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Molecularly Defined Antibody Conjugation through a Selenocysteine Interface[†]

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ABSTRACT: Antibody conjugates have broad utility in basic, preclinical, and clinical applications. Conventional antibody conjugation through the amine group of lysine or the thiol group of cysteine residues yields heterogeneous products of undefined stoichiometry and considerable batch-to-batch variability. To preserve the two hallmarks of the antibody molecule, precision and predictability, methods that enable site-specific antibody conjugation are in high demand. On the basis of a mammalian cell expression system, we describe the utilization of the 21st natural amino acid selenocysteine for the generation of IgG and Fab molecules with unique nucleophilic reactivity that affords site-specific conjugation to electrophilic derivatives of biotin, fluorescein, and poly(ethylene glycol). The resulting antibody conjugates were found to fully retain their antigen binding capability and, in the case of IgG, the ability to mediate effector functions. Gain of function was demonstrated in vitro and in vivo. While these antibody conjugates are relevant for a variety of proteomic, diagnostic, and therapeutic applications, they also constitute a proof of principle for the generation of molecularly defined antibody−drug conjugates and radioimmunoconjugates. Compared to other site-specific antibody conjugation methods, selenocysteine interface technology (i) only involves a minor modification at the C-terminus that does not interfere with disulfide bridges, (ii) does not require activation, and (iii) generates unique 1:1 stoichiometries of biological and chemical components. Collectively, our method affords the generation of highly defined antibody conjugates with broad utility from proteomic applications to therapeutic intervention.

In addition to their key role in basic research as reagents with exceptional specificity and affinity, monoclonal antibodies (mAbs)[1] are increasingly used in the prevention, diagnosis, and therapy of human diseases (*1*). To date, 21 mAbs are approved by the Food and Drug Administration (FDA), and roughly 10 times more are at various stages in clinical development (*2*, *3*). Sometimes considered the second generation of mAbs, antibody conjugates that combine a biological component (the antibody molecule) and a chemical component (generally a small synthetic molecule) were developed to merge their particular properties in one hybrid molecule. For example, while the antibody molecule provides specificity, affinity, and increased circulatory half-life, the small synthetic molecule affords imaging capability or cytotoxicity.

Conventional antibody conjugation randomly utilizes the ε-amino group of lysine (Lys) residues or the thiol group of cysteine (Cys) residues. The resulting antibody conjugate is a mixture of molecules with a range of stoichiometries and characterized by substantial batch-to-batch variability. In addition, random conjugation can impair the antibody molecule with respect to antigen binding, circulatory half-life, and effector functions. Site-specific antibody conjugation, on the other hand, affords molecules of defined stoichiometry in reliable and reproducible batches and aims to fully preserve the integrity of the antibody molecule. Thus, the challenge of site-specific antibody conjugation is to introduce a unique chemical reactivity without impact on the overall structure and function of the antibody molecule.

Recently, we reported the development a unique class of antibody derivatives that consist of an IgG-derived Fc fragment as a generic biological component covalently linked to a variable chemical component through a selenocysteine (Sec) interface (*4*). We showed that both the biological and chemical components are endowed with pharmacological advantages through this conjugation. On the basis of an FDA-approved therapeutic mAb, we here demonstrate that Sec interface technology can be applied to whole antibody molecules (IgG) and antibody fragments (Fab), vastly broadening the utility of this method.

MATERIALS AND METHODS

Cloning of Rituximab-Based Antibody Derivatives. (*i*) *PIGG-rituximab-Sec-His*. On the basis of amino acid sequences of the variable domains of rituximab (U.S. Patent 5,736,137),

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[1]Abbreviations: mAb, monoclonal antibody; FDA, Food and Drug Administration; Sec, selenocysteine; SECIS, selenocysteine insertion sequence; UTR, untranslated region; HEK, human embryonic kidney; IMAC, immobilized metal affinity chromatography; PEG, poly(ethylene glycol); HRP, horseradish peroxidase; FcRn, neonatal Fc receptor; ADCC, antibody-dependent cellular cytotoxicity; PBMC, peripheral blood mononuclear cells; CDC, complement-dependent cytotoxicity.

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DNA sequences encoding the mouse variable domain of the heavy chain (V_H) and the chimeric mouse/human κ light chain (V_κ-C_κ) were optimized for expression in human cells by custom synthesis (GenScript) and cloned by SacI/ApaI and HindIII/XbaI ligation, respectively, into mammalian cell expression vector PIGG. In this plasmid, heavy and light chains are expressed by an engineered bidirectional CMV promoter cassette (*5*). For the expression of a C-terminal Sec in the heavy chain, a SacII/SalI fragment of the previously described (*4*) mammalian cell expression vector pCEP4-Fc-Sec-His was cloned into PIGG-rituximab by SacII/SalI ligation. This fragment consisted of a sequence encoding a C-terminal portion of heavy chain constant domain C_H3 downstream from a natural SacII site, fused to a TGA codon, followed by a His_6-encoding sequence, a TAA stop codon, a selenocysteine insertion sequence (SECIS) element from the 3′-untranslated region (UTR) of the cDNA of human thioredoxin reductase 1, and an engineered SalI site. The resulting plasmid was designated PIGG-rituximab-Sec-His.

(*ii*) *PIGG-rituximab-Sec*. To express rituximab with a C-terminal Sec but without a His tag, we first generated mammalian cell expression vector pCEP4-Fc-Sec in close analogy to previously described pCEP4-Fc-Sec-His (*4*). Using pCEP4-Fc-Sec-His as a template, a PCR fragment was amplified with primer pair VIII-5′/VIII-3′ and cloned into pCEP4-Fc (*4*) by HindIII/XhoI ligation. The resulting plasmid was designated pCEP4-Fc-Sec. An Fc-Sec encoding portion of pCEP4-Fc-Sec was subsequently transferred into PIGG-rituximab by SacII/SalI ligation, resulting in PIGG-rituximab-Sec.

(*iii*) *PIGG-rituxifab-Sec-His*. To shorten the IgG1 expression cassette to a Fab expression cassette, an ApaI/SalI fragment of PIGG-rituximab-Sec-His was replaced with a fragment that consisted of a sequence encoding the portion of heavy chain constant domain C_H1 downstream from a natural ApaI site, fused to a TGA codon, followed by a His_6-encoding sequence, a TAA stop codon, the above-described SECIS element, and an engineered SalI site. This fragment was generated by overlap extension PCR of two PCR fragments that had been amplified with primer pairs IX-5′/IX-3′ and X-5′/X-3′ and PIGG-rituximab-Sec-His as a template.

(*iv*) *Primer sequences*: VIII-5′, gcctaagcttgtctccgggtgcctgataagccccagtgtggatgctgttg; VIII-3′, agctctcgaggccaaatgagatgaggacgtgag; IX-5′, ccaagggcccatcggtcttccccctggcaccctcctccaagagcacctctgggggca; IX-3′, atgtcatgtgtgagttttgtcacaagatttgggctcaactttctt; X-5′, tcttgtgacaaaactcacacatgacatcaccatcaccatcactaagccccagtgtggatgctgttgcca; X-3′, ctaggtcgactttatttgccaaatgagatgaggacgtgag.

Expression and Purification of Rituximab-Based IgG-Sec-His and Fab-Sec-His. The mammalian cell expression vectors described above were transiently transfected into human embryonic kidney (HEK) 293F cells (Invitrogen) with 293fectin (Invitrogen) using conditions detailed in the manufacturer's protocol. Transfected HEK 293F cells were cultured in FreeStyle serum-free medium (Invitrogen), supplemented with 1 μM Na_2SeO_3 (Sigma), in spin flasks (Integra Biosciences) under constant rotation at 75 rpm (Integra Biosciences Cellspin stirring platform), in a humidified atmosphere containing 8% CO_2 at 37 °C. Three days after transfection, the medium was collected after centrifugation, replaced for an additional 2 days, and collected again. This procedure was repeated once for an additional 2 days. The combined supernatants were filtered through a 0.45 μm membrane and 10-fold concentrated using an ultrafiltration device with a 10 kDa cutoff membrane (Millipore). Whereas the concentrate containing IgG-Sec-His was loaded on a 1 mL recombinant Protein G HiTrap column (GE Healthcare), Fab-Sec-His was purified using a 1 mL NHS-activated HiTrap column coated with goat anti-human Fab polyclonal IgG (Bethyl Laboratories) as described previously (*6*). PBS was used for column equilibration and washing, 0.5 M acetic acid (pH 3.0) for elution, and 1 M Tris-HCl (pH 8.0) for immediate neutralization. The neutralized eluate was dialyzed at 4 °C overnight against PBS using Slide-A-Lyzer cassettes with a 10 kDa cutoff (Pierce) and concentrated with 10 kDa cutoff centrifugal filter devices (Millipore). To separate IgG-Sec-His and Fab-Sec-His from IgG-stop and Fab-stop, respectively, the purified proteins were 10-fold diluted in loading/washing buffer (500 mM NaCl and 25 mM imidazole in PBS) and loaded on a 1 mL immobilized metal affinity chromatography (IMAC) column (HisTrap, GE Healthcare). After the flow-through that contained IgG-stop and Fab-stop proteins had been collected, the column was washed with 50 mL of loading/washing buffer. Bound IgG-Sec-His and Fab-Sec-His proteins were subsequently eluted with elution buffer (500 mM NaCl and 500 mM imidazole in PBS). Both eluate and flow-through were dialyzed and concentrated as described above.

Selective Conjugation. For selective conjugation at the Sec interface, rituximab-based IgG-Sec-His and Fab-Sec-His as well as the negative controls rituximab-based IgG-stop and Fab-stop were diluted in 15 mL of 100 mM sodium acetate (pH 5.2) and concentrated to 4 μM using a 10 kDa cutoff centrifugal filter device. DTT at 0.1 mM followed by either (+)-biotinyl-iodoacetamidyl-3,6-dioxaoctanediamine ("biotin-iodoacetamide"), maleimide-PEO_2-biotin ("biotin-maleimide"), or fluorescein 5-maleimide ("fluorescein-maleimide") (all from Pierce) at 40 μM or a 7.5 kDa biotin-PEG-maleimide (JenKem Technology) at a final concentration of 10 μM was added to the protein and incubated for 1−2 h at room temperature in the dark. Following conjugation to biotin-iodoacetamide, biotin-maleimide, or fluorescein-maleimide, the proteins were diluted in 15 mL of 100 mM sodium acetate (pH 5.2) and concentrated to 250 μL as described above. This step was repeated once with 15 mL of 100 mM sodium acetate (pH 5.2) and subsequently twice with 15 mL of PBS to remove unconjugated compounds. After conjugation to the 7.5 kDa biotin-PEG-maleimide, which cannot be removed with a centrifugal filter device, size-exclusion chromatography (see below) was used to separate conjugate and unconjugated compound. Following sterile filtration, purified conjugates in PBS were stored refrigerated (4 °C) for short-term use and frozen (−80 °C) in aliquots for long-term use.

Purification of Rituximab-Based IgG-Sec. Rituximab-based IgG-Sec without a His tag was expressed and purified by Protein G affinity chromatography as described above. The purified mixture of IgG-Sec and IgG-stop was reacted with maleimide-PEO_2-biotin using the selective conjugation conditions described above. IgG-Sec/biotin and IgG-stop were subsequently separated by affinity chromatography using the Monomeric Avidin Kit (Pierce) according to the manufacturer's protocol.

Purification of Rituximab-Based Fab-Sec-His/PEG-biotin by Size-Exclusion Chromatography. A Superdex 200 10/300 GL size-exclusion column (GE Healthcare) was connected to an AKTAfplc liquid chromatography system (GE Healthcare) and equilibrated with 2 column volumes of PBS. For the separation of PEGylated protein from unconjugated poly(ethylene glycol) (PEG), the conjugation reaction mixture

(total volume of ~400 μL) was injected onto the column and separated with PBS using a flow rate of 0.5 mL/min. UV absorbance was read at 280 nm. After an initial 10 mL of buffer had passed through the column, 0.5 mL fractions were collected continuously. The fractions of each peak were pooled and concentrated with a 10 kDa cutoff centrifugal filter device.

ELISA. All incubations were conducted for 1 h at 37 °C.

(*i*) *Analysis of Selective Conjugation*. To confirm selective biotinylation at the Sec interface through maleimide-PEO_2-biotin, wells of a 96-well Costar 3690 plate (Corning) were incubated with 200 ng of rituximab-based IgG-Sec-His/biotin, IgG-stop, Fab-Sec-His/biotin, Fab-stop, or Rituxan (Genentech) in 25 μL of PBS. After being blocked with 3% (w/v) BSA/PBS, the plate was incubated with either horseradish peroxidase (HRP)-coupled streptavidin (50 ng/well) or a 1:1000 dilution of HRP-coupled donkey anti-human IgG polyclonal antibodies (Jackson ImmunoResearch Laboratories) in 1% (w/v) BSA/PBS. After the sample had been washed with H_2O (10 × 200 μL/well), colorimetric detection was performed using 2,2′-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid (Roche) as a substrate according to the manufacturer's protocol.

(*ii*) *Analysis of Fc Receptor Binding*. To analyze and compare the binding of selectively conjugated rituximab-IgG-Sec-His and Rituxan to commercially available recombinant human FcγRI, FcγRIIA, and FcγRIIIA (all from R&D Systems) as well as to our previously described (*4*) recombinant human neonatal Fc receptor (FcRn), 500 ng of each Fcγ receptor and 200 ng of FcRn were coated and blocked on a 96-well plate as described above. In the FcRn binding assay, the plate was then incubated with various concentrations (0.625−5 μg/mL) of rituximab-based IgG-Sec-His/biotin and IgG-stop; in the Fcγ receptor binding assay, the plate was incubated with 8 μg/mL (400 ng/well) rituximab-based IgG-Sec-His/biotin and Rituxan. Subsequent washing, incubation with HRP-coupled streptavidin (50 ng/well), HRP-coupled donkey anti-human IgG polyclonal antibodies (1:1000), or HRP-coupled goat anti-human Fab polyclonal antibodies (1:1000; Jackson ImmunoResearch Laboratories) in 1% (w/v) BSA/PBS, washing, and colorimetric detection were conducted essentially as described above. For the FcRn binding assay, however, all steps were conducted side by side in acidic PBS (pH 6.0) or neutral PBS (pH 7.4). For detection of multimeric IgG binding in the Fcγ receptor binding assay, 1 μg of rituximab-based IgG-Sec-His/biotin and Rituxan (negative control) were preincubated with 250 ng of HRP-coupled streptavidin followed by incubation with coated and blocked FcγRIIA and FcγRIIIA, washing, and colorimetric detection as described above.

Electrophoresis. (*i*) *SDS−PAGE*. Loaded at a level of 5 μg/lane, Rituxan and rituximab-based IgG-Sec-His/biotin, IgG-stop, Fab-Sec-His, Fab-Sec-His/biotin, and Fab-stop in NuPAGE LDS sample buffer (Invitrogen) were electrophoresed on a NuPAGE 4 to 12% gradient gel (Invitrogen) followed by staining with Simply Blue SafeStain (Invitrogen).

(*ii*) *Western Blotting*. SDS−PAGE was conducted as described above with 200 ng/lane and using NuPAGE LDS sample buffer supplemented with 10% (v/v) β-mercaptoethanol. Nitrocellulose membrane blotting and processing were continued as previously described in detail (*4*).

Flow Cytometry. All incubations were conducted for 1 h on ice. Cells from the human Burkitt's lymphoma cell line Raji and human mantle cell lymphoma cell line JeKo-1 (American Type Culture Collection) maintained in 10% FCS in RPMI 1640 medium supplemented with penicillin-streptomycin (all Invitrogen) were collected by centrifugation and resuspended in 1% (v/v) FCS/PBS, and aliquots of 50 μL containing 5×10^5 cells were distributed into a V-bottom 96-well plate (Corning). The cells were then incubated with 0.6 μM Rituxan or rituximab-based IgG-Sec-His/biotin, IgG-Sec-His/fluorescein, Fab-Sec-His/biotin, and corresponding negative controls. After being washed twice with 1% (v/v) FCS/PBS, the cells were incubated with a 1:25 dilution of PE-coupled streptavidin (BD Biosciences) or with FITC-coupled goat anti-human Fab polyclonal antibodies (Jackson ImmunoResearch Laboratories). This step was skipped for cells that had been incubated with IgG-Sec-His/fluorescein. After being washed twice as described before, the cells were resuspended in 400 μL of 1% (v/v) FCS/PBS and analyzed using a FACScan instrument (Becton-Dickinson). For the competition experiment, the cells were first incubated with 0.6 μM Rituxan or with the humanized anti-human CD52 mAb Campath (Genzyme) and then processed as described above using PE-coupled streptavidin. Flow cytometry with IgG-Sec/biotin and Fab-Sec-His/PEG-biotin was conducted analogously using a 1:25 dilution of PE-coupled donkey anti-human Fab polyclonal antibodies (Jackson ImmunoResearch Laboratories) for detection.

Antibody-Dependent Cellular Cytotoxicity (ADCC) Assay. (*i*) *Preparation of Effector Cells*. Human peripheral blood mononuclear cells (PBMC) were prepared from whole blood of healthy volunteers obtained from the Department of Transfusion Medicine at the National Institutes of Health by density gradient separation on lymphocyte separation medium (Lonza) and incubated at 37 °C overnight in 10% FCS in RPMI 1640 medium supplemented with 20 mM Hepes (pH 7.4), penicillin-streptomycin (all Invitrogen), and 100 units/mL human IL-2 (PeproTech).

(*ii*) *Preparation of Target Cells*. JeKo-1 cells were plated at a density of 1×10^5 cells per well in a 96-well round-bottom plate (Corning) in 5% FCS in RPMI 1640 medium supplemented with 15 mM Hepes (pH 7.4) and penicillin-streptomycin. The cells were incubated with 10 μg/mL rituximab-based IgG-Sec-His, Rituxan, or nonspecific polyclonal human IgG (Pierce) for 30 min at 37 °C. Unbound antibody was removed by aspiration.

(*iii*) *Combination of Effector and Target Cells*. The prepared target cells (T) were incubated with the prepared effector cells (E) in 5% FCS in RPMI 1640 supplemented with 15 mM Hepes (pH 7.4) and penicillin-streptomycin for 4 h at 37 °C at E:T ratios of 1:1 and 20:1. Target cell lysis was assessed by the release of lactate dehydrogenase using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's protocol.

Complement-Dependent Cytotoxicity (CDC) Assay. Aliquots of 50 μL containing 5×10^5 Raji cells were prepared as described above and distributed into a V-bottom 96-well plate. After incubation with 0.6 μM Rituxan, rituximab-based IgG-Sec-His/fluorescein, or Fab-Sec-His/biotin for 1 h on ice, the cells were washed twice with 1% (v/v) FCS/PBS and incubated with 10% complement from 3−4-week-old rabbits (Pel-Freez) for 2 h at 37 °C. After the addition 100 μg/mL propidium iodide, dead cells were detected by propidium iodide accumulation using a FACScan instrument (Becton-Dickinson).

In Vivo Study. The mouse study was conducted by Biocon, Inc. (Rockville, MD), in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. Three groups of two or three adult

C57BL/6 mice each were injected intravenously (tail vein) with 100 μL of 3 mg/mL Fab-Sec-His/PEG-biotin (two mice), Fab-stop (three mice), or Rituxan (three mice) in PBS. Sera from retro-orbital bleeds were prepared 30 min, 24 h, 48 h, 72 h, and 96 h after injection. Sera were diluted 10-fold in 1% (v/v) FCS/PBS and analyzed by flow cytometry using Raji cells and Cy5-coupled goat anti-human $F(ab')_2$ polyclonal antibodies (Jackson ImmunoResearch) or PE-coupled streptavidin as described above. For the sandwich ELISA, 30 min and 24 h sera from the Fab-Sec-His/PEG-biotin and Fab-stop groups were diluted 1:100 in 1% (v/v) BSA/TBS, captured with goat anti-human $F(ab')_2$ polyclonal antibodies (Jackson ImmunoResearch Laboratories) coated at a density of 200 ng/well, and detected with HRP-coupled goat anti-human κ light chain polyclonal antibodies (SouthernBiotech) diluted at 1:1000 in 1% BSA/TBS. Blocking, washing, and colorimetric detection were conducted essentially as described above. The difference between signals (mean $\pm$ the standard deviation of triplicates) obtained from 30 min and 24 h sera of individual mice was calculated.

RESULTS

Cloning, Expression, and Purification of Rituximab-Based IgG-Sec-His and Fab-Sec-His. We previously reported a mammalian cell expression system for the generation of a recombinant Fc protein with a C-terminal Sec followed by a His tag (Fc-Sec-His) (*4*). The corresponding mammalian cell expression vector consisted of an exon−intron gene sequence encoding the human IgG1-derived Fc fragment fused to a TGA codon, followed by a His_6-encoding sequence, a TAA stop codon, and a SECIS element from the 3′-untranslated region of the cDNA of human thioredoxin reductase 1. To convert the Fc-Sec-His cassette to an IgG-Sec-His cassette, we replaced the Fc-encoding sequence in the previously published (*5*) mammalian cell expression vector PIGG with the Fc-Sec-His-encoding sequence (Figure 1A). As a prototype for this study, a newly generated PIGG vector was used that contained heavy and light chain variable domain encoding sequences of the chimeric mouse/human anti-human CD20 mAb rituximab. Designated PIGG-rituximab-Sec-His, the vector was transiently transfected into HEK 293F cells that were maintained in suspension in serum-free medium supplemented with 1 μM sodium selenite (Na_2SeO_3). Analogous to Fc-Sec-His, rituximab-based IgG-Sec-His protein was purified from the supernatant by a tandem column chromatography process (*4*). Protein G affinity chromatography was used to purify the total IgG protein, followed by IMAC to separate IgG-Sec-His (the product of Sec insertion at UGA) from IgG-stop protein (the product of termination at UGA). IgG-stop protein is an inevitable byproduct of our mammalian cell expression system as termination at the UGA codon typically dominates Sec insertion and read-through, despite the presence of a SECIS element (Figure 1B) (*7*). Although the yield of total protein was 5-fold lower for rituximab-based IgG (2 mg/L) than what we previously reported for Fc (10 mg/L) (*4*), it was comparable to those of other chimeric mouse/human IgGs we have conventionally expressed (without C-terminal Sec) in this system. Interestingly, the ratio of IgG-Sec-His to IgG-stop (1:1 to 1:2) was found to be substantially higher than the ratio of Fc-Sec-His to Fc-stop (1:4). Thus, higher incorporation rates of Sec may be favored by lower levels of protein expression, indicating the limiting influence of *cis* and *trans* factors (*7*, *8*) in the Sec insertion machinery of HEK 293F cells.

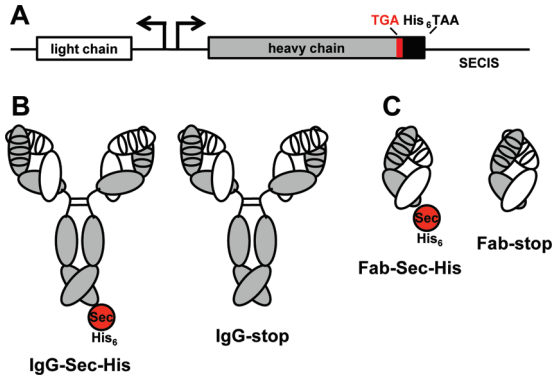

FIGURE 1: IgG-Sec-His and Fab-Sec-His engineering. (A) For the expression of rituximab-based IgG-Sec-His, the sequences of the light chain (white) and the heavy chain (gray) were cloned downstream from the engineered bidirectional CMV promoter cassette in the previously described (*5*) mammalian cell expression vector PIGG. For the expression of the corresponding Fab-Sec-His, the sequences encoding heavy chain constant domains C_H2 and C_H3 were deleted. In both constructs, a C-terminal Sec was introduced by genetically combining a TGA codon (red) with a SECIS element derived from the 3′-untranslated region of human thioredoxin reductase 1 cDNA. In the proximity of a SECIS element, the TGA codon (UGA in the transcribed mRNA) instructs the translational insertion of the 21st natural amino acid Sec. A His_6-encoding sequence followed by stop codon TAA was inserted between the TGA codon and SECIS element. Because termination at the UGA codon competes with and typically dominates Sec insertion, a mixture of IgG-stop and IgG-Sec-His proteins (B) or Fab-stop and Fab-Sec-His proteins (C) was expressed. Note that the IgG-Sec-His fraction is a statistical mixture of a larger proportion of heterogeneous dimers that display one Sec residue (shown) and a smaller proportion of homogeneous dimers with two Sec residues (not shown). The His tag enabled detection and purification of the IgG-Sec-His and Fab-Sec-His fractions. The six complementarity-determining regions, three provided by each variable domain, are shown as ovals and mark the antigen binding site on the opposite end of the antibody molecules.

In addition to IgG-Sec-His, we also developed a mammalian cell expression system for the generation of Fab-Sec-His (Figure 1C), essentially by deleting the exon−intron gene sequence encoding the human IgG1-derived Fc fragment in PIGG-rituximab-Sec-His. The engineered C-terminal sequence encoded a shortened IgG1 hinge region with Sec replacing the second Cys that normally forms one of two interchain disulfide bridges between the heavy chains. The first Cys that is involved in the only interchain disulfide bridge between the heavy and light chain was conserved. The shortened vector, designated PIGG-rituximab-Sec-His, was transiently transfected into HEK 293F cells as described previously. The total Fab protein consisting of rituximab-based Fab-Sec-His and Fab-stop was first purified by affinity chromatography using goat anti-human Fab polyclonal antibodies and then separated by IMAC. The total yield of protein was 1 mg/L with a Fab-Sec-His to Fab-stop ratio of 1:1 to 1:2.

Selective Conjugation. To demonstrate that the engineered Sec displays unique chemical reactivity allowing selective conjugation, rituximab-based IgG-Sec-His and Fab-Sec-His were probed with commercially available biotin-iodoacetamide and biotin-maleimide compounds using our previously established mildly acidic and reducing conjugation conditions (*4*). As summarized in Table 1, ELISA and Western blotting demonstrated that rituximab-based IgG-Sec-His and Fab-Sec-His were biotinylated. By contrast, Rituxan and rituximab-based IgG-stop and Fab-stop remained nonbiotinylated under these conditions.

Conjugation of IgG-Sec-His and Fab-Sec-His appeared to be as selective as the quantitatively analyzed conjugation of Fc-Sec-His (*4*). Nonreducing SDS−PAGE followed by Coomassie staining revealed that the molecular integrity of the tetrameric 150 kDa IgG and the dimeric 50 kDa Fab protein was preserved following selective conjugation at the Sec interface (data not shown). We next investigated whether selective conjugation at the Sec interface influenced antigen binding. Using FITC-coupled goat anti-human Fab polyclonal antibodies for detection, equimolar concentrations of rituximab-based IgG-Sec-His/biotin and Fab-Sec-His/biotin were compared to Rituxan with respect to their binding to the human CD20+ B cell lines Raji (Figure 2A) and JeKo-1 (data not shown) by flow cytometry. No difference in binding was detected for rituximab-based IgG-Sec-His/biotin and Rituxan, indicating that neither conjugation conditions nor conjugation at the Sec interface affected antigen binding. As expected, a somewhat weaker binding was observed for rituximab-based Fab-Sec-His/biotin, likely reflecting its lower avidity (*9*). As confirmation that cell surface binding was mediated through CD20, rituximab-based IgG-Sec-His/biotin could be competed with Rituxan but not with the humanized anti-human CD52 mAb Campath (Figure 2B). Using a commercially available fluorescein-maleimide compound, we also demonstrated that rituximab-based IgG-Sec-His can be directly and selectively labeled with a fluorescent tag that does not impair antigen binding and facilitates flow cytometry without secondary reagents (Figure 2C).

Table 1: Summary of Selective Biotinylation at the Sec Interface[a]

biological component	chemical component	reactivity with Strep-HRP[b]	reactivity with α hIgG-HRP[c]
rituximab-based IgG-Sec-His	biotin-iodoacetamide	+[d]	+
rituximab-based IgG-stop	biotin-iodoacetamide	−	+
Rituxan	biotin-iodoacetamide	−	+
rituximab-based IgG-Sec-His	biotin-maleimide	+	+
rituximab-based IgG-stop	biotin-maleimide	−	+
Rituxan	biotin-maleimide	−	+
rituximab-based Fab-Sec-His	biotin-maleimide	+	+
rituximab-based Fab-stop	biotin-maleimide	−	+

[a]Reaction conditions: 4 μM biological component incubated with 40 μM chemical component in 100 mM sodium acetate (pH 5.2) and 0.1 mM DTT for 1−2 h at room temperature. [b]As determined by ELISA and Western blotting using HRP-coupled streptavidin. [c]As determined by ELISA and Western blotting using HRP-coupled donkey anti-human IgG polyclonal antibodies. [d]Indicates ELISA and Western blotting signals at least 3-fold above background.

Although selective conjugation at the Sec interface did not influence antigen binding, it remained to be shown whether various protein interactions mediated by the Fc domain of the IgG1 molecule were also preserved. Compared to the antigen binding site, binding sites on the Fc domain for FcRn, Fcγ receptors, and complement protein C1q are in the proximity of the Sec interface (Figure 1B). Given the importance of the FcRn in IgG recycling and transcytosis across endothelial and epithelial barriers (*10*), we first compared the pH-dependent binding of various concentrations of rituximab-based IgG-Sec-His/biotin and IgG-stop to immobilized recombinant human FcRn by ELISA (Figure 3). Both bound equally well to FcRn at pH 6.0, but only weakly at pH 7.4. The same was found for Rituxan and rituximab-based IgG-Sec-His/fluorescein (data not shown). The conserved pH-dependent binding of IgG conjugates to FcRn confirmed our previous findings that were based on Fc conjugates (*4*).

ELISA was also used to compare the binding of rituximab-based IgG-Sec-His/biotin and Rituxan (which are both of the IgG1 isotype) to immobilized human Fcγ receptors FcγRI (CD64), FcγRIIA (CD32a), and FcγRIIIA (CD16a) (Figure 4A). The high-affinity IgG1−FcγRI and the intermediate-affinity IgG1−FcγRIIIA interactions were readily detectable in this assay and did not reveal any difference between rituximab-based IgG-Sec-His/biotin and Rituxan. As expected, the low-affinity

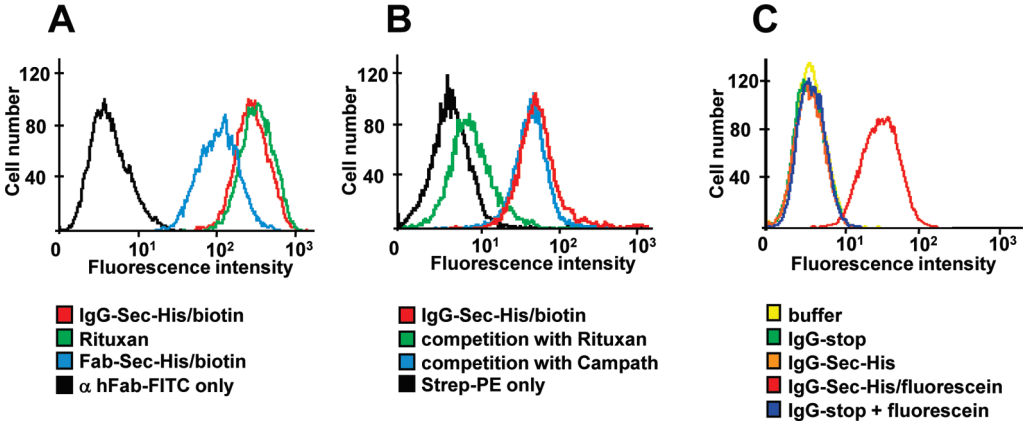

FIGURE 2: Conserved antigen binding of selectively conjugated IgG-Sec-His and Fab-Sec-His. (A) Flow cytometry analysis of the binding of equimolar concentrations of rituximab-based IgG-Sec-His/biotin (red) and Fab-Sec-His/biotin (blue) to human CD20+ Raji cells in comparison to Rituxan (green). FITC-coupled goat anti-human Fab polyclonal antibodies (α hIgG-FITC) were used for detection. The *y*-axis gives the cell number in linear scale and the *x*-axis the fluorescence intensity in logarithmic scale. (B) Flow cytometry analysis of the binding of rituximab-based IgG-Sec-His/biotin to Raji cells that were preincubated with Rituxan (green), Campath (blue), or buffer alone (red). PE-coupled streptavidin (Strep-PE) was used for detection. (C) Binding of rituximab-based IgG-Sec-His/fluorescein (red) to Raji cells and its direct detection by flow cytometry without secondary reagents. Unconjugated rituximab-based IgG-Sec-His (orange) and IgG-stop (green) as well as IgG-stop that had been incubated with fluorescein-maleimide under our mildly acidic and reducing conditions (blue) did not reveal any staining above background (yellow).

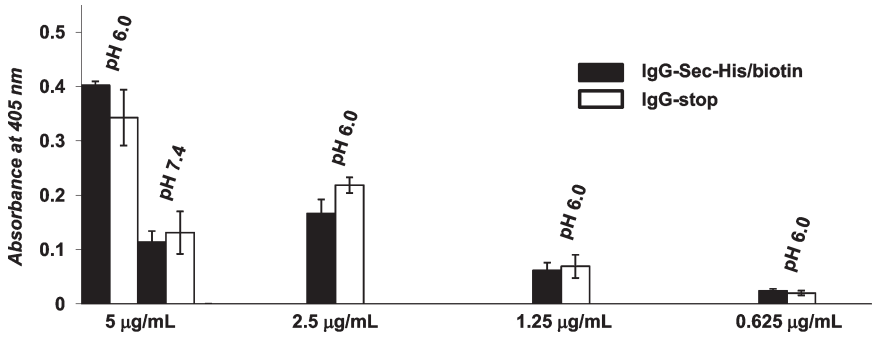

FIGURE 3: Conserved FcRn binding of selectively conjugated IgG-Sec-His. Analysis of rituximab-based IgG-Sec-His/biotin and IgG-stop for binding to immobilized recombinant human FcRn at pH 6.0 and 7.4 by ELISA using HRP-coupled goat anti-human Fab polyclonal antibodies for detection. Shown are mean ± standard deviation values after background depletion (n = 3).

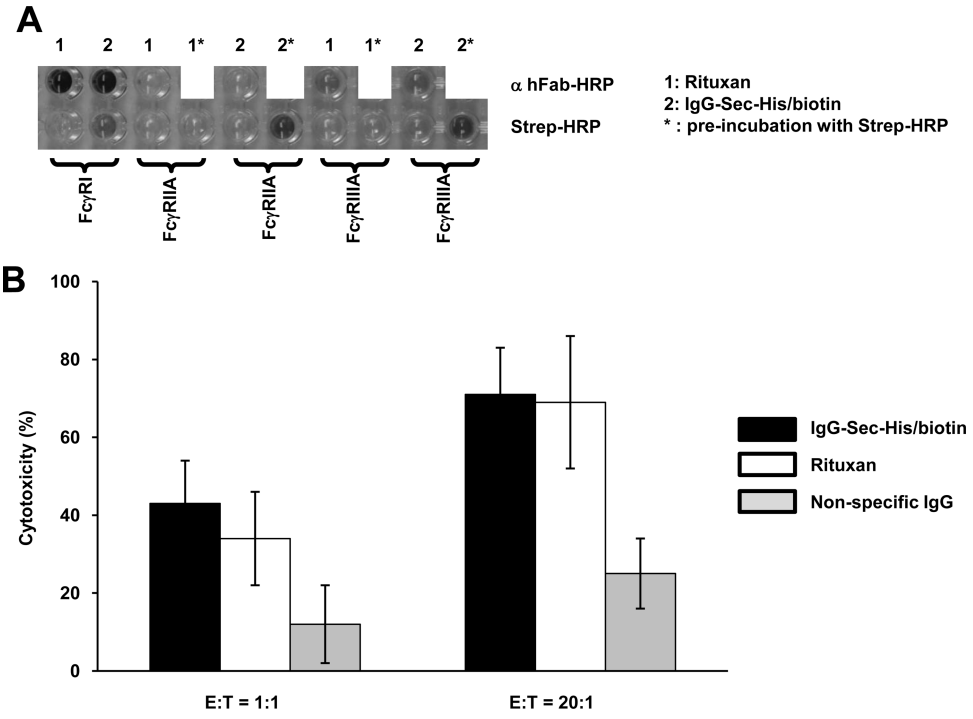

FIGURE 4: Fc functionality of selectively conjugated IgG-Sec-His. (A) Following selective biotinylation at the Sec interface, Rituxan (1) and rituximab-based IgG-Sec-His/biotin (2) were analyzed for binding to immobilized recombinant human FcγRI, FcγRIIA, and FcγRIIIA by ELISA using HRP-conjugated goat anti-human Fab polyclonal antibodies (top row) and HRP-coupled streptavidin (bottom row). Preincubation (asterisk) with HRP-coupled streptavidin increased the avidity of rituximab-based IgG-Sec-His/biotin but not Rituxan. (B) ADCC mediated by rituximab-based IgG-Sec-His/biotin, Rituxan, and nonspecific polyclonal human IgG (from left to right) was measured by lactate dehydrogenase release using JeKo-1 cells as target cells (T) and human PBMC as effector cells (E) at E:T ratios of 1:1 (left) and 20:1 (right). Lactate dehydrogenase release is proportionate to target cell lysis and shown as percent cytotoxicity with mean ± standard deviation values after background depletion (n = 3).

IgG1−FcγRIIA interaction was not detectable. FcγRIIA and FcγRIIIA are expressed on macrophages and NK cells and play a key role in mediating antibody effector functions through phagocytosis and antibody-dependent cellular cytotoxicity (ADCC), respectively (*11*). The low to intermediate affinity of FcγRIIA and FcγRIIIA defines their preferred recognition of IgG1 aggregates surrounding multivalent antigens over recognition of monomeric IgG1. To mimic IgG1 aggregation, we incubated rituximab-based IgG-Sec-His/biotin with streptavidin. On the basis of the monomeric biotin display of rituximab-based IgG-Sec-His/biotin and the four biotin binding sites of streptavidin, the resulting complex was expected to be a molecularly defined IgG1 aggregate with an increased avidity for FcγRIIA and FcγRIIIA. In fact, our ELISA revealed the strong binding of a mixture of rituximab-based IgG-Sec-His/biotin and streptavidin to FcγRIIA and FcγRIIIA (Figure 4A). No binding was detected for a control mixture of (nonbiotinylated) Rituxan and streptavidin. The conserved features of binding of rituximab-based IgG-Sec-His/biotin to human Fcγ receptors suggested that conjugation at the Sec interface does not impair ADCC. To show this directly, we performed ADCC assays using human PBMC as effector cells and JeKo-1 cells as target cells. Rituximab-based IgG-Sec-His/biotin was found to mediate ADCC as potently as Rituxan (Figure 4B), confirming the Fc functionality of selectively conjugated IgG-Sec-His.

Another effector function mediated by the Fc domain of the IgG1 molecule is complement-dependent cytotoxicity (CDC). Like ADCC, CDC is a mechanism that contributes to the antitumor activity of therapeutic mAbs, including rituximab (*12*, *13*). To test whether conjugation at the Sec interface influences CDC, Raji cells were incubated with rituximab-based IgG-Sec-His/fluorescein followed by rabbit complement. Cell death was

quantified by propidium iodide staining using flow cytometry. As shown in Table 2 for a typical experiment, rituximab-based IgG-Sec-His/fluorescein had a potency similar to that of Rituxan in mediating CDC. Both antibodies required the presence of complement to induce cell death. By contrast, rituximab-based Fab-stop, which does not contain an Fc domain, was found to be incapable of mediating CDC. Taken together, neither conjugation conditions nor conjugation at the Sec interface was found to impact effector functions mediated by the Fc domain of the IgG1 molecule. This suggests that Sec interface technology can be applied to therapeutic mAbs without impairing their common mode of action.

His Tag Omission. Although we have shown in this study and our previous study (*4*) that adding a His tag provides a convenient device for purifying recombinant proteins with C-terminal Sec, its tendency to promote protein aggregation, elicit immunogenicity, and sequester potentially toxic metal ions is a concern for certain applications. In addition, it may not be necessary to separate IgG with and without C-terminal Sec prior to conjugation. To investigate the feasibility of expressing, purifying, and selectively conjugating IgG-Sec proteins without a His tag, we cloned a mammalian cell expression vector otherwise identical to PIGG−rituximab-Sec-His but without the His_6-encoding sequence. The mixture of IgG-Sec and IgG-stop protein was purified from supernatants of transiently transfected HEK 293F cells by Protein G affinity chromatography.

Table 2: CDC Measured by Flow Cytometry[a]

antibody	complement	cytotoxicity (%)
none	−	9.4
none	+	7.2
Rituxan	−	20.3
Rituxan	+	90.1
rituximab-based IgG-Sec-His/fluorescein	−	17.7
rituximab-based IgG-Sec-His/fluorescein	+	79.6
rituximab-based Fab-stop	−	9.3
rituximab-based Fab-stop	+	13.3

[a]Raji cells were incubated with the indicated components, and dead cells were quantified by propidium iodide staining. The outcome of a typical experiment is shown.

In addition to enabling detection, biotin can also serve as a handle for protein purification by affinity chromatography using monomeric avidin or monomeric streptavidin resins that bind biotin reversibly (*14*−*16*). To demonstrate that a mixture of proteins with and without C-terminal Sec could be separated following selective biotinylation, the purified mixture of rituximab-based IgG-Sec (without the His tag) and IgG-stop was subjected to selective conjugation to biotin-maleimide followed by monomeric avidin affinity chromatography. Subsequent analyses by Western blotting (Figure 5A) and flow cytometry (Figure 5B) revealed efficient (>99%) separation of IgG-Sec/biotin (eluate) and IgG-stop (flow-through). Western blotting analysis also confirmed selective conjugation at the Sec interface as reflected by a biotinylated heavy chain and a nonbiotinylated light chain band (Figure 5A). Flow cytometry analysis using PE-coupled streptavidin showed that purified rituximab-based IgG-Sec/biotin binds to Raji cells as efficiently as rituximab-based IgG-Sec-His/biotin (Figure 5B). Collectively, these findings suggest that a His tag is convenient but not necessary for the separation of mixtures of proteins with and without the C-terminal Sec as long as the conjugated compound contains a handle for protein purification. Thus, trifunctional compounds that consist of (i) a functional core linked to (ii) a biotin group for detection and purification and (iii) an electrophilic moiety for conjugation are of particular utility in Sec interface technology (*4*, *17*).

PEGylation. As an advance beyond proof of concept, we next investigated the selective conjugation of PEG to rituximab-based Fab-Sec-His with the goal of increasing its circulatory half-life without impairing antigen binding. PEGylated Fab have

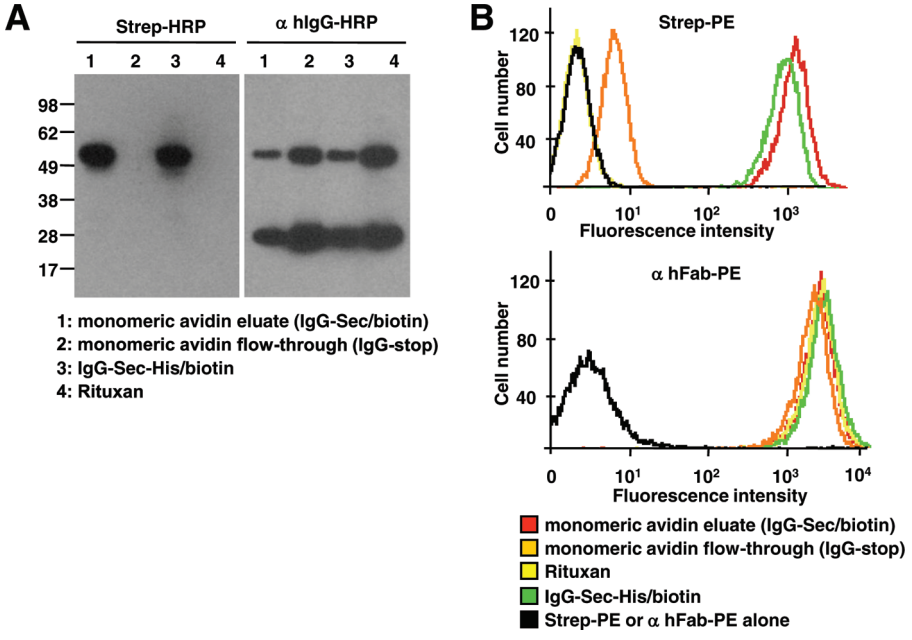

FIGURE 5: Monomeric avidin-mediated purification of IgG-Sec/biotin without the His tag. Following Protein G affinity chromatography, a mixture of rituximab-based IgG-Sec (without the His tag) and IgG-stop was selectively biotinylated and separated by monomeric avidin affinity chromatography. Eluate and flow-through were analyzed by (A) Western blotting and (B) flow cytometry using Raji cells and the indicated secondary reagents. Rituximab-based IgG-Sec-His/biotin and Rituxan served as controls. Note that only the heavy chain (~50 kDa) of IgG-Sec/biotin and IgG-Sec-His/biotin was biotinylated, confirming selective conjugation at the Sec interface.

become a therapeutically relevant competitor of IgG, particularly in indications that do not require or desire Fc-mediated antibody effector functions (*18*). Previously, Fab have been PEGylated randomly through accessible Lys residues or more selectively through Cys residues following reduction. It was found that random PEGylation typically results in a substantial loss of antigen binding whereas selective PEGylation at the C-terminus generally retains antigen binding (*19*). On the basis of the greater nucleophilic reactivity of the selenol group compared to the thiol group, utilizing a C-terminal Sec instead of Cys residue can be anticipated to enhance both selectivity and efficacy of Fab PEGylation.

Using our selective conjugation conditions, purified rituximab-based Fab-Sec-His was reacted with a commercially available 7.5 kDa biotin-PEG-maleimide compound. Because of its large hydrodynamic volume, the 7.5 kDa PEG was anticipated to increase the functional size of the Fab molecule by a factor of at least 2. Size-exclusion chromatography following selective conjugation resulted in the separation of two protein fractions, indicated by peak 1 and peak 2 in a ratio of approximately 2:1 (Figure 6A). By contrast, rituximab-based Fab-stop, subjected to the same conjugation conditions in the presence of biotin-PEG-maleimide, revealed only peak 2 (Figure 6A). This suggested that peak 1 contained the rituximab-based Fab-Sec-His/PEG-biotin fraction and peak 2 contained the unconjugated Fab fraction. This was confirmed by flow cytometry, using the concomitantly conjugated biotin group for detection. As shown in Figure 6B, peak 1, but not peak 2, revealed strong binding to Raji cells. As previously observed for rituximab-based Fab-Sec-His/biotin and attributed to lower avidity (Figure 2A), the binding of Fab-Sec-His/PEG-biotin was found to be somewhat weaker than the binding of IgG-Sec-His/biotin. The remaining weak binding activity in peak 2, which was slightly above the background defined by negative control conjugate Fc-Sec-His/PEG-biotin (Figure 5B), can be explained by an incomplete separation of peak 1 and peak 2. The presence of rituximab-based Fab in both peak 1 and peak 2 was confirmed by flow cytometry (Figure 6B) and Western blotting (data not shown), using donkey anti-human Fab polyclonal antibodies for detection.

The selective and efficient PEGylation of rituximab-based Fab-Sec-His allowed us to investigate whether PEGylation extended the circulatory half-life of the Fab molecule. For this purpose, 300 μg of rituximab-based Fab-Sec-His/PEG-biotin, Fab-stop, or Rituxan was injected intravenously into C57BL/6 mice. Sera prepared from retro-orbital bleeds were collected after 30 min and every 24 h for 4 days and analyzed for binding to Raji cells by flow cytometry (Figure 7A). As expected, and in agreement with a previously reported half-life of 199 h for human IgG1 in SCID mice (*20*), Rituxan revealed only marginal weakening over the course of the experiment. Although the signals obtained for both Fab preparations indicated much shorter circulatory half-lives, the 7.5 kDa PEG group was found to delay the clearance of the Fab molecule. Whereas the difference was most pronounced 30 min after intravenous injection, Fab-Sec-His/PEG-biotin was still detectable at the 24 h time point in contrast to Fab-stop (Figure 7A). Delayed clearance was confirmed by a sandwich ELISA in which the difference of Fab retention between the 30 min and 24 h time points was compared for sera from two mice treated with rituximab-based Fab-Sec-His/PEG-biotin and three mice treated with rituximab-based Fab-stop (Figure 7B). The retained Fab-Sec-His/PEG-biotin conjugate was detectable with both donkey anti-human Fab

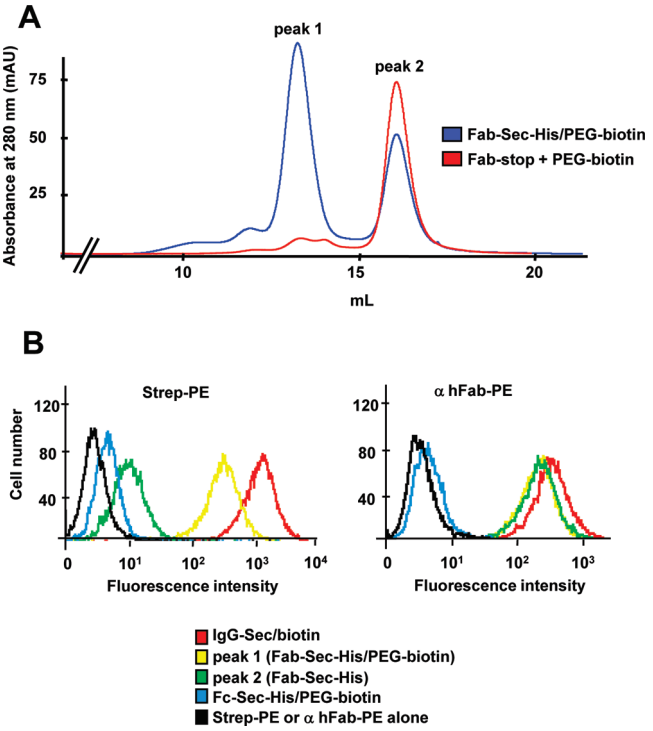

FIGURE 6: In vitro characterization of selectively PEGylated Fab-Sec-His. (A) After reaction with a commercially available 7.5 kDa biotin-PEG-maleimide compound, conjugated (peak 1) and unconjugated (peak 2) rituximab-based Fab-Sec-His were separated by size-exclusion chromatography (blue line). Rituximab-based Fab-stop subjected to the same conjugation and separation conditions was included for the sake of comparison (red line). (B) The separated fractions were analyzed by flow cytometry using Raji cells and the indicated secondary reagents. Rituximab-based IgG-Sec-His/biotin and Fc-Sec-His/PEG-biotin served as controls.

polyclonal antibodies (Figure 7A,B) and streptavidin (data not shown), confirming the previously noted stability of C-terminal Sec conjugates in vivo (*4*).

DISCUSSION

We previously reported the generation of an IgG1-derived Fc fragment with a C-terminal Sec as a molecularly defined conjugation site between biological and chemical components (*4*). Here we investigate the suitability of a C-terminal Sec for the generation of molecularly defined IgG and Fab conjugates. A Sec interface would provide a unique platform for the generation of a variety of antibody conjugates with broad utility for the imaging, diagnosis, prevention, and therapy of human diseases. For example, antibody−drug conjugates (*21*) and radioimmunoconjugates (*22*), which constitute one-third of FDA-approved mAbs for cancer therapy (*23*), have been shown to enhance the potency of mAbs while preserving their specificity.

All FDA-approved and most investigational antibody conjugates in clinical trials are based on random conjugation chemistries involving the ε-amino group of Lys residues or the thiol group of Cys residues. By contrast, the unique chemical reactivity provided by the selenol group of Sec would allow the production of antibody conjugates with defined stoichiometry and limited batch-to-batch variability. Other approaches, including one in clinical trials (*24*, *25*), also exploit or engineer unique chemical reactivity for the generation of defined antibody conjugates (*26*, *27*). On the basis of preclinical animal models, it was recently shown that the site-specific conjugation of drugs to

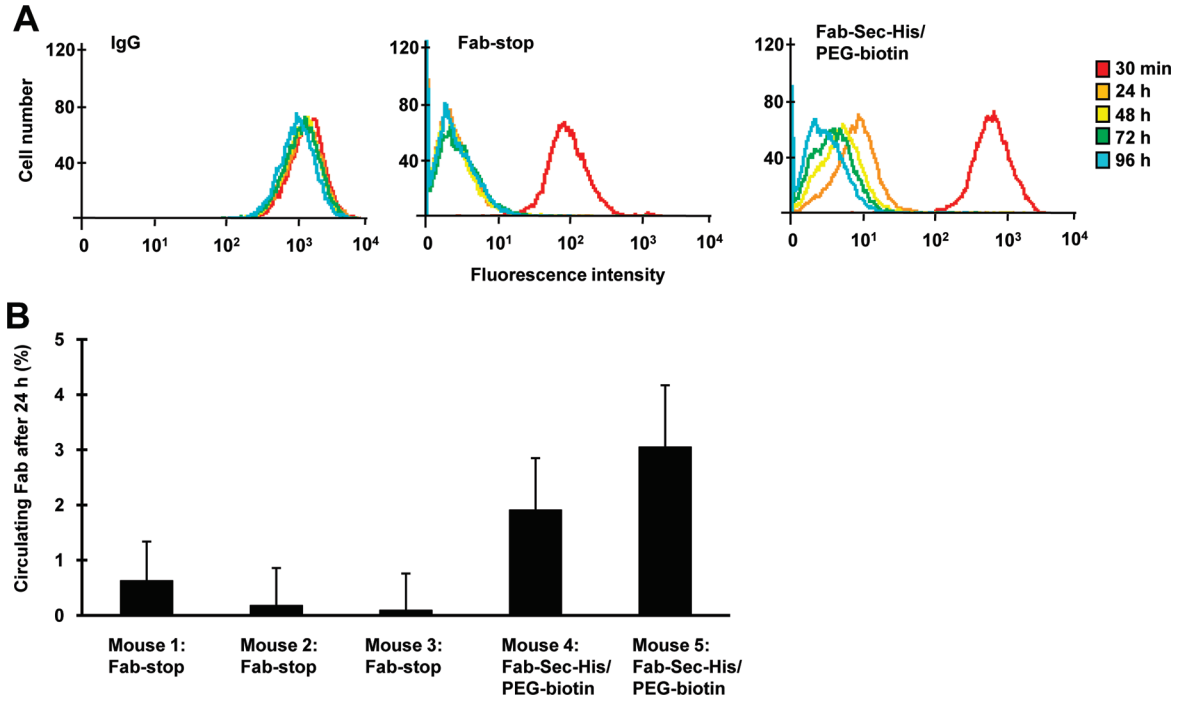

FIGURE 7: In vivo characterization of selectively PEGylated Fab-Sec-His. (A) Comparison of the circulatory half-life of Rituxan (left), rituximab-based Fab-stop (middle), and Fab-Sec-His/PEG-biotin (right). Sera from C57BL/6 mice treated intravenously with 300 μg of protein were prepared from retro-orbital bleeds taken 30 min (red), 24 h (orange), 48 h (yellow), 72 h (green), and 96 h (blue) after injection; 10-fold diluted sera were analyzed by flow cytometry using Raji cells and Cy5-coupled goat anti-human $F(ab')_2$ polyclonal antibodies. Typical results based on two to three individual mice in each treatment group are shown. (B) Sera from all five mice treated with Fab-stop (mouse 1, 2, or 3) or Fab-Sec-His/PEG-biotin (mouse 4 or 5) were analyzed by a sandwich ELISA using goat anti-human $F(ab')_2$ polyclonal antibodies for capturing and HRP-coupled goat anti-human κ light chain polyclonal antibodies for detection. The difference between signals found at 24 and 30 min time points is given as the percentage of circulating Fab after 24 h with mean $\pm$ standard deviation values ($n = 3$).

mAbs with engineered Cys residues can lower the systemic toxicity of antibody−drug conjugates while preserving their therapeutic efficacy (*28*, *29*). Site-specific conjugation to mAbs with an engineered Sec residue provides additional advantages over mAbs with engineered (*28*, *30*, *31*) or confined (*32*) Cys residues. First, compared to the thiol group, the selenol group has higher nucleophilic reactivity, permitting higher (>90%) conjugation efficacy (*4*). Second, the conjugation of engineered Cys residues requires their activation through reduction, dialysis, and oxidation steps to preserve the four interchain and 12 intrachain disulfide bridges in the IgG1 molecule (*28*). By comparison, our antibody conjugates are anticipated to simplify manufacturing because the engineered Sec residue is readily available for conjugation in a one-step reaction under mildly reducing and acidic reaction conditions that preserves native disulfide bridges. Third, due to the fact that termination at the UGA codon typically dominates Sec insertion (*7*), the majority of Fc-Sec-His (*4*) and IgG-Sec-His proteins from our mammalian cell expression system are anticipated to display a single Sec residue in an otherwise dimeric protein. This facilitates the generation of antibody−drug conjugates with unique stoichiometry, i.e., one drug molecule per antibody molecule, potentially further improving the therapeutic index and pharmacokinetic profile (*33*).

Interestingly, the ratio of Sec insertion (yielding IgG-Sec-His) and termination (yielding IgG-stop) at the UGA codon was found to be substantially higher (1:1 to 1:2) than what we previously reported for Fc-Sec-His and Fc-stop (1:4). This higher ratio appeared to correlate inversely with the yield of total protein in our mammalian cell expression system based on rituximab-based IgG-Sec-His, IgG-Sec, Fab-Sec-His (this report), and IgG-Sec-His derivatives of other chimeric, humanized, and human mAbs (unpublished data). Thus, the lower protein expression yields of total IgG and total Fab compared to total Fc may be, to some extent, compensated by higher Sec insertion ratios. A further increase in the Sec insertion ratio may be possible through identifying and compensating the limiting influence of both *cis* and *trans* factors of the Sec insertion machinery (*34*). In theory, a higher Sec insertion ratio also increases the proportion of IgG-Sec-His proteins with two Sec residues over IgG-Sec-His proteins with a single Sec residue, i.e., from 1% (1:4 ratio) to 3% (1:2 ratio) and 9% (1:1 ratio) of total protein, assuming a binomial distribution ($p + q = 1$) with free pairing of the heavy chains ($p^2 + 2pq + q^2 = 1$). Whether this small fraction of IgG-Sec-His proteins with two Sec residues can mediate dual, single, or no conjugation events may depend on the formation of a corresponding diselenide bridge (*35*) and its stability under our mildly reducing and acidic reaction conditions. We anticipate, but have not yet shown, that higher expression levels in stable cell lines will lower both the Sec insertion ratio and the potential heterogeneity of IgG-Sec-His proteins and conjugates.

Using rituximab as a prototype mAb, we showed that IgG-Sec with and without the His tag as well as Fab-Sec-His proteins can be expressed and purified in sufficient yields for preclinical investigations. Selective and efficient conjugation at the Sec interface was demonstrated with maleimide derivatives of biotin and fluorescein. The resulting conjugates were found to have retained their antigen binding capability and, in the case of IgG, their ability to mediate ADCC and CDC. Remaining challenges for clinical applications that need to be addressed are large-scale expression and purification of the proteins from stable cell lines and the potential immunogenicity of the conjugates.

Site-specific biotinylation of IgG and Fab constitutes a potentially important advancement for proteomic applications, in particular, antibody arrays (*36*), which still suffer from chip-to-chip variability due to heterogeneous antibody conjugation and antigen binding. Likewise, the site-specific conjugation of IgG and Fab to fluorescein and other fluorophores or chromophores could overcome the batch-to-batch variability of mAb reagents that are increasingly used for imaging and diagnosis in basic, preclinical, and clinical applications. In addition, our antibody conjugates establish a proof of principle for the generation of molecularly defined antibody−drug conjugates and radioimmunoconjugates. Maleimide derivatives of cytotoxic drugs and chelates commonly used for antibody−drug conjugates (*37*, *38*) and radioimmunoconjugates (*22*), respectively, are readily available for conjugation to the Sec interface. Another potential application of our Sec interface technology is the generation of molecularly defined bispecific reagents.

In addition to antibody−drug conjugates and radioimmunoconjugates, PEGylated mAbs, in particular mAb fragments in the Fab format, have become clinically relevant competitors of unconjugated mAbs in certain applications. PEGylation can increase the solubility, stability, and circulatory half-life and decrease the immunogenicity and toxicity of biologics in general (*39*) and mAbs and mAb fragments in particular (*19*, *40*). A recently FDA-approved PEGylated Fab, certolizumab pegol (Cimzia) (*41*) for the treatment of Crohn's disease and rheumatoid arthritis, is an anti-human TNFα humanized Fab with a C-terminal Cys residue expressed in *Escherichia coli* and conjugated to ∼40 kDa PEG. The extended circulatory half-life of certolizumab pegol has allowed a recommended maintenance dosing frequency of two 200 mg subcutaneous injections every 4 weeks. To test whether Sec interface technology would provide an alternative route for generating PEGylated Fab, we used a trifunctional biotin-PEG-maleimide with a molecular mass of 7.5 kDa for proof of concept. We demonstrated that rituximab-based Fab-Sec-His retains its antigen binding capability after selective PEGylation and purification by size-exclusion chromatography. In vivo, the PEGylated Fab revealed an extended circulatory half-life compared to that of the unconjugated Fab. Given the relatively low molecular mass of the PEG compound (7.5 kDa), it is not surprising that the circulatory half-life was still much shorter than what we measured for the corresponding unconjugated IgG. It is conceivable, however, that PEG compounds with higher molecular masses, such as those used for certolizumab pegol (40 kDa), could be used to generate Fab-Sec-His conjugates with circulatory half-lives that match or exceed that of IgG as has been demonstrated for Fab conjugates (*40*). Although improved methods (*42*) have allowed more selective utilization of the C-terminal Cys for the PEGylation of Fab, our Sec interface technology provides the advantage of high conjugation efficacy in one step under mildly reducing and acidic reaction conditions that preserve the only interchain disulfide bridge in the Fab molecule.

In summary, Sec interface technology provides a new method for the generation of molecularly defined antibody conjugates with distinctive stoichiometry. Previous efforts to exploit the unique chemical reactivity of Sec in recombinant proteins have been confined to *E. coli* expression and are therefore generally not suitable for whole antibody molecules and other large or glycosylated proteins (*43*). As our method is amendable to mAbs in IgG, Fab, and virtually any alternative antibody format (*44*), it affords broad utility ranging from proteomic applications to therapeutic intervention.

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