsequently confirmed by sequencing by GATC. Sequenced fragments containing mutations were subcloned into unmanipulated vectors as HindIII-BglII, BglII-PmlI, PmlI-NsiI, or PmlI-SphI (Fig. 1B) to exclude possible amplification errors outside the sequenced areas. Complete mutant H chain genes were assembled in pLNOH2_{i3}, which encode IgD-specific H chains, as described elsewhere (11, 27). The corresponding L chain gene, encoded on pLNOK_{i3} (11, 27) and each of the 18 pLNOH2_{i3} variants were transiently cotransfected in 293E cells (28). Portions of supernatant were harvested and replaced with fresh medium every 2–3 days for 14 days as described. A substitution of loop 4C_{i1} with 91–101 A_{155} has been described elsewhere (8) and the complete H chain gene was assembled in pLNOH2_{i3}. The corresponding loop 6C_{i1} substitution in an IgD-specific H chain has been described previously (11). Both H chain genes were transiently cotransfected with pLNOK_{i3} and supernatant harvested as described above. The complete IgD-specific rAbs are denoted “loop 4 or loop 6 C_{i1}” (91–101), respectively.

### Sandwich ELISA for detection of hlgG3 variant concentrations

The amounts of IgD-specific rAb mutants secreted after each transfection were measured as follows: 96-well microtiter plates were coated with a hlgG3-specific Ab and incubated overnight at room temperature (RT). Then, samples of 100 μl of diluted supernatants were added to each well and detected with a second hlgG3-specific Ab. A hlgG3wt preparation was diluted in a 3-fold series and used as standard. Three different Ab combinations were used as coat and detecting agent: s303 (2 μg/ml) and 132c8-bio (1/6000), s12 (10 μg/ml) and 132c8-bio (1/6000), as well as K13 (2 μg/ml) and s303-bio (1/6000), respectively. Detection was done with the substrate for alkaline phosphatase, p-nitrophenyl phosphate (Sigma-Aldrich) diluted in diethanolamine buffer to 1 mg/ml.
Isolation of mutant hIgG3

Proteins in supernatants were precipitated by 1:1 addition of portions of a saturated ammonium sulfate solution. Incubation at RT for 20 min was followed by a 10-min centrifugation at 17,000 g at 4°C before sterile filtration. Absorbance at 405 nm was read after 15–60 min.

Western blotting

Western blots were performed using Criterion XT Precast Gels (Bio-Rad). Briefly, the Ab samples were preheated at 95°C for 3 min before they were loaded onto the gel and separated at 140 V for 100 min. By Semi Dry electrophoresis, proteins were blotted onto filters, and counted using the TopCount detector. Ab concentrations were normalized to a known concentration of purified IgG3.

Binding to soluble FcγRIIA or protein G

The extracellular domains of hFcγRIIA were cloned and expressed as soluble fusion to GST (FcγRIIA-GST) as described elsewhere (29). Proteins were dialyzed against PBS/0.05% Tween 20, and incubated for 1 h at RT with the mutant Ab samples at 1 μg/ml. After washing, the wells were incubated for 1 h at RT with protein G-conjugated to HRP (VWR) or FcγRIIA-GST at 1 μg/ml followed by anti-GST conjugated to HRP (GE Healthcare). After three washes, the plates were developed in ABTS (Sigma-Aldrich) in citrate buffer at pH 2.2. Absorbance at 405 nm was read after 15–60 min.

Table II. Mutagenesis primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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</thead>
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<tr>
<td>1CH(+)</td>
<td>CCCCACCTGGTTTTCCAGGGACACGACGGCTGACGGGTCG</td>
</tr>
<tr>
<td>1CH(-)</td>
<td>GGCCGCAAGCTGCACTGGCAACGACGGCTGACGGGTCG</td>
</tr>
<tr>
<td>2CH(+)</td>
<td>CCCTCTGGTTTTCCAGGGACACGACGGCTGACGGGTCG</td>
</tr>
<tr>
<td>2CH(-)</td>
<td>CGCCGCAAGCTGCACTGGCAACGACGGCTGACGGGTCG</td>
</tr>
<tr>
<td>3CH(+)</td>
<td>ACCAACCTGGTTTTCCAGGGACACGACGGCTGACGGGTCG</td>
</tr>
<tr>
<td>3CH(-)</td>
<td>CGCCGCAAGCTGCACTGGCAACGACGGCTGACGGGTCG</td>
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</tr>
<tr>
<td>5CH(+)</td>
<td>ACCAACCTGGTTTTCCAGGGACACGACGGCTGACGGGTCG</td>
</tr>
<tr>
<td>5CH(-)</td>
<td>CGCCGCAAGCTGCACTGGCAACGACGGCTGACGGGTCG</td>
</tr>
</tbody>
</table>

Letters in bold indicate nucleotides encoding the (89–105) A213 peptide while underlining indicates nucleotide substitutions for the introduction of a silent DraIII site. All primers were designed with the 33 nt encoding (89–105) A213 in the center flanked by 18–21 nt complementary to the Cy5 swt sequence. Name according to loop of epitope grafting and domain, e.g. IC11+/- indicates template or complementary direction, respectively.

In vivo Ag presentation assays

**Growth inhibition assay** Growth inhibition assays were performed as described by Bogen et al. (7). All Ab mutants were diluted in 5-fold series starting at 1 μg/ml and added as triplicates in flat-bottom 96-well microtiter plates. 7A10B2 T cells on day 10 after the last stimulation were irradiated (20 Gy) and 10^5 cells were added to each well along with 5000 A206 cells in exponential growth expressing the IgD^+ allotype (13). Synthetic 89–107 A213 peptide was included as positive control. Supernatants from cells that had been treated with transfection reagent without DNA addition were also tested. After 72 h, supernatants from each well were filtered, and counted using a TopCount NXT scintillation counter (GMI). The experiments were repeated with 1 μg of [^3H]TdT in 16–24 h, harvested onto filters, and counted using the TopCount1 counter (GMI).

**T cell proliferation assay** Samples of 5 × 10^5 irradiated (20 Gy) BALB/c spleen APC were cultured with 2 × 10^5 7A10B2 T cells and various amounts of rAb or synthetic 89–107 A213 peptide in triplicates for 48 h. The cultures were then pulsed with 1 μCi of [^3H]TdT for the 16–24 h, harvested onto filters, and counted using the TopCount1 counter (GMI).

**In vivo experiments** BALB/c mice were injected i.v. in the tail with titrated amounts of loop 6C11 and loop 4C12 rAbs. Two mice received PBS only. Ninety minutes after i.v. injections, the mice were killed by cervical dislocation and the spleens were removed. Irradiated (8 Gy) spleen cells (5 × 10^7/well) were cultured with responder T cells, namely, 7A10B2 or T cell hybrids BW4 × 2A1 (both 2 × 10^5/well). An optimal concentration of the A213^+ synthetic peptide (10 μg/ml) was added to the positive control. After 72 h, portions of 100 μl of supernatant were collected for cytokine measurements, and the cultures were pulsed for 24 h with 1 μCi of [^3H]TdT. The cultures were harvested and incorporated [^3H]TdT was measured using a TopCount counter. The incorporation of IFN-γ and IL-2 in supernatants was performed in microtiter plates coated with AN18 or JES6-1A12 (both 2 μg/ml in PBS), respectively. XMG1.2-bio (1 μg/ml in PBS) was used as detection Ab for IFN-γ, whereas JES6-5H4 (1 μg/ml in PBS) was used as a detection Ab for IL-2.
for IL-2, followed by streptavidin-alkaline phosphatase (1/3000) and 1 mg/ml p-nitrophenyl phosphate in diethanolamine buffer. Standard curves were prepared from a 3-fold dilution starting at 35 ng/ml IFN-γ H9253 supernatant or a 2-fold dilution starting at 2 ng/ml rIL-2 (BD Pharmingen).

Prediction of asparaginyl endopeptidase (AEP) cleavage sites

Prediction of AEP cleavage sites within every mutated hIgG3 H chain was performed with NetAEP (http://theory.bio.uu.nl/kesmir/AEP/) as provided by C. Kesmir at Utrecht University (Utrecht, The Netherlands). Distribution of every asparagine residue, from those positively identified as AEP cleavage sites to totally overlooked residues, were positioned relative to the inserted epitope and within the original secondary structure.

Prediction of MHC II peptide presentation

The presence of I-E^b binding peptides in hIgG3 H chains was predicted using the PredBALB/c server (30). Briefly, each mutant IgG3 H chain was scanned for I-E^b binding nonameric peptides. Nonamers of lower score than aa 92–100 in the K^b epitope (31) were excluded.

Results

Construction of mutant Cγ3 genes and secretion of recombinant Abs

Because Abs are stable molecules and may be given unique specificities that allow targeting to APC, they are ideal vehicles for delivery of amino acid sequences that contain T cell epitopes. We therefore wished to study how such a sequence of 17 aa that contains a model epitope could be introduced into a hIgG molecule. It is essential that the fusion proteins are secreted from producing cells. We focused on loop sequences that link β strands in the Cγ3.

FIGURE 1. A. Graphic representation of a model C domain. The loops are numbered 1–6, β strands are labeled A–F. B. Human Cγ3 gene (ID: X03604). Exons are marked as arrows. For simplicity, the four hinge exons are shown as one exon. Restriction sites used for subcloning are indicated.

FIGURE 2. A. Secretion of rAb compared with wt, as determined by ELISA. Error bars indicate SD of triplicates. The graph is derived from one representative experiment of three. rAbs are named according to loop of epitope grafting and domain, i.e., 1C1 = epitope in loop 1 of C1γ1. B. Hydrophobicity; C. Variability; and D. accessibility analysis of the C1γ1 domain. Loop positions are indicated as L1–L6.
domains of hIgG3. The mutant molecules should necessarily pass
the endoplasmic reticulum quality control and be secreted as com-
plete H2 plus L2 Ig (32). We did an analysis of the domain archi-
tecture and then took an empirical approach and exchanged every
loop in every CH domain with the model sequence and estimated
the amounts of rAbs secreted (Figs. 2A, 3A, and 4A).

Since no crystal structure of a hIgG3 has been published, sec-
ondary structure analysis of individual C region domains was
based on knowledge of the structure of representative IgGs, as
described in Materials and Methods. Amino acids located in loops
connecting $\beta$ strands were determined and the results are presented
in Table I. Although loops 1, 4, and 6 in each domain connect $\beta$
strands within the same sheet, loops 2, 3, and 5 connect $\beta$ strands
on two opposing sheets. One-half of the 18 loops had a proline at
or close to the N- or C-terminal boundary and these were defined
as part of the loop rather than the framework. Hydropathicity and
solvent exposure of each amino acids in all three domains were
analyzed as described in Materials and Methods. The results are
described for hydropathicity (Figs. 2B, 3B, and 4B) and solvent
exposure (Figs. 2C, 3C, and 4C). We found that in $\mathrm{C}_{\mathrm{H}} 1$, four of six
loops are mostly hydrophilic while two are hydrophobic. All loops
in $\mathrm{C}_{\ell 2}$ are either neutral or mostly hydrophilic. In $\mathrm{C}_{\ell 3}$, five loops
are mostly hydrophilic and one is neutral in character.

The loops in the $\mathrm{C}_{\ell 2}$ domain have by far the largest number of
amino acids that are solvent exposed, while the loops in the $\mathrm{C}_{\ell 3}$
domain have the largest number of buried amino acids. The ma-
jority of the loop amino acid residues with low solvent exposure
point toward the domain core rather than an interdomain interface.

Exceptions are single amino acid residues in loop 2 and loop 4$\mathrm{C}_{\ell 3}$
that point toward the $\mathrm{C}_{\ell 3}-\mathrm{C}_{\ell 3}$ interface and one amino acid lo-
cated in loop 2$\mathrm{C}_{\ell 1}$ that point into the V region. As expected,
hydrophobic regions contain many buried amino acids, mainly in
frameworks, while the loops, mostly hydrophilic in nature, for the
most part are exposed to the solvent.

The T cell epitope selected for study, characterized by the spec-
ificity of the T cell line 7A10B2 (7), encompasses aa 91–101 of
$\mathrm{V}_{\ell 2}$. Notably, the aa 91–97 sequence constitutes the CDR3 loop
of the $\mathrm{V}_{\ell}$ chain of the myeloma protein of MOPC315 (33). In this
study, this sequence was extended by two amino acids N-termi-
"
FRNHVF GGTTK-105, and denoted 89–105. Compared with the loop sequences that were exchanged, this is rather hydrophobic. Studies with synthetic peptides show that the Cys90Ala substitution does not influence T cell responsiveness (B. Bogen, unpublished data).

We assumed that residues involved in maintaining structural stability would be rather conserved in various Ab molecules and that amino acids that were highly variable might well be substituted. We therefore performed the variability analysis for all three CH region domains that has previously been described for CH1 (9) (Figs. 2D, 3D, and 4D). In short, 144 different sequences of all isotypes from 30 species were aligned and the variability at each position was calculated as the number of different residues observed divided by the frequency of the most common residue. We found that both framework and loop sequences in all three domains contained both variable and conserved amino acids. For the most part, the loop amino acids were more variable than the framework residues. Conserved loop residues that in addition had low solvent exposure were in particular phenylalanine in loop 2CH1, a tryptophan residue in loop 5CH2 in addition to residues in all loops in CH3.

All loops were substituted with aa 89–105. Initially, 18 different mutants were made. Supernatants from transfectants were ammonium sulfate precipitated and dialyzed. As observed in earlier substitution experiments involving CH1, the IgD specificity was retained in all mutants (data not shown). Secretion levels were detected by three different sandwich ELISAs using pairs of hIgG3-specific Ab. In each pair, one Ab was used as coat and the other, which was biotinylated, as detection reagent. The Abs s303, s12, and K13 used as coat had specificity for Fab, hinge, or κ L chain, respectively, whereas the detecting Abs 132c8-bio and s303-bio had specificity for hinge or Fab, respectively. The results in Figs. 2A, 3A, and 4A are from a representative ELISA with anti-Fab as coat and anti-hinge as detection reagent. The secretion level of wild-type (wt) IgG3 was defined as 100% and compared with the mutants. ELISAs with the two other Ab pairs gave approximately the same results. We found that all mutants were secreted at levels below wt. One mutant only, that had the mutation in loop 2 of CH1, was completely retained. Samples of the secreted rAb run on SDS-PAGE and Western blot demonstrated that all were secreted as ~165-kDa proteins characteristic of complete H2L2 molecules (data not shown).

**FIGURE 4.** A, Secretion of rAb compared with wt, as determined by ELISA. Error bars indicate SD of triplicates. The graph is derived from one representative experiment of three. rAbs are named according to loop of epitope grafting and domain i.e., 1Cys3 = epitope in loop 1 of Cys3. B, Hydrophobicity; C, Variability; and D, accessibility analysis of Cys3. Loop positions are indicated as L1–L6. Amino acids in IgG known to be involved in effector molecule binding are indicated: ★, FcRn.
Comparing the secretion levels of the mutants, those with $89-105$ in loop $6CH1$ and loop $6CH2$ were secreted in relatively high amounts. Excluding loop $2CH1$, all mutants with substitutions in $CH1$ were secreted rather well at levels between 20 and 80% of wt. The $CH2$ mutants were all secreted at 20 – 60% of wt, whereas for $CH3$, the loop $6$ mutant only was secreted in high amounts. The remaining $CH3$ rAbs were secreted at levels below 10% of wt. All in all, 17 of 18 loop positions may be exchanged without complete retention and 12 without more than a 5-fold reduction in secretion.

Activation of specific T cells

It is crucial that the mutants are internalized by APC so as to enter the Ag-processing pathway and that the specific epitopes are properly excised from the rAb carrier to bind MHC and transported as peptide-MHC complexes to the cell surface. In this study, the mutants hlgG3s were tested in two different in vitro T cell activation assays, namely, a growth inhibition assay and a T cell proliferation assay.

Growth inhibition assay

The $T_H$ 1 cell clone used (7A10B2) has cytotoxic activity toward A20 lymphoma APC upon ligand recognition (7). The assay read-

out, incorporated radioactivity, reflects growth of the lymphoma cell APC only, as the T cells are irradiated. The assay is at least 10-fold more sensitive than a conventional T cell proliferation assay. The APC had been transfected with genes encoding IgDa (13) and were thus targets for the IgD-specific mutants. Irradiated 7A10B2 Th1 cells were mixed with secreted rAb mutants and APC as described in Materials and Methods (Fig. 5).

We found that the APC mixed with all rAb-harboring epitopes in $CH2$ or $CH3$ loops activated specific T cells, with $CH2$ replacements being more effective and with less variation than $CH3$ replacements. Thus, the epitope was presented to T cells from all positions in $CH2$ and $CH3$. However, from $CH1$, the epitope was detectably presented from loop 6 only. Notably, a high amount of loop $6CH1$ was needed to obtain the effect. At $0.01 \mu g/ml$, a concentration that was sufficient to induce the effect of all $CH2$ and $CH3$ mutants, no growth inhibition was detected. Thus, the epitopes in loops of $CH1$ seemed to be somewhat protected from presentation.

T cell proliferation assay

To confirm the results from the growth inhibition assay, eight mutants were selected, namely, loop $6CH1$, all $CH2$ mutants, and loop
One of the most active CH2 mutants, namely, loop 4CH2, was selected for further characterization in vivo and compared side-by-side with the corresponding mutant with a short sequence (Fig. 6). In each case, the mutant with short sequence was indeed more efficient than the corresponding mutant with a long sequence (Fig. 6). Assays in normal mice that have an important structural feature.

Induction of T cell activation after rAb injection in BALB/c mice and in vivo targeting. Titrated amounts of loop 4CH2 and loop 6CH1 rAbs were injected i.v. (two mice per point). After 90 min, the spleens were removed and the splenocytes were irradiated and used as APC in coculture with specific T cells: 7A10B2 for 48 h (Fig. 6A). The cells were pulsed with [3H]dThd and T cell proliferation was measured as incorporation of radioactivity. © IFN-γ production was measured by ELISA. © IL-2 production was measured by ELISA.

A loop 4CH1 mutant has previously proved to induce T cell activation (8). This mutant was identical to the one tested here, and T cell activation induced by the corresponding mutant.

6CH1, 6CH2, and 6CH3, all of which were secreted at levels above 20% of wt. These were further tested in a T cell proliferation assay. BALB/c spleen cells as APC, T cells, and rAbs were combined. The APC were irradiated and, thus, in this case, incorporation of radioactivity reflects T cell proliferation upon Ag stimulation. All mutants tested induced proliferation of the specific T cells (Fig. 6A). As in the growth inhibition assays, the mutants with epitopes in either of loops 2, 3, or 4CH2 were the most efficient T cell activators, followed by those with epitope in loops 1, 5, or 6CH2, which showed intermediate activation ability. Both loop 6CH1 and loop 6CH3 mutants required ~100× higher concentration than the best mutants to induce proper activation. A hierarchy appeared where loop 2CH2 = 3CH2 = 4CH2 > 5CH2 > 1CH2 > 6CH2 > 6CH3 > 6CH1. The results correspond well to those obtained in the APC growth inhibition assay. A summary of results from the secretion and T cell activation assays are presented in Table III.

A loop 6CH1 mutant has previously proved to induce T cell activation (8). This mutant was identical to the one tested here, except for the fact that a short sequence of 11 aa, namely, 91–101: A213, was introduced. To test whether the 89–105 sequence of 17 aa was less efficient than 91–101, two mutants with the long and two mutants with the short sequence in loop 4 or 6 of CH2, respectively, were compared side-by-side in the T cell proliferation assay. In each case, the mutant with short sequence was indeed found to be more efficient than the corresponding mutant with a long sequence (Fig. 6, B and C).

T cell activation in vivo targeting

One of the most active CH2 mutants, namely, loop 4CH2, was selected for further characterization in vivo and compared side-by-side with the loop 6CH1 mutant. Both were injected in BALB/c mice at various concentrations, and after 90 min the spleens were removed and whole spleen cell preparations mixed with specific T cells in vitro. T cell activation was measured as proliferation, IFN-γ secretion or IL-2 secretion, as a function of dose injected. Thus, in vivo APC targeting and processability of the two rAbs was compared in normal mice that express the relevant MHC class II molecule as previously described (11). Two different T cell preparations were used, 7A10B2 as described above and a T cell hybridoma derived from a mouse transgenic for the TCR of the T cell line 4B2A1. 7A10B2 and 4B2A1 have the same specificity (7). The dose-response curves are shown in Fig. 7. In all cases, whether measuring proliferation (Fig. 7A), IFN-γ production (Fig. 7B), or IL-2 production (Fig. 7C), the loop 4CH2 mutant induced specific T cell activation at concentrations at least 102-fold lower than loop 6CH1, and detectable activation was found after injection of ~1 µg of loop 4CH2 rAb per mouse.

Binding studies

The eight rAbs were tested for binding to FcyRIIA and protein G. In general, IgGs bind ligands in either of two Fc locations, namely, the lower hinge (all FcyRI as well as Clq) or the elbow region between CH12 and CH3 (protein A, protein G, FcRn) (39). To investigate the integrity of these two regions in the mutants, they were tested in ELISA for binding to recombinant soluble FcyRIIA and protein G, respectively. Mouse IgD was coated in the wells followed by the various IgD-specific rAbs. As was to be expected, the loop 6CH1 mutant bound both protein G and FcyRIIA (Fig. 8, A and B). Although none of the other seven mutants bound FcyRIIA, the loop

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**Table III. List of hIgG3 mutants**

<table>
<thead>
<tr>
<th>Domain</th>
<th>Loop</th>
<th>Loop Sequence</th>
<th>Amino Acid Difference</th>
<th>Secretion</th>
<th>Activation</th>
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<tr>
<td>Cγ1</td>
<td>1</td>
<td>APC5–10</td>
<td>7</td>
<td>++</td>
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<tr>
<td>2</td>
<td>F5–10</td>
<td>13</td>
<td>–</td>
<td>ND</td>
<td></td>
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<tr>
<td>3</td>
<td>NS4–10</td>
<td>9</td>
<td>++</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>NSS1–10</td>
<td>12</td>
<td>++</td>
<td>–</td>
<td></td>
</tr>
<tr>
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<td>PS1–10</td>
<td>8</td>
<td>++</td>
<td>–</td>
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<tr>
<td>6</td>
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<td></td>
</tr>
</tbody>
</table>

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*a* Underlining indicates an amino acid is displaying <10% variation. Super- *script numbers indicate that an amino acid is involved in binding an effector molecule or has an important structural feature. 1, H-L chain pairing (34); 2, ball and socket joint (35); 3, FcRn binding (36); 4, FcγRI binding (37); 5, C1q binding (38).

*b* Indicates loop length differences after replacement of the native loop with the corresponding mutant.

*c* Indicates secretion at 50–100%, ++ indicates secretion at 10–49%, and + indicates secretion at 1–9% of wild-type level.

*d* T cell activation induced by the corresponding mutant.

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**FIGURE 7.** Induction of T cell activation after rAb injection in BALB/c mice and in vivo targeting. Titrated amounts of loop 4CH2 and loop 6CH1 rAbs were injected i.v. (two mice per point). After 90 min, the spleens were removed and the splenocytes were irradiated and used as APC in coculture with specific T cells: 7A10B2 for 72 h (Fig. 6A). The cells were pulsed with [3H]dThd and T cell proliferation was measured as incorporation of radioactivity. © IFN-γ production was measured by ELISA. © IL-2 production was measured by ELISA. Error bars indicate SD of triplicates.

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Discussion

We expected that substitution of constant region loops with a different amino acid sequence would influence the stability and, therefore, secretion, of the fusion molecules. In general, amino acids that are important for thermodynamic stability can be involved in packing of the hydrophobic core, charge interactions, hydrogen bonding, desolvation upon folding, and be more or less compatible with the enforced local structure (40). Regarding C4H1, this domain interacts with C4 through a large, well-packed hydrophobic interface that enhances the stability of both domains (34). Furthermore, the variability and surface accessibility of loop amino acids in the C4H1 domain is notable. Therefore, it was not surprising that the mutants with substitution of loops in this domain were well secreted. An exception is the loop 2C4H1 mutant that was completely retained. This is in agreement with previous results (9) and confirms that removal of the ball-and-socket joint (35) between V4H and C4H1 that involves this loop, is detrimental to secretion of IgG3. A corresponding mutant in mlgG2b is secreted, however (9), and, therefore, the requirement for the interaction is not absolute. The amino acids involved in the C4H1/C4 interface are mostly situated in the A, B, D, and E strands, separated by loops 1 and 4, respectively. Substitution of these loops was less well tolerated than substitution of loops 3 and 6.

The C4H2 domains do not physically interact with each other, they are heavily glycosylated on the Asn297 residue situated in loop 4, and the carbohydrate moiety normally fills the space between the domains. Mutating loop 4 decreased the secretion level 4-fold. However, all C4H2 loop mutants were secreted in amounts between 20 and 60% of wt level, and the reduction may therefore not easily be attributed to removal of the carbohydrate. Of the C4H2 loop mutants, that in loop 5 showed the lowest secretion, probably due to removal of the conserved and buried tryptophan in the native loop sequence.

The C4H3 domain contains several amino acids that contribute to either the interface between C4H2 and C4H3 or dimerization through C4H3-C4H3 domain interaction and consequently have low accessibility scores. Variability analysis demonstrated the presence of many conserved residues in this domain, both in frameworks and in loops. The C4H3-C4H3 interface involves 16 residues that make interchain contacts (41). Of these, dimer stabilization is largely mediated by two loop 4 residues and four β strand residues. Thus, substitution of the involved loop 4C4H3 may have destabilized the domain interface, whereas the other loop mutations may well have altered the configuration of β strands to reduce overall domain stability. It is therefore not surprising that the secretion of all but one mutant, namely, that involving loop 6, was very low. In this study, the new sequence is introduced at the outer edge of the domain and destabilizing effects introduced may have left the domain core mostly unaffected. The introduced 17 aa increased the loop length of all mutants from 3 to 13 aa. Those secreted in high amounts were all elongated from 10 to 13 aa (Table III). In a previous study, loop 6C4H1 was exchanged with epitopes as long as 37 aa without interrupting secretion (42). Thus, long sequences may be introduced.

Proline residues at the framework/loop boundaries were defined as part of the loop by the Swiss-Pdb Viewer program and removed upon introduction of the 89–105 L2315 sequence. In one case, the loop 5C4H1 mutant was made both with and without an N-terminal proline and the presence of proline improved secretion 4-fold (M. Flobak, unpublished data). Thus, secretion may be further improved by keeping or possibly adding prolines to the framework/loop boundaries.

As we addressed ligand binding, we observed that the mutant with the new sequence in Fab bound both FcyRIIA and protein G. None of the mutants with insert in the Fc region bound FcyRIIA, whereas two bound protein G, namely, the loop 4C4H2 and the loop 6C4H1 mutants. A lack of FcyR binding may be an advantage, as the function of IgG in the context of the studies reported here, is solely to target APC by way of V region specificity. Protein G binds in a region remote from the FcγR binding site, namely, at the interface of C4H2 and C4H3 which is also the site of FcRn binding, and alterations here are likely to affect biodistribution and half-life of the mutant in vivo (36).

Specific growth inhibition and T cell proliferation assays were used to monitor peptide presentation on MHC class II and thus processing and loading of all mutants in vitro. The epitope was excised and presented to T cells from 13 of 18 positions in the rAb, and there were variations in activation efficiency. The mutants with substitution in loops of C4H1 did not induce T cell activation, with the exception of loop 6C4H1, which did so with a relatively low activity. All of the C4H2 mutants induced with a high activity and, in particular, the mutant with epitope in loop 4, which was the better inducer in both the growth inhibition and T cell proliferation experiment. To induce the same in vitro effect, 100× less rAb of the loop 4C4H2 than the loop 6C4H1 variant was needed. The mutants with epitope in either of the C4H3 domain loops activated at various levels. Of these, the loop 6C4H3 mutant, which unlike the other C4H3 mutants, was secreted well, had low in vitro activity, almost as low as loop 6C4H1.

The C4H1 domain seemed to be somewhat resistant to intracellular processing. This was not an effect of the epitope chosen, as the same result was obtained for the unrelated sequence of the
OVA epitope, aa 323–339. Although an OVA323–339 loop 6C\textsubscript{H}1 mutant induced activation (10), an OVA323–326 loop 1C\textsubscript{H}1 mutant did not (M. Flobakk, unpublished data). Furthermore, we have previously observed very poor presentation of the A2\textsuperscript{155} sequence from loop 2 and 4C\textsubscript{H}1 in mlgG2, and the observation is therefore not restricted to hlgG3 (9) (Note that mutant terminology differs in this article.) In all cases were the C\textsubscript{H}2 domain mutants well secreted from producing cells and induced T cell activation at low concentrations. Again, the finding was not restricted to the amino acid sequence chosen for the systematic substitution experiments, as analogous fusion molecules with aa 110–120 from the hemagglutinin epitope in loop 1C\textsubscript{H}2 or aa 46–61 from the hen egg lysozyme in loop 6C\textsubscript{H}2 were well secreted and induced T cell activation in vitro at the same low rAb concentrations (M. Flobakk, I. B. Rasmussen, E. Lunde, T. E., Michaelsen, B. Bogen, and I. Sandlie, submitted for publication). In conclusion, a total of eight loop mutants were found to be useful for T cell activation purposes when both secretion from producing cells and the ability to induce activation in vitro were considered.

Of these, two were chosen for in vivo investigation, namely, loop 6C\textsubscript{H}1 and loop 4C\textsubscript{H}2. One harbors the epitope in Fab and one in Fc and they have a remarkable 100-fold difference in T cell activation efficiency in vitro. Importantly, this difference was also seen in vivo. Others have demonstrated that stability is inversely related to processing and, thus, to presentation in vitro. In vivo, the situation was reversed and the stable molecule induced a stronger T cell response than the unstable variant (43, 44). In the experiment presented here, on the other hand, the mutants were actively targeted to APC in vivo. Because we have previously shown that presentation peaks when the targeted spleen cells are removed and tested for T cell activation ability after 1–2 h (11), it is reasonable to believe that both mutants are stable in serum until internalized.

We were intrigued by the fact that the C\textsubscript{H}1 domain mutants were poor inducers of T cell activation and speculated that there might not be recognition sites for proteolytic enzymes adjacent to the epitope in the C\textsubscript{H}1 domain. Although most proteases have broad specificity, making processing predictions difficult, AEP has restricted specificity (45). It cleaves at selected asparagine residues and unlocks the globular protein structure for MHC class II screening (46). There are 14 asparagine residues in the C\textsubscript{3}3 amino acid sequence (26), and the AEP cleavage server (http://theory.bio.unnl.nl/keshmir/AEP) predicts five cleavage sites in the Fc region and only one in C\textsubscript{H}1. Thus, additional recognition sites in C\textsubscript{H}1 might improve the activation potential of the C\textsubscript{H}1 mutants. Notably, the 89–105 A2\textsuperscript{155} sequence also contains an asparagine residue (Asn\textsuperscript{96}), but according to the AEP cleavage server, it is not susceptible to AEP cleavage regardless of its position within any C\textsubscript{H}1 domain.

Peptides presented on I-Ed are anchored at positions P1, P4, P6, and P9 (31). The anchor residues in 89–105 A2\textsuperscript{155} are Leu\textsuperscript{92}, Phe\textsuperscript{94}, Arg\textsuperscript{95}, and Phe\textsuperscript{100} within the epitope (91–101). Competing I-Ed binding motifs could disturb 91–101 epitope loading. The Pred\textsubscript{BABLc} (http://Ag.ig2.r Osaka-edu.sg/Pred\textsubscript{BABLc}/) predicts nonamer peptide binding to the H-2\textsuperscript{d} haplotype of BALB/c mice (30). Focusing on nonamers with an equal to or higher score than 92–100, 25 peptides in C\textsubscript{3}3wt were identified that may compete for I-Ed binding, but there does not seem to be an obvious correlation between the location of these peptides and activation efficiency. Importantly, the 89–105 sequence was found to encompass an additional nonamer, namely, 97–105 (HVFPVGGTGKT) that fits better in I-Ed\textsubscript{d} than 92–100. These two are mutually exclusive and this may explain the finding that the long peptide of 17 aa induced activation of aa 91–101-specific T cells at a lower level than the short peptide of only 11 aa in two different mutants (loop 4C\textsubscript{H}1 and loop 6C\textsubscript{H}1).

In the experiments shown here, APC were targeted by anti-mouse IgG\textsuperscript{d}, i.e., to the Ag receptor of B cells (13). This is an endocytic receptor that directs Troybodies to intracellular vesicles where Ag processing and epitope loading on MHC class II occur. However, the B cell receptors are not optimal targets in vivo and a major disadvantage is the possibility for polyclonal activation of B cells (47). Alternative targets are surface molecules such as CD19 on B cells (48), MHC class II (48, 49), and CD14 (50). Receptors on dendritic cells may be even better targets since dendritic cells are able to prime naive T cells and induce Th1, Th2, and B cell responses. CD40 (51, 52), TLR (53, 54), CD91 (55), several chehomokine receptors (56, 57), and C-type lectins, such as DC-SIGN (58, 59), mannose receptor (60), and DEC-205 (61, 62), have all been shown to be attractive candidates for targeting.

Our results are clear regarding an activation hierarchy in vitro and in vivo. rAbs with epitope loaded in the C\textsubscript{H}2 domain are secreted from producing cells and processed very efficiently after targeting to APC. Thus, whenever the target is strong enhancement of T cell responses, epitope grafting in the C\textsubscript{H}2 domain seems preferable. For vaccination purposes, integration of several different sequences may be desirable. The results presented here suggest that multiple loop replacements within a single rAb may be a useful strategy using a combination of loop substitutions in two or possibly three domains.

Acknowledgments
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Disclosures
Inger Sandlie and Bjørn Bogen are coinventors on U.S. patent 6.249.654 granted September 25, 2001. Title: Modified immunoglobulin molecule incorporating antigen in a non-CDR loop region.

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