Expression vector-derived heterogeneity in a therapeutic IgG4 monoclonal antibody

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ABSTRACT

While characterizing a therapeutic IgG4 monoclonal antibody (mAb), we observed a variant with a mass 1177 Da larger than the predominant mAb form that could not be ascribed to previously described modifications. Through successive rounds of experimentation, we localized the mass addition to the C-terminus of the heavy chain (HC). During this process we observed that when the mAb was broken down into separate domains, the Fc and the 1177 Da-modified Fc could be chromatographically separated. Separation allowed collection of native and modified Fc fractions for LC/MS peptide mapping. A unique peptide present in the modified fraction was de novo sequenced and demonstrated to be a modified form of the HC C-terminus lacking two native residues (GK) and gaining twelve additional non-native residues (EAEAASASELFQ). Aware of other mAb variants with genetic origins, we sought to understand whether this modification too had a genetic basis. In silico translation of the expression vector encoding the mAb demonstrated that a normally non-coding section of nucleotides in the +1 reading frame relative to the HC C-terminal coding region could have led to a transcript with the non-native C-terminal extension. Two potential mechanisms for how this nucleotide sequence might have fused to the native HC coding region and led to expression of the extension product are presented.

Introduction

Microheterogeneity is a common feature of therapeutic monoclonal antibodies (mAbs) that manifests in many forms. 1–4 Non-enzymatic post-translational modifications (PTMs) such as asparagine deamidation, aspartic acid isomerization, or accumulation of succinimides of either residue may occur spontaneously in mAbs, with reaction rates that are sensitive to pH, local amino acid sequence, and higher order structure. 5–9 Side chain oxidation at susceptible residues, such as histidine, methionine, or tryptophan, is another non-enzymatic PTM. While local sequence and higher order structure also play roles in oxidation, cell culture components, formulation additives, transition metal impurities, or light stress are often root causes. 10–14 Glycation, the first step of which is a sugar aldehyde (often from glucose in cell culture) undergoing Schiff base formation with a deprotonated lysine side chain, is yet another common non-enzymatic source of mAb heterogeneity. 15–17 Heterogeneity in mAbs may also arise from variability in biosynthetic pathways (e.g., glycosylation 18–20), or a combination of non-enzymatic and biosynthetic factors (e.g., disulfide isoforms 21–23). Also derived from both non-enzymatic and biosynthetic origins, variability at the N- and C-termini of mAb chains is common. Cell culture carboxypeptidases extensively cleave C-terminal lysine, whereas C-terminal amidation tends to be present at much lower levels. 24,25 On N-termini, glutamine is predominantly converted to pyroglutamic acid (pE), but mAb chains initiating with glutamate only partially convert to pE. Likewise, since antibodies are secreted proteins, translation of mAb chains is initiated by signal peptides that are enzymatically removed from the product by proteases. Variability in this proteolysis may lead to N-terminal heterogeneity, generally as a small number of extra or fewer N-terminal amino acids. 26–28 Sequence variants are another type of mAb heterogeneity with biosynthetic origins. Incorrect amino acids can be mis-incorporated into proteins during translation due to aminoacyl tRNA mis-charging or due to wobble in aminoacyl tRNA recognition. 29–36 Single amino acid sequence variants may also derive from genetic sources as point mutations in one or more copies of the protein coding cassette in the host cell genome. 37–40 Whether non-enzymatic or biosynthetic in origin, the aforementioned forms of heterogeneity are all relatively simple and frequently easy to identify. In some instances, however, more significant chemical alterations to mAb structures have been reported. Fusions of mAb chains with other sequences, large-scale truncations, and combinations of fusions and truncations have been observed. 41–46 These types of variants typically have genetic origins, but the extent to which any such form persists through purification is likely species- and process-dependent. Here, we describe evidence for an additional contributor to mAb heterogeneity: an expression vector-derived twelve amino acid C-terminal mass spectrometry; sequence variant; LC/MS; biopharmaceutical; biologic; monoclonal antibody; mass spectrometry; sequence variant; LC/MS; expression vector; heterogeneity; splicing; recombination

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extension on mAb heavy chains (HCs). Evidence for this variant was first observed from intact mass spectrometry (MS) of the purified mAb and in subsequent analyses incrementally localized to more defined regions of the protein until the contributing altered amino acid sequence could be identified. Knowledge of this modified amino acid sequence and the genetic makeup of the protein expression construct indicates a vector-based mechanism for the modified species formation.

**Results**

Intact reverse phase liquid chromatography/MS (RP-LC/MS) was performed on the IgG4 mAb as part of extended characterization to confirm the intended amino acid sequence, intended polypeptide stoichiometry, and PTMs typical of a mAb. While most of the observed intact masses were consistent with an IgG4 mAb of the intended amino acid sequence, with intended stoichiometry, and typical PTMs; an additional species with an apparent mass 1177 Da larger than the primary intact mAb form was also detected (Figure 1, Supplemental Table 1).

To determine whether the observed mass adduct was present on the heavy or light chain, or if it was otherwise disulfide related, intact RP-LC/MS of the antibody after disulfide reduction was also performed. While the reduced light chain deconvoluted mass spectrum showed no atypical masses (data not shown), the mass adduct was observed unperturbed on the reduced HC (Figure 2, Supplemental Table 2). This observation indicated both that the mass adduct was due to HC chemical heterogeneity and was not disulfide related.

Having identified the unknown adduct as relating to HC heterogeneity, we deglycosylated the HC, and used the hinge-specific protease IdeS followed by disulfide reduction and RP-LC/MS analysis to further localize the mass adduct. The resultant total ion chromatogram is presented in Figure 3A. While light chain, Fc, and Fd regions of the reduced IdeS-treated sample were well resolved, additional peak signal was observed above the baseline at ~ 8.4 minutes. The deconvoluted mass spectrum for this peak was consistent with the Fc mass plus 1177 Da (Figure 3(B, C)). Based on the IdeS IgG4 hinge cleavage site, CPPCPAPEFLG/GPSVFLFPKP, this finding effectively localized the mass adduct to the C-terminal 210 amino acids of the HC.

That the modified species could be chromatographically separated provided a route for enrichment and further analysis. A scaled preparation of IdeS-treated sample was generated, but, due to concerns of stability and sample recovery, it was not deglycosylated or reduced. Iterative RP separation and fraction collection for the native and modified Fc target species was then performed. Materials corresponding to each of the two retention time windows for the native and modified domains were subsequently pooled to two final representative fractions. Aliquots were taken from each fraction to gauge their compositional purities by RP-LC/MS, while the remainder of each fraction was dried to concentrate the material by vacuum concentration. MS analysis of the native fraction revealed that it predominantly contained native Fc with typical glycoforms (Supplemental Figure 1 and Supplemental Table 4). The modified Fc fraction, however, contained the target modified species and two additional forms eluting in the same region, an Fc with an unpaired disulfide bond, and an Fc that was aglycosylated (Supplemental Figure 2 and Supplemental Table 4). Despite containing additional species, this fraction was significantly enriched in the 1177 Da modified form and was deemed suitable for additional studies.

The dried fractions were subsequently reconstituted in a denaturation/solubilization buffer, reduced, alkylated, and subjected to endopeptidase Lys-C digestion prior to RP-LC/MS peptide mapping. The comparative peptide maps for the two fractions are shown in Figure 4.
While differences in relative intensities for some peaks were observed throughout the chromatograms, attributable to differences in digestion, glycosylation, and oxidation between the samples, a unique peak at 52.8 minutes was observed in the peptide map from the 1177 Da-modified Fc fraction (Figure 4B) that was not observed in the native Fc fraction (Figure 4A). Mass spectrometric analysis of this retention time region revealed that while co-elution of some identical species existed in both maps, a distinct ion at 926.96 m/z (2+), was present in the map from the modified Fc fraction (Figure 5). To enhance the quality of the collision-induced dissociation (CID) spectrum for this species, re-injection of the sample with targeted isolation, CID fragmentation, and high-resolution MS/MS data acquisition was performed. The resultant CID mass spectrum was of high quality and, together with the high-resolution mass for the 2+ parent ion at 926.96 m/z, could be used for de novo peptide sequencing. The CID mass spectrum and the resultant de novo-derived peptide sequence along with supporting b and y ions are presented in Figure 6. Correlations between observed and theoretical ion masses for the derived sequence are presented in Supplemental Table 5.

Peptide map MS and CID data suggested that a unique peptide with a sequence of SLSLSLEAEAASASELFQ was present in the modified Fc fraction. Upon identification of this peptide, we noted that the N-terminal amino acids were consistent with the first six of seven C-terminal residues of the native IgG4 HC, SLSLSLG. Also noteworthy was that substitution of the proposed modified sequence for the native sequence accounted exactly for 1177 Da intact mass shift observed through the various stages of our investigation (Table 1).

Aware of other examples of mAb heterogeneity with genetic origins,41–45 we sought to identify whether a similar mechanism could have led to C-terminal modification observed here. To explore this possibility, we performed in
in silico translation of the expression construct in all 6 possible reading frames. A nucleotide sequence was identified in the expression vector that accounted for the C-terminal modification (EAEAASASELFQ) in the peptide. The coding sequence begins 2482 nucleotides after the natural HC stop codon in a +1 position reading frame (Figure 7). The identified peptide sequence suggests that the modified product was created by fusing the coding region for the C-terminus of the HC with this otherwise non-coding region in the vector. In the process, the code for the final two residues of the native HC was also disrupted, leading to replacement of a C-terminal GK with EAEAASASELFQ (Figure 7). From our protein-level data, it is unclear whether the new code formed at the level of DNA or mRNA. If occurring from DNA double-strand breakpoint recombination, a break before (or after) the first guanosine of the newly added glutamic acid codon. Either outcome would lead to deletion of the native C-terminal GK residues and addition of EAEAAASASELFQ as the new C-terminus. Following translation and secretion, the new C-terminal lysine would be proteolytically removed by carboxypeptidases in the cell culture just as native C-terminal lysines are removed (Figure 7). Alternatively, if originating at the mRNA level, a modified transcript may have arisen as a splice variant. The C-terminal glycine in the native chain is coded as ggt in the DNA (Figure 7) and transcribed to ggu in the pre-mRNA. Meanwhile, the first glutamic acid of the C-terminal addition is coded by gag (same bases in pre-mRNA), but preceded immediately by adenosine, a. As gu is a potential splice donor and ag is a potential splice acceptor, it is possible that alternate splicing between the gu normally in the glycine codon and the ag at the beginning of the glutamic acid codon could excise a 2494 nucleotide sequence giving rise to an mRNA analog of the fused DNA shown in Figure 7. Interestingly, this latter potential mechanism would require generation of a very large pre-mRNA transcript, necessitating transcription through at least three potential termination/polyadenylation signals in the DNA prior to reaching the new C-terminal code. Whether the modification originated at the DNA or mRNA level is unclear from our data, as either mechanism would lead to the same modified protein found in the product and have originated from the production cell line.

Discussion

Here, we describe the observation, localization, isolation, and identification of an IgG4 mAb variant bearing a C-terminal oligopeptide extension. Conclusive evidence was presented tracking the modification to the C-terminus of a sub-population of mAb HCs in the purified product. As the modified C-terminal portion of the molecule appears to derive from an otherwise non-coding sequence of the expression vector, two potential mechanisms for how this sequence might have fused with the native HC C-terminal coding region are presented. Both potential mechanisms would lead to an identical amino acid sequence (the sequence identified in the product). Further attempts to decipher which mechanism led to the modified product had not been undertaken at the time our characterization activity concluded, as our focus shifted to functional assessment and process impacts on the variant. Nevertheless, since neither alternate splicing nor DNA damage can be entirely predicted, our observation and identification further highlight the importance of screening for unexpected mass adducts and sequence variants throughout cell line development for therapeutic mAbs.

Materials and methods

The IgG4 mAb was expressed in a Chinese hamster ovary K1-derived cell line and purified in-house at Gilead Sciences in Oceanside, CA. The 1 M Tris-HCl (pH 7.4) used in deglycosylation, IdeS treatment, and intact mAb reduction was from Teknova (T9074). PGNase F was from New England Biolabs.
IdeS was from Genovis (A0-FR1-050). Lys-C was from Wako (125-0506). The guanidine hydrochloride (G9284) and tris base (T6791) used for peptide mapping, along with the EDTA (E4884), Chromasolv HPLC-grade 2-Propanol (IPA, 34863), iodoacetamide (I6125), and calcium chloride (C5670) were from Sigma Aldrich. The guanidine HCl (24115) used for reduced mass analysis, trifluoroacetic acid (TFA, 28904), and dithiothreitol (DTT, 20290) were from Thermo Scientific. Optima LC/MS grade solvents, water (W6-4) and acetonitrile (ACN, A955-4), were from Fisher Scientific. Sephadex G-25-packed NAP-5 gel filtration columns were from GE Life Sciences (17085301).

**Intact and reduced mAb RP-LC/MS preparation and analysis**

For intact mass analysis, formulated mAb was diluted to 1 mg/mL in intact mass RP-LC/MS mobile phase A (see below). Four micrograms of material were injected per intact analysis. Reduced mass analysis samples were denatured using final concentrations of 4 M guanidine HCl, 50 mM DTT, and 50 mM Tris (pH 7.4) and heated for 20 minutes at 60° C. One microgram of sample was injected per reduced mAb analysis. RP-LC/MS of intact and reduced mAbs was performed on a Waters BEH C4 300 Å 2.1 × 150 mm column (186004497) maintained at 70° C. For intact mass analysis,
mobile phase A was 0.1% TFA in water; and mobile phase B was 0.1% TFA, 10% IPA, 90% ACN. A linear gradient was applied from 32% to 46% B at 0.3 mL/min for 4.5 minutes prior to purging and re-equilibrating the column. For reduced mass analysis, mobile phase A was 0.1% TFA in water, whereas mobile phase B was 0.2% TFA, 50% IPA, 50% ACN. A linear gradient was applied from 28.5% to 48% B at 0.3 mL/min over 6 minutes prior to purging and re-equilibrating the column. Chromatography was performed on a Waters H-Class Bio system. The chromatographic region of interest was eluted into the electrospray ionization source of a Waters Synapt G2-Si mass spectrometer operating in positive ion, sensitivity mode. MS parameters were applied that had previously been optimized for either intact or reduced mAbs, respectively. Raw combined mass spectra were deconvoluted using Maximum Entropy 1 in MassLynx software (Waters Corporation) by applying appropriate parameters given the raw data and analyte masses.

**IgG4 deglycosylation**

Two mg of formulated mAb was deglycosylated at 10 mg/mL in 50 mM Tris (pH 7.4) using 15,000 units of PGNase F over a 3-hour incubation at 37° C. Complete deglycosylation was verified by intact mass analysis.

**IdeS digestion of deglycosylated IgG4**

5000 units of IdeS (one vial) were reconstituted at 50 units/µL with 100 µL of water. 8 µL of the reconstituted IdeS were mixed with 400 µg (40 µL) of deglycosylated mAb and 50 µL of 50 mM Tris (pH 7.4) prior to incubation at 37°C for 45 minutes. This material was then diluted using a denaturation buffer to a final volume of 252 µL, with 3 M guanidine HCl and 40 mM Tris (pH 7.4) as final concentrations. 5 µL of 0.5 M DTT was added prior to a 60-minute incubation at 37°C to reduce the samples.

**RP-LC/MS of deglycosylated, IdeS-treated, reduced mAb**

RP-LC/MS of the reduced IdeS-treated sample was performed on the same system and column as used for intact and reduced mAbs described above. For analytical purposes, 1 µg of material was injected. Mobile phase A was water with 0.1% TFA. Mobile phase B was 10% IPA, 90% ACN, and 0.1% TFA. Separation was achieved over a linear gradient from 33% to 46% B at 0.250 mL/min applied over 20 minutes (70°C C column temperature) prior to purging and re-equilibrating the column. The chromatographic region of interest was eluted into the electrospray ionization source of a Waters Synapt G2-Si mass spectrometer. Mass spectrometer settings were identical to those used for reduced mAb chains and MaxEnt 1 deconvolution parameters were identical to those developed and applied for reduced mAb light chains.

**Native and modified Fc collection and Lys-C peptide mapping**

To enable fraction collection, IdeS-treated, IgG4 was prepared by scaling IdeS and substrate concentrations in the procedure described above with the exception that this material was not deglycosylated or reduced prior to RP separation. 350 µg of the prepared sample was injected onto an Agilent Zorbax 300 Å, 3.5 µm, 4.6 × 150 mm, C8 reverse-phase column (863973.906) applying the same mobile phases, and at the same column temperature as used for analytical RP-LC/MS. On this column, separation was achieved with a linear gradient from 29% to 45% B at 0.250 mL/min applied over 38 minutes prior to purging and re-equilibrating the column. Peaks eluted and fractions were collected corresponding to the relative retention times of the native and modified Fc elution regions. This process was repeated for several cycles to enable collection of enough material for peptide mapping of the major and minor forms. Corresponding fractions from each injection were pooled. From each pooled fraction aliquots were taken to assess compositional purity by RP-LC/MS on the same chromatography system coupled to a Sciex Q-Star Elite mass spectrometer. Raw data was deconvoluted using Applied Biosystems Analyst QS 2.0, while the remainder of each pooled fraction was dried via Beckman Speed Vac Concentrator.

The dried native and modified Fc fractions were reconstituted to 1 mg/mL using 7.5 M guanidine hydrochloride, 1 mM EDTA, and 100 mM Tris (pH 7.5). 300 µL of this material was taken forward and reduced using 5 µL of 0.5 M DTT prior to a 60-minute incubation at 37°C. After allowing the samples to cool, alkylation was conducted by adding 15 µL of 0.5 M iodoacetamide with a 30 minute, light-protected, room temperature incubation. 180 µL of 25 mM Tris, 2 mM CaCl₂ (pH 8.3) was then added to each sample to achieve a final volume of 500 µL. Samples were buffer exchanged into 990 µL of 25 mM Tris, 2 mM CaCl₂ (pH 8.3) with a final protein concentration of 0.3 mg/mL using a Sephadex G-25-packed NAP-5 gel filtration column. Lys-C was added at a 1:10 (w:w) protein ratio and incubated for 6 hours at 37° C. Enzymatic digestions were quenched by adding 10% TFA to a final concentration of 0.25%. Native and modified Fc digests were subsequently analyzed by RP-LC/MS as described below, while excess proteolyzed samples were stored at ~80° C.

**Native and modified Fc Lys-C peptide map RP-LC/MS analysis**

Lys-C peptide maps prepared from the native and modified Fc fractions were chromatographically separated using a Waters Acquity UPLC HSS T3, 100 Å, 1.8 µm, 2.1 x 100 mm, C18 reverse-phase column (186003539). Peptide map mobile phase A consisted of water and 0.1% TFA. Mobile phase B was 90% ACN, 10% water, and 0.1% TFA. A linear gradient was applied from 1% to 45% B over 80 minutes while the flow rate and column temperature were maintained at 250 µL/min and 60° C, respectively. 20 µg of digest was injected per run. Separated peptides were monitored by spectrophotometry at...
214 nm prior to elution into the electrospray ionization source of a Thermo Scientific LTQ-Orbitrap XL mass spectrometer. Prior to this investigation the tuning and MS/MS parameters were optimized using Glu-Fibrinopeptide B and mAb digests to increase signal to noise and to achieve a suitable balance between quantity of MS/MS events and quality of MS/MS data. For each of the fraction digests the instrument was operated in positive ion, data-dependent acquisition mode over a mass range of 150 to 2000 m/z. After observing the unique peptide ion at 926.96 m/z in the modified Fc digest, a second injection was performed applying targeted MS/MS settings to specifically isolate and fragment the ion enabling collection of high-resolution CID data for subsequent de novo sequencing of the peptide. Peptide assignments were based on matching observed masses in the LC/MS analysis with theoretical masses based on a Lys-C digest of the IgG4 mAb. De novo sequencing of non-matched peptides was carried out using Thermo Pepfinder 2.0 with manual verification and correction as necessary.

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Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| Å            | Angstrom    |
| ACN          | acetonitrile|
| °C           | Celsius     |
| mM           | millimolar  |
| m/z          | mass to charge ratio |
| µl           | microliter  |
| pm           | picrometer  |
| µm           | micrometer  |
| mm           | millimeter  |
| mL           | milliliter  |
| CL/MS        | liquid chromatography-mass spectrometry |
| mAb          | monoclonal antibody |
| mRNA         | messenger ribonucleic acid |
| MS/MS        | mass spectrometry/mass spectrometry |
| µg           | microgram   |
| N-terminal/terminus | N-terminal/terminus |
| PTM          | post-translational modification |
| RP           | reverse phase |
| TFA          | trifluoroacetic acid |
| tRNA         | transfer ribonucleic acid |
| w/w          | weight/weight |

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