A general approach to antibody thermostabilization

Audrey D McConnell, Xue Zhang, John L Macomber, Betty Chau, Joseph C Sheffer, Sorena Rahmanian, Eric Hare, Vladimir Spasojevic, Robert A Horlick, David J King, and Peter M Bowers*

AnaptysBio, Inc.; San Diego, CA USA

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Abbreviations: SPR, surface plasmon resonance; CDR, complementarity-determining region; SHM, somatic hypermutation; HC, heavy chain; LC, light chain; DSC, differential scanning calorimetry; VL, light chain variable region; VH, heavy chain variable region; Tm, melting temperature; CH2, heavy chain constant domain 2; CH3, heavy chain constant domain 3; NGF, β-nerve growth factor; TNF, tumor necrosis factor

Antibody engineering to enhance thermostability may enable further application and ease of use of antibodies across a number of different areas. A modified human IgG framework has been developed through a combination of engineering approaches, which can be used to stabilize antibodies of diverse specificity. This is achieved through a combination of complementarity-determining region (CDR)-grafting onto the stable framework, mammalian cell display and in vitro somatic hypermutation (SHM). This approach allows both stabilization and maturation to affinities beyond those of the original antibody, as shown by the stabilization of an anti-HA33 antibody by approximately 10°C and affinity maturation of approximately 300-fold over the original antibody. Specificities of 10 antibodies of diverse origin were successfully transferred to the stable framework through CDR-grafting, with 8 of these successfully stabilized, including the therapeutic antibodies adalimumab, stabilized by 9.9°C, denosumab, stabilized by 7°C, cetuximab stabilized by 6.9°C and to a lesser extent trastuzumab stabilized by 0.8°C. This data suggests that this approach may be broadly useful for improving the biophysical characteristics of antibodies across a number of applications.

Introduction

The use of antibodies for many applications is greatly facilitated by good biophysical characteristics, including high thermostability. Therapeutic antibodies must exhibit favorable pharmaceutical properties, including high thermostability and low aggregation propensity, in order to facilitate manufacturing and storage, as well as to promote long serum half-life. Antibodies used for diagnostic purposes and antibody-based biosensors also benefit from increased thermostability, reducing the need for cold-chain storage, extending shelf life, and expanding the range of applications for practical use. Biophysical properties such as thermostability are often limited by the antibody variable domains, which differ greatly in their intrinsic properties. Unfavorable biophysical characteristics have been more prevalent in antibodies and antibody fragments derived from in vitro display technologies such as phage display. Such approaches to antibody generation require selection in microbial expression systems, and in some cases have resulted in the selection of antibodies with a propensity to aggregate, poor expression in mammalian cells, and low stability. It has been suggested that these methods lack an inherent selection for thermostability that occurs in vivo.

To take advantage of the full breadth of specificity existing in both native and synthetic antibody repertoires, a general approach is needed for antibody stabilization that can be applied across multiple antigen specificities and antibody formats, without compromising antigen binding affinity. The previously reported stabilization and affinity maturation of an anti-MS2 antibody was achieved by a combination of grafting complementarity-determining regions (CDRs) onto known stable frameworks, consensus design, disulfide bond engineering, and computational design. The antibody was stabilized to a $T_m$ of over 90°C and retained significant antigen binding activity after heating to 90°C for one hour. Stabilizing mutations were located in framework regions, avoiding the antigen-binding CDR loops. Additionally, binding affinity was optimized >100-fold via in vitro somatic hypermutation (SHM) with selection by mammalian cell display. This suggested that the stabilized IgG framework identified may provide a scaffold for grafting CDR loops from other antibodies and antibody fragments to improve their stability while maintaining the desired antigen binding specificity.

We investigated whether the stabilized IgG framework reported would allow a general approach for improving the stability and solution behavior of a varied panel of reagent and...
therapeutic antibodies. Grafting specificities onto the engineered stable IgG framework improved the thermostability of antibodies derived from either in vitro or in vivo sources, including mouse and human. Stabilization of an anti-HA33 antibody by approximately 10°C was achieved alongside affinity maturation of approximately 300-fold to generate a potent, highly stable antibody. In addition, the specificities of 10 other antibodies, including five therapeutic antibodies, were grafted onto the stable framework. Four of the five therapeutic antibodies were successfully improved in stability, with minimal effect on antigen binding affinity.

Results

Stabilization and Affinity Maturation of an anti-HA33 antibody

The extreme stabilization and affinity maturation of an antibody via a combination of antibody engineering and in vitro SHM has been described previously.9 In this study, we undertook to determine whether the framework developed during stabilization could be used to stabilize antibodies of diverse origin, to allow a broadly useful technique. This was attempted via a CDR-grafting approach in which CDRs of an antibody of desired specificity are transferred to the stable framework and the resulting stabilized antibody is subject to mammalian cell display and affinity maturation via in vitro SHM (Fig. 1).

As a challenging initial case, a mouse monoclonal antibody to the neurotoxin-associated protein hemagglutinin 33 (HA33) from Clostridium botulinum was chosen. This antibody, provided by Dr. P. Buckley of the US Army Edgewood Chemical Biological Center, is intended for use in advanced biosensors, and increasing both stability and affinity of the antibody is of interest for improved biosensor performance. The CDRs of the mouse monoclonal antibody to HA33, possessing low homology to the human stable framework, were grafted into the stable human variable-region framework and formatted as a full-length immunoglobulin using human IgG1 kappa constant regions. The stable human variable regions contained heavy chain (HC) mutations L5V, R19I, S49C and I69C introduced into IgHV3–23, and with mutations at M4L, P12A, T14L, F36Y, R46L, and Y87F introduced into IgKV2D-30.9 The IgG1 HC also contained an added intra-domain disulfide bond, residues, L12C and K104C in the C3d2 domain, which has been shown to improve stability of both the CH2 domain and the IgG overall.9 The anti-HA33 antibody heavy chain was of the VH14–3 framework, 61% identical to the stable hV14–3 HC framework, excluding the CDRs. Similarly, the starting mouse VH, IGKV12–4 was 62.5% identical to the stable hV12D-30 framework (Table 1). A chimeric IgG comprising the anti-HA33 mouse V-region with the same human constant regions, excluding the added CH2 domain disulfide, was generated for use as a control (Table 2).

The stability of the grafted antibody (APE1146) was compared with chimeric IgG (APE1148) and a Fab fragment of the original mouse antibody (APE1136) using differential scanning calorimetry (DSC). The stable grafted antibody demonstrated a 10°C increase in Fab Tm in comparison to the original mouse Fab, from 82.1 to 92.1°C (Fig. 2A). Similar analysis of the chimeric IgG revealed a 3.8°C increase in the Fab Tm over that of the original antibody Fab, indicating that some of the stabilization resulted from reformattng the Fab as a full-length human IgG. The full-length IgGs unfolded with three characteristic transitions representing the Fab, CH2, and CH3 domains,17 whereas the Fab unfolded with the expected single transition. Binding affinity measurements obtained by surface plasmon resonance (SPR) indicated that grafting into the stable framework gave a 1.5-fold loss in HA33 antigen binding affinity with a KD of 9 nM (Fig. 2B; Table 2). Both the starting mouse Fab and the chimeric IgG gave an identical KD of 6 nM. In a separate experiment, stabilization was also attempted by transferring a subset of stabilizing mutations from the stable framework at analogous positions in the mouse V-region framework of the chimeric antibody. These mutations, three in the HC (Q5V, G49C, I69C) and one in the light chain (LC; M4L), resulted in a 3.1°C increase in Tm (APE1196, Table 2), and the antibody retained wild-type HA33 binding affinity, suggesting that these framework stabilizing mutations may be transferrable to other antibody frameworks in cases where CDR grafting is less successful.

To determine whether stabilization via transfer of CDRs into the stable framework was compatible with further improvement in antigen binding, affinity maturation of the stabilized anti-HA33 antibody was performed by mammalian cell surface display and SHM in vitro as described.16 Affinity-improving mutations were identified, including HC substitutions H35N, A53L, and Q64R, and LC substitutions N50D and G66E. The antibody containing these mutations, termed APE1553, was expressed, purified and shown to be approximately 300-fold improvement in antigen binding affinity (K D = 30 pM) from the starting antibody (Fig. 2B). DSC of APE1553 revealed a Tm of 88.2°C, representing a 3.9°C loss in Tm relative to the stabilized antibody APE1146. Removal of the G66E LC mutation from the affinity matured antibody resulted in APE1854, which was found to completely restore stability to a Fab Tm of 92°C, with minimal effects on affinity, binding HA33 with a KD of 45 pM (Table 2).

The stabilized and affinity matured antibodies were subsequently assessed for stability by thermal challenge. Starting and stabilized antibody samples were heated at 70°C at a concentration of 1 µg/mL in phosphate-buffered saline (PBS) for 1 h, cooled to 4°C, and subsequently analyzed for HA-33 binding activity by SPR and compared with unheated samples to determine percent activity remaining after thermal challenge. Both APE1553 and APE1854 maintained 100% activity after an hour at 70°C (Fig. 2C). In contrast, the starting antibody showed a complete loss in activity under these conditions. The improvements made to the thermal stability, affinity, and durability (thermal stress) of anti-HA-33 antibody using the stabilized framework should significantly improve its utility as a detection reagent.
exhausting CDRs, and the grafted antibody demonstrated a 9.2°C improvement in melting temperature as measured by Thermostat fluor assay, with a stabilized $T_m$ of 94°C (Table 1). Affinity analysis of the stabilized antibody revealed a modest 2-fold loss in β-NGF binding affinity (Table 1). Similarly, the anti-C5 antibody was 93% identical to the stable HC framework and 72.5% identical to the stable LC framework. The grafted anti-C5 antibody exhibited a 7.9°C improvement in $T_m$ and this antibody maintained full binding affinity for C5 (Fig. 3; Table 2). The mouse anti-TNF antibody was more divergent from the stable framework with 56% and 66% identity to the HC and LC. The $T_m$ of this antibody was improved by 7°C when CDR grafted into the stable framework, and maintained TNF binding affinity within 1.5-fold (Fig. 3; Table 2). The anti-IL-17A antibody, APE508, was the only antibody of this set not stabilized by the CDR graft that in this case resulted in a 3.2°C loss in $T_m$. This antibody was 87% identical to the stable HC framework and 72.5% identical to the stable LC framework. The grafted IL-17A antibody demonstrated a slight 1.6-fold improvement in antigen binding affinity.

To further demonstrate the practical application of this approach, the stable framework described above was combined with the CDRs from five therapeutic antibodies including denosumab, trastuzumab, adalimumab, cetuximab, and omalizumab. These antibodies were selected to compare the effectiveness of grafting CDRs from highly homologous (e.g., denosumab with 91.5% HC and 70% LC identities to the stable framework) and from increasingly divergent starting frameworks (e.g., cetuximab with 60% HC and 66% LC identities), and from human and non-human origins. Four of the five stable-grafted therapeutic antibodies were improved in thermostability (Fig. 3B). Denosumab, cetuximab, and adalimumab displayed a 6.9–9.9–10°C improvement in $T_m$. Trastuzumab was slightly stabilized, giving a relative 0.8°C $T_m$ increase. Only one antibody in this set was not stabilized by CDR grafting: Omalizumab showed a 3.1°C decrease in $T_m$ upon CDR grafting into the stable framework. These data indicate that CDRs from highly divergent antibodies can be successfully grafted into the stable framework; even with low sequence homology, cetuximab, with mouse variable regions, was stabilized by 6.9°C and retained most of its antigen binding affinity.

Each of the CDR-grafted antibodies was tested for antigen binding affinity using the relevant antigens by SPR in comparison to the respective starting antibodies (Fig. 3; Table 1). In all cases but one, $K_D$ measurements for the stable-grafted antibodies were within 3-fold of the starting antibody. Cetuximab displayed an 8-fold loss in antigen binding affinity despite its 7°C increase in $T_m$. Omalizumab, the only antibody in this group for which a $T_m$ improvement was not observed, had high HC homology to the stable framework (92.4%), yet the LC sequence was among the lowest homology to the stabilized framework of all antibodies tested (65%). Poor LC homology combined with the effects of omalizumab CDR sequences on the overall structure of the antibody may explain the failed stabilization. It may also be possible to improve the stability of omalizumab by incorporating stabilizing mutations into its framework regions while maintaining a

Stabilization of antibodies of diverse origin
To investigate how broadly applicable this approach might be, we grafted CDRs from four antibodies with varied levels of homology to the stable scaffold and assessed stability and affinity of each resulting antibody. These included three human antibodies targeting human proteins β-nerve growth factor (NGF), complement protein C5, and interleukin (IL)-17A, respectively, and a fourth mouse antibody targeting tumor necrosis factor (TNF). The anti-β-NGF antibody was 94% identical to the stable HC framework and 64% identical to the stable LC framework excluding CDRs, and the grafted antibody demonstrated a 9.2°C improvement in melting temperature as measured by Thermostat fluor assay, with a stabilized $T_m$ of 94°C (Table 1). Affinity analysis of the stabilized antibody revealed a modest 2-fold loss in β-NGF binding affinity (Table 1). Similarly, the anti-C5 antibody was 93% identical to the stable HC framework and 72.5% identical to the stable LC framework. The grafted anti-C5 antibody exhibited a 7.9°C improvement in $T_m$ and this antibody maintained full binding affinity for C5 (Fig. 3; Table 2). The mouse anti-TNF antibody was more divergent from the stable framework with 56% and 66% identity to the HC and LC. The $T_m$ of this antibody was improved by 7°C when CDR grafted into the stable framework, and maintained TNF binding affinity within 1.5-fold (Fig. 3; Table 2). The anti-IL-17A antibody, APE508, was the only antibody of this set not stabilized by the CDR graft that in this case resulted in a 3.2°C loss in $T_m$. This antibody was 87% identical to the stable HC framework and 72.5% identical to the stable LC framework. The grafted IL-17A antibody demonstrated a slight 1.6-fold improvement in antigen binding affinity.

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more wild type framework environment for the CDRs, as was demonstrated with the anti-HA33 antibody APE1196.

Long-term stability analysis

Increasing the melting temperature of an antibody might be expected to translate to improved biophysical characteristics, including extended shelf life, and offer the potential to reduce or eliminate cold chain storage requirements. To demonstrate this, long-term stability analysis was performed on both original and stabilized forms of the therapeutic antibody adalimumab. Antibodies were heated at 1 μg/mL in PBS at 70°C, with samples taken over the 72 h time course for the binding activity analysis based on ELISA. The original adalimumab antibody lost all activity after 1 h at 70°C (Fig. 4). In contrast, the stabilized adalimumab variant maintained full activity at 1 h, and displayed measurable activity up to 48 h at 70°C. These data suggest a significant improvement in stability of adalimumab, and that the stable framework is not only useful in improving the T<sub>m</sub> of a given antibody, but may also translate to enhanced long-term stability. It is important to note that other properties and forms of degradation of the antibody may also be limiting to overall shelf-life, and these have not been tested to date. However, the stabilization approach described here is likely to translate to other antibodies, proving a useful tool for extending the long-term stability of a variety of antibodies.

Discussion

Antibodies are used broadly both as therapeutics for human diseases and as specific and sensitive reagents for diagnostics. The utility of antibodies can be limited by solution properties such as solubility, aggregation and thermal stability, which are often related to each other. Poor thermal stability may affect both antibody solubility and aggregation, although antibody aggregation may also be independent of thermal stability. Rational engineering and selection-based methods have demonstrated that solubility and aggregation can be improved independently of thermal stability. However, protein unfolding even in the absence of aggregation can allow for unwanted protein degradation, and high thermal stability is often associated with high expression level and other desired properties. Moreover, a general relationship has been observed in which antibodies with enhanced thermal stability are less susceptible to aggregation. Studies have attempted to improve antibody aggregation and

Table 1. Summary of stable grafted antibodies

| Antibody     | Variable domain framework | CDR3 Lengths (HC, LC) | Mutations from germline (excluding CDRs) | V<sub>H</sub> % Identity to stable framework | V<sub>L</sub> % Identity to stable framework | Starting<sup>a</sup> T<sub>m</sub> (°C) | Stabilized<sup>a</sup> T<sub>m</sub> (°C) | ΔT<sub>m</sub> (°C) | KD Stabilized (nM) |
|--------------|---------------------------|-----------------------|------------------------------------------|-------------------------------------------|-------------------------------------------|---------------------------------|---------------------------------|-----------------|-----------------|
| Denosumab    | hIgH3-23                  | 13, 9                 | 1                                        | 91.5                                      | 70                                        | 78.1 ± 0.09                     | 85.1 ± 0.04                    | 7               | 1.5, 0.82       |
| Omalizumab   | hIgH3-66                  | 12, 9                 | 5                                        | 92.4                                      | 65                                        | 83.6 ± 0.3                      | 80.5 ± 0.09                    | -3.1           | 2.1, 5.2        |
| Trastuzumab  | hIgH3-66                  | 11, 9                 | 5                                        | 90.1                                      | 62.5                                      | 85.1 ± 0.18                     | 85.9 ± 0.04                    | 0.8             | 10, 9           |
| Adalimumab   | hIgH3-9                   | 12, 9                 | 2                                        | 92.6                                      | 73.8                                      | 75.0 ± 0.1                      | 84.9 ± 0.08                    | 9.9             | 0.49, 1.5       |
| Cetuximab    | mlGKV2-2-3                | 11, 9                 | 5                                        | 59.8                                      | 66.2                                      | 77.6 ± 0.08                     | 84.5 ± 0.02                    | 6.9             | 1.9, 16         |
| Anti-TNFα    | mlGKV9-3-1                | 8, 9                  | 9                                        | 56.1                                      | 66.2                                      | 69.7 ± 0.04                     | 76.5 ± 0.06                    | 6.8             | 0.3, 0.55       |
| Anti-NGF     | hIgH3-23                  | 14, 9                 | 1                                        | 93.9                                      | 63.7                                      | 84.8 ± 0.07                     | 94.0 ± 0.06                    | 9.2             | 6, 12           |
| Anti-IL17A   | hIgH3-7                   | 13, 9                 | 5                                        | 86.6                                      | 72.5                                      | 77.5 ± 0.04                     | 74.3 ± 0.16                    | -3.2            | 0.34, 0.21      |
| Anti-C5-C34SC| hIgH3-23                  | 10,10                 | 1                                        | 92.7                                      | 72.5                                      | 79.5 ± 0.11                     | 87.4 ± 0.01                    | 7.9             | 6.5             |
| Anti-HA33    | mlGKV14-3                 | 8, 9                  | 3                                        | 61                                        | 62.5                                      | 82.1 ± 0.06                     | 92.3 ± 0.16                    | 9.9             | 133.3*          |

*Affinity maturation using SHM in vitro. <sup>a</sup>Mean and standard deviation of n ≥ 2 independent Tm measurements by ThermoFluor.

Table 2. Properties of anti-HA33 antibody variants

| Antibody   | Description                                           | Mutations improving binding affinity | Fab T<sub>m</sub> (°C)<sup>a</sup> | K<sub>D</sub> (nM) |
|------------|-------------------------------------------------------|------------------------------------|---------------------------------|-----------------|
| APE1136    | Originating mouse Fab                                 | NA                                 | 82.1                            | 6 nM            |
| APE1148    | Chimeric antibody with APE1136 variable domain and hlgG1/kappa constant regions | NA                                 | 85.9                            | 6 nM            |
| APE1196    | APE1148 with V<sub>H</sub> (Q5V, G49C, I69C) and V<sub>L</sub> (M4L) mutations from stabilized framework | NA                                 | 89                              | 6 nM            |
| APE1146    | V<sub>H</sub> and V<sub>L</sub> CDRs from APE1136 grafted on stabilized IGHV3–23/LC framework and hlgG1/kappa constant regions | NA                                 | 92.1                            | 9 nM            |
| APE1553    | Affinity matured APE1146                               | V<sub>H</sub> H35N, A53L, Q64R; V<sub>L</sub> N50D, G66E | 88.2                            | 30 pM           |
| APE1854    | Affinity matured APE1146                               | V<sub>H</sub> H35N, A53L, Q64R; V<sub>L</sub> N50D | 92                              | 45 pM           |

<sup>a</sup> T<sub>m</sub> values determined by DSC as described in Materials and Methods.
solubility while minimizing the effect on antigen binding using a variety of engineering approaches, including the addition of negative and positive charges within the V region,\textsuperscript{15,23} the addition of charged residues at antibody termini,\textsuperscript{24,25} and the introduction of hydrophilic residues that improve VH/VL pairing and reduce exposure of the hydrophobic interface.\textsuperscript{26}

Our study sought to demonstrate the general applicability of a stable IgG framework for the grafting of diverse antibody CDRs and specificities, simultaneously providing improvement in thermal stability and retention of high-affinity antigen binding affinity. As the basis of this framework, we chose germline HC and LC V regions that are commonly utilized in vivo and that are well expressed.\textsuperscript{16,27} The stabilized framework was shown to consistently improve the thermostability of diverse antibodies with little or no effect on antigen binding affinity, and has the potential to expand the practical use of antibodies in applications where high thermostability is required.\textsuperscript{28}

This work builds upon previously reported antibody stabilization methodologies, combining rational and computational design to generate a human IgG that was demonstrated to have a $T_m$ of 90°C in the context of a single high-affinity antibody.\textsuperscript{9} The approach was applied to improve the stability of a mouse anti-HA33 Fab possessing low sequencing homology to the thermostable IgG scaffold. The resulting CDR-grafted IgG was approximately 10°C stabilized.
relative to the starting Fab with minimal loss of binding affinity for antigen. A common finding with grafting CDRs from one antibody framework to another is a loss in antigen-binding affinity. In this case, we were able to demonstrate that the stabilized antibody could be matured approximately 300-fold while maintaining stability using in vitro SHM. Mutations that improve affinity may be detrimental to stability of an antibody, and vice versa. This may be especially true of antibodies generated and matured via in vitro selection systems frequently used to optimize antibody affinity in which there is no inherent selection for thermal stability.7,8

The thermostabilized scaffold was then applied to improve the stability of 11 antibodies, including a number of FDA-approved therapeutics, resulting in as much as a 10°C improvement in $T_m$ with only minimal loss in antigen binding affinity. Stabilized antibodies were of either mouse or human origin, and originated from in vitro- or in vivo-based antibody generation methodologies. Homology between donor Vh and Vl sequences and the stablized frameworks ranged from 90% to as low as 59%, and improvements in $T_m$ and perturbations with affinity were only modestly correlated with sequence similarity (Table 1), suggesting that most antibodies and antibody fragments may be candidates for this approach. Thermal challenge and accelerated stress studies described here suggest that the biophysical properties of both reagent and therapeutic antibodies can be significantly improved. Combining the stable scaffold with high affinity-antibodies derived from in vitro selection has the potential to generate high affinity antibodies with enhanced

![Figure 3. Application of stable framework to improve antibody stability. (A) Thermofluor assay analysis comparing wild-type (dashed lines) and stable framework, CDR-grafted versions (solid lines) of four different antibodies of differing specificities. Unfolding temperatures are indicated, with arrows representing change in $T_m$ resulting from grafting into the stable framework. Anti-NGF, anti-CS, and anti-TNF antibodies demonstrated increased $T_m$, while the $T_m$ of the anti-IL17-A antibody was decreased. (B) Summary of Thermofluor assay results comparing the $T_m$ of original (blue) and stabilized (red) therapeutic antibodies. (C) Graph depicting change in $T_m$ of five therapeutic antibodies upon CDR grafting into the stable framework.](#)
biophysical characteristics for a variety of antigen binding specificities and applications.

**Materials and Methods**

**Protein expression and purification**

The mouse monoclonal antibody to HA33 and variable region sequences of the antibody were obtained from Dr. P. Buckley (US Army Edgewood Chemical Biological Center). The antibody was reconstructed and expressed in HEK293 c-18 cells as a chimeric IgG with a human IgG1 Fc using standard methodology. The same HEK293 c-18 cell expression system was used for expression of all human IgG antibodies that were purified using a protein A/G agarose resin (Thermo Scientific) by standard techniques. Mutagenesis was performed using the Quick-Change II site-directed mutagenesis kit (Agilent Technologies). The Kabat numbering scheme is employed throughout this manuscript.

The HA33 antigen was expressed in *E. coli* and purified via a C-terminal his tag using standard procedures. Antigen was labeled with DyLight650 amine-reactive dye (Thermo Scientific). C5-C345C was similarly produced in *E. coli* as described. βNGF and IL-17A with N-terminal his6 tags were expressed transiently in HEK293 c-18 cells and purified using standard Ni-chelate affinity purification. TNF, RANKL, HER2, and EGFR were purchased from R&D Systems and IgE was purchased from Abcam.

**Differential scanning calorimetry**

Thermal unfolding profiles of antibody variants were measured by differential scanning calorimetry (DSC) using the VP-Capillary DSC system (GE Healthcare). All antibodies were tested in phosphate buffered saline (PBS), pH 7.4 at protein concentrations ranging from 0.7–1.0 mg/ml at a scan rate of 1°C/minute. Samples were heated from 20–110°C. Data analysis was performed using Origin 7 software. Transition mid-point values ($T_m$) were determined from the thermogram data using the non-two-state model that employs the Levenberg-Marquardt nonlinear least-square method.

**Thermofluor assay**

Thermofluor assay analysis was performed using the ProteoStat Thermal Shift Stability Assay (Enzo Life Sciences). This assay measures thermostability using a fluorescent dye that binds to hydrophobic patches exposed as the protein unfolds. Antibody concentration was normalized to 0.2 mg/mL and fluorescence was read using the LightCycler 480 system (Roche) at 480 nm excitation and 610 nm emission. Samples were heated from 20–99°C at 5°C/minute. The aggregation temperature measured by this assay matches closely with $T_m$ as measured by DSC, and the rank-order of protein stability was found to be highly consistent between the two methods.

**Affinity characterization of antibody variants**

Kinetic analysis was performed by SPR using a Biacore T200 (GE Healthcare). A capture assay was used to allow accurate assessment of antibody affinity and to minimize potential avidity effects due to the bivalent nature of the full-length antibodies. Antibody was captured on a surface of approximately 3000 RU mouse anti-human IgG Fc for 60 s at a flow rate of 10 mL/min, resulting in low capture levels between 50–100 RU. The relevant antigen was flowed over the captured IgG surface for 300–600 s. Trastuzumab and cetuximab were evaluated in the

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**Figure 4.** Thermal stability of therapeutic and stabilized antibodies. Adalimumab (right) and stabilized adalimumab (left) were heated at 70°C for up to 3 d with samples taken at indicated time points for TNF binding analysis by ELISA. Heated and unheated antibodies were evaluated by ELISA to determine activity. Plates were coated with 1 μg/mL TNF (R&D Systems) and incubated for 1 h with heated and unheated antibodies, each over a 1 μg/mL–100 pg/mL concentration range. Bound antibody was detected using a 1:10,000 dilution of goat anti-human IgG-HRP conjugate (Southern Biotech).
opposite orientation with 300 RU antigen immobilized on the surface and antibody flowed over with a concentration range of 1.4 nM-1000 nM. Surfaces were regenerated using 3 M MgCl$_2$. Association and dissociation kinetic values ($k_a$ and $k_d$) were determined from a best fit of the data with the 1:1 Langmuir global fitting procedure to sensograms using the Biacore T200 Evaluation Software, version 1.0.

**Mammalian cell display and in vitro SHM**

Stable HEK293 c18 cell lines displaying IgG of either the chimeric anti-HA33 antibody APE1148 or the stable CDR-grafted antibody APE1146, were generated as described. 16 Antibody surface expression was confirmed by staining with FITC-labeled goat-anti-human CH1. Cells were transiently transfected with an activation-induced cytidine deaminase (AID) expression vector to initiate SHM as described by Bowers et al. After five days, cells were subjected to selection by fluorescence-activated cell sorting (FACS) using fluorescently labeled HA33 antigen. Co-expression of heavy and light chain genes with the AID enzyme induced SHM in the antibody resulting in in situ generation of genetic diversity in the antibody variable domain. Cells were stained by incubating for 30 min at 4°C with HA33-DyLight-650 starting at 100 pM for early sorts and decreasing to 5 pM in the later sort rounds. Cells were simultaneously stained for IgG expression using FITC-labeled goat anti-human IgG for 30 min at 4°C. The highest antigen binding cells, normalized for antibody expression, were sorted using a BD Influx cell sorter (BD Biosciences). Sequencing of approximately 30 HCs and LCs from sorted cells subsequent to each FACS round revealed enriching SHM-induced mutations.

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**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
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