Stability engineering of scFvs for the development of bispecific and multivalent antibodies

Brian R. Miller1,5, Stephen J. Demarest1, Alexey Lugovskoy2, Flora Huang1, Xiufeng Wu1, William B. Snyder1, Lisa J. Croner1, Norman Wang4, Aldo Amatucci2, Jennifer S. Michaelson2 and Scott M. Glaser1

1Biogen Idec, Inc., 5200 Research Place, San Diego, CA 92122, USA, 2Biogen Idec, Inc., 12 Cambridge Center, Cambridge, MA 02142, UK, 3Present address: Biosite Inc., 9975 Summers Ridge Road, San Diego, CA 92121, USA and 4Present address: Molecular Biosciences and Bioengineering, University of Hawaii-Manoa, 1955 East-West Road, Ag. Science 218, Honolulu, HI 96822, USA

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Single-chain Fvs (scFvs) are commonly used building blocks for creating engineered diagnostic and therapeutic antibody molecules. Bispecific antibodies (BsAbs) hold particular interest due to their ability to simultaneously bind and engage two distinct targets. We describe a technology for producing stable, scalable IgG-like bispecific and multivalent antibodies based on methods for rapidly engineering thermally stable scFvs. Focused libraries of mutant scFvs were designed using a combination of sequence-based statistical analyses and structure-, and knowledge-based methods. Libraries encoding these designs were expressed in E. coli and culture supernatants-containing soluble scFvs screened in a high-throughput assay incorporating a thermal challenge prior to an antigen-binding assay. Thermally stable scFvs were identified that retain full antigen-binding affinity. Single mutations were found that increased the measured $T_m$ of either the $V_H$ or $V_L$ domain by as much as 14°C relative to the wild-type scFv. Combinations of mutations further increased the $T_m$ by as much as an additional 12°C. Introduction of a stability-engineered scFv as part of an IgG-like BsAb enabled scalable production and purification of BsAb with favorable biophysical properties.

Keywords: antibody therapeutics/bispecific antibodies/protein engineering/protein stability/scFv

Introduction

Bispecific antibodies (BsAbs) are an emerging class of biological therapeutics designed to bind to two distinct epitopes present on either the same target molecule or two entirely dissimilar molecules. Over the last decade, a spectrum of innovative BsAb formats has been constructed with single-chain Fv (scFv) fragments being extensively used as fundamental building blocks (Hollinger and Hudson, 2005; Kontermann, 2005; Marvin and Zhu, 2005; Fischer and Leger, 2007). ScFv fragments are single polypeptide antigen-binding domains consisting of a $V_H$ domain connected to a $V_L$ domain by way of a synthetic peptide linker, usually a 15 amino acid (Gly/Ser)$_3$ peptide. Full-length IgG-like BsAbs have been constructed as fusion proteins with scFvs located at either the amino or carboxyl termini of the parent antibody (Coloma and Morrison, 1997; Alt et al., 1999; Lu et al., 2005; Michaelson et al., 2009). Because IgG-like BsAbs retain an intact Fc domain and therefore can interact with immune system receptors (e.g. neonatal Fc receptor and Fcγ receptors) as well as Fc-specific ligands, there is the potential for this subclass of BsAbs to retain acceptable pharmacokinetic properties, engage immune effector function, and be readily amenable to scalable manufacturing using well-established protein expression and purification processes. While many scFv-based BsAb designs have shown promising biological activity in early stage research programs, low production yields and attenuation of activity in vivo has hampered preclinical and clinical development (Marvin and Zhu, 2005). It is well established that the physical stability of scFv fragments can vary widely often leading to protein aggregation, precipitation, proteolysis, or hydrolysis resulting in poor quality and low yields (Glockshuber et al., 1990; Worn and Pfüllkühn, 2001). Indeed, several strategies have been described for stabilizing scFv fragments and have led to the successful expression of these fragments in prokaryotic and simple eukaryotic hosts (Reiter et al., 1996; Shusta et al., 2000; Worn and Pfüllkühn, 2001; Ewert et al., 2003; Jespers et al., 2004; Graff et al., 2004; Brockmann et al., 2005). Recently, we described the construction and characterization of a stability-engineered IgG-like BsAb targeting two TNF family member receptors, TRAIL-R2 (TRAIL-Related Apoptosis Inducing Ligand Receptor-2) and LTβR (Lymphotoxin-β Receptor) created by appending a stabilized BHA10 (anti-LTβR) scFv at either the amino or carboxyl terminus of the 14A2 (anti-TRAIL-R2) antibody heavy chain (Michaelson et al., 2009). In these constructs a disulfide bond was introduced at scFv variable domain positions $V_H$ 44 and $V_L$ 100 (Kabat numbering system) (Kabat et al., 1991; Reiter et al., 1996), in combination with a longer (Gly/Ser)$_4$ linker designed to relieve structural constraints imposed by a shorter, more commonly used (Gly/Ser)$_3$ linker as suggested by molecular modeling. While this approach enabled production of high quality, active IgG-like TRAIL-R2 x LTβR BsAbs we subsequently observed contrary results in stability engineering studies with two scFvs of different antigen specificities implying that disulfide-bond stabilization was not a universally applicable solution (X. Wu, B. Miller and S. Glaser, unpublished data). This prompted us to explore developing a broader, library strategy for creating and identifying stability-engineered scFvs for use in constructing BsAbs.

Here, we use a combination of sequence-based statistical analyses and structure-based design to create focused libraries of mutant BHA10 scFvs for the purpose of...
identifying scFvs with improved stability. Expressed, soluble, mutant BHA10 scFv proteins were screened in a high-throughput thermal challenge antigen-binding assay and thermally stable, antigen-active scFvs were isolated. Amino acid mutations identified among lead scFv ‘hits’ were examined in combination for further improvements in stability and a final stability-engineered scFv was selected for creating a new TRAIL-R2 x LTBR BsAb. This BsAb was produced in high yield in a CHO mammalian protein expression system and purification yielded a highly pure, monomeric, biologically active BsAb. To further test the robustness of this approach, we applied this strategy to stability engineer a second scFv that initially exhibited extremely poor thermal stability. Together, these studies validate that high-throughput thermal stability screening can be used to isolate stable variant scFvs that serve as useful building blocks for constructing stable, scalable bispecific and multivalent antibodies.

Materials and methods

Library design identification of stabilizing mutations

Sequence-based and structure-guided methods were used to identify the target residues for mutagenesis as previously described (Jordan et al., 2009). The sequence-based methods included residue frequency analysis consensus methods for finding residues in the BHA10 V\textsubscript{H} and V\textsubscript{L} sequences whose occurrence in natural sequences are rare. Custom databases of V\textsubscript{H} and V\textsubscript{L} sequences were used to derive the consensus sequence for scoring and the individual amino acid frequencies at each residue position (Demarest et al., 2004, 2006; Jordan et al., 2009). The amino acid frequency of every residue within the BHA10 V\textsubscript{H} and V\textsubscript{L} sequences was calculated and mutations were included in the scFv library at BHA10 positions whose frequency at that position divided by the most common residue frequency (consensus) at that same position was <0.3.

Sequence-based covariation analyses were also applied to the BHA10 V\textsubscript{H} and V\textsubscript{L} sequences. Covariation data were derived from a large and separate database of highly diverse V-gene sequences (Wang et al., 2009). In short, mutations within BHA10 V\textsubscript{H} and V\textsubscript{L} domains were included in the scFv library if they generated positive correlations with other residue positions existing within the native sequence.

The DEEK structure-based protein engineering method has been described (Jordan et al., 2009). The homology models used for computing calculations were built in MODELLER (Sali and Blundell, 1993) based on the proprietary structures of the anti-VLA4 antibody HP1/2 and the BHA10 antibodies and refined using SCWRL3.0 (Canutescu et al., 1993) and CHARMM (Brooks et al., 1983).

Library construction and screening

ScFvs (V\textsubscript{H}-(Gly\textsubscript{4}Ser\textsubscript{4}) linker-V\textsubscript{L} orientation) cloned into the pBAD vector (Invitrogen, Carlsbad, CA) were used for constructing expression libraries (Michaelson et al., 2009). ScFvs contained a C-terminal His\textsubscript{6} tag for detection and protein purification. ScFv libraries were generated by introducing variant codon sequences using the QuikChange II Site-Directed Mutagenesis Kit according to the manufacturer’s instructions (Stratagene, La Jolla, CA). Polyacrylamide gel electrophoresis-purified oligonucleotides were obtained from Valuegene, Inc. (San Diego, CA). Mutagenesis reactions were transformed into E. coli strain XL10-GOLD® (Stratagene, La Jolla, CA).

Libraries were plated onto LB agar plates containing 50 \textmu g/ml carbenicillin (Teknova, Hollister, CA). Pooled plasmid DNA was prepared from the library and used to transform E. coli strain W3110 (ATCC, Manassas, Va, Cat. no. 27 325) and prepare arrayed libraries as described (Miller et al., 2009). Briefly, individual transformed colonies were picked into deep-well 96 well dishes (Corning, Corning, NY) containing 400 \textmu l/well LB plus 50 mg/ml carbenicillin and grown overnight at 37°C. Master plates were created by adding an equal volume of LB containing 20% glycerol to each well of the deep-well 96 well dishes, transfusing 50 \mu l aliquots of the bacterial suspension to sterile V-bottom polypropylene microtiter plates (Corning, Corning, NY) and storing the plates frozen at ~8°C until use. Master plates were used to inoculate deep-well microtiter plates containing 250 \textmu l of expression media per well and cultures grown overnight at 32°C as described (Yang et al., 1998; Michaelson et al., 2009). ScFv cultures grown under the same conditions and processed simultaneously as the library were used as controls. Bacteria were removed by centrifugation and 50–100 \mu l aliquots of test supernatants were placed in either duplicate PCR strip tubes or 96-well plates (Applied Biosystems, Foster City, CA). One plate was held at room temperature (reference temperature) and the second plate was thermally challenged for 60–90 min in a heating incubator (Torrey Pines Scientific, San Diego, CA). The thermal challenge temperature for primary screens is set to +2–3°C above the predetermined mid-point transition temperature (T\textsubscript{50}) for the parent scFv, for example in the case of wild-type BHA10 scFv was 50°C. Plate samples were transferred to fresh 96-well V-bottomed plates and insoluble material removed by centrifugation in a refrigerated clinical centrifuge at 2000 rpm for 30 min (IEC model 8R, Thermo Electron, Waltham, MA). Supernatants were transferred to standard micotiter plates for analysis in a binding assay. Thermally challenged and reference wild-type and mutant BHA10 scFv samples were assayed for binding to LT\textbeta R-Fc antigen by DELFIA assay as described (Michaelson et al., 2009). Plates were read using the Europium method on a Victor 2 (Perkin Elmer, Boston, MA). Assay data were processed using Spotfire DecisionSite software (Spotfire, Somerville, MA) and expressed as the ratio of the DELFIA counts observed at challenge temperature to the reference temperature for each mutant. Mutants that reproducibly gave ratios greater than or equal to twice what was observed for the parental plasmid were considered ‘hits’. Plasmid DNAs from these positive clones were isolated by mini-prep (Wizard Plus, Promega, Madison, WI) and retransformed back into E. coli W3110 for confirmation secondary thermal challenge assays. DNA sequence analyses determined the sequence of all constructs.

T\textsubscript{50} binding assay

Replicate supernatant samples from confirmed scFv ‘hits’ were challenged in a thermal gradient and tested for binding to LT\textbeta R-Fc for BHA10 scFv and to soluble CD23 ectodomain for the anti-CD23 scFv using a DELFIA assay to derive a numerical value corresponding to the temperature where 50% binding activity is retained (Michaelson et al.,...
were determined using an 8 with 0.1 M glycine, pH 3.0. Purified scFvs were dialyzed washed with phosphate-buffered saline (PBS) and eluted 300 mM imidazole, pH 8.0. Eluted scFv was loaded onto a stabilized BHA10 V H S16E scFv gene fragments using the oligonucleotide primers: 5'-GGGGTGATCCGGTGGAGGGGGCTCCG GCGGTGCGGGGTCCCCAGTCCACCTGTCGGTCGATCTG-3' and 5'-GTAAACGATCCTTATGGATCCTACCACTTGG-3'. The mutant BHA10 scFv gene fragment was gel isolated, digested with PpuM I and Kpn I restriction endonucleases, and ligated into plasmid pN5KGI harboring the chimeric anti-TRAIL R2 IgG (chi14A2) predigested with the same restriction endonucleases resulting in a fusion product of the stabilized BHA10 V H S16E + V L S46L scFv to the carboxyl terminus of the chi14A2 antibody CH3 domain through a 16 amino acid Ser (Gly) 3 linker. Correct sequence was confirmed by DNA sequence analysis. Plasmid DNA was used to transform DHFR-deficient CHO DG44 cells for stable production of antibody protein. Transfected cells were grown in alpha minus MEM medium containing 2 mM glutamine supplemented with 10% dialyzed fetal bovine serum (Invitrogen Corporation) and enriched as a stable bulk culture pool using fluorescently labeled antibodies and reiterative fluorescent-activated cell sorting (FACS) (Brezinsky et al., 2003). FACS was also used to generate individual cell lines. Cell pools or cell lines were adapted to serum-free conditions and scaled for antibody production in a WAVE bioreactor. Supernatant was harvested, precleared by ultrafiltration and BsAb titer determined by ELISA (Michaelson et al., 2009). The BsAb was purified by Protein A chromatography followed by anion exchange chromatography. Purity and molecular mass of the BsAb was assessed by non-reduced and reduced SDS-PAGE and analytical size-exclusion chromatography with in-line static light scattering as described (Michaelson et al., 2009). Preparation of wild-type TRAIL-R2 x LTBR BsAb has been described (Michaelson et al., 2009).

Tumor cell proliferation assay

WiDr colon carcinoma (ATCC, Manassas, VA) and MDA-MB-231 breast carcinoma (Dr. Dajun Yang, Ascenta Therapeutics, San Diego, CA) cell lines and BHA10 and chi14A2 IgGs were used in cell proliferation assays as described (Michaelson et al., 2009). Tumor cells were treated with 3-fold serial dilutions of antibodies prepared in the complete media. The final concentrations of antibodies typically ranged from 5000 to 0.07 pM. Cells were grown for 4 days (WiDr) or 3 days (MDA-MB-231) at 37°C in a 5% CO2 humidified chamber and cell killing assessed by the addition of 20 μl/well Promega CellTiter 96 Aqueous One

Production of scFvs

E. coli strain W3110 transformants were grown in 4 × 250 ml SB media (Teknova, Half Moon Bay, CA) containing 50 μg/ml carbenicillin in 11 baffled flasks to OD600 ≈ 0.8, induced by adding to 0.02% arabinose and cultured overnight. Bacteria were collected by centrifugation. The pellets were solubilized and lysed using 40 ml B-PER protein extraction reagent (Pierce). Solubilized scFv was applied to a 5 ml Ni-NTA-Superflow column (Qiagen). Bound scFv was washed with 60 mM imidazole, pH 8.0 and eluted with 300 mM imidazole, pH 8.0. Eluted scFv was loaded onto a 6 ml Protein L agarose column (Pierce). Bound protein was washed with phosphate-buffered saline (PBS) and eluted with 0.1 M glycine, pH 3.0. ScFvs were dialyzed against PBS and stored at −20°C. Protein concentrations were determined using an ε280nm = 2.1 ml/mg/cm. ScFvs were characterized by analytical size-exclusion chromatography with in-line static light scattering as described (Michaelson et al., 2009).

Characterization of scFvs

The thermal unfolding profiles of purified scFv proteins were measured by differential scanning calorimetry (capDSC, MicroCal, LLC, Northampton, MA) and 1-anilino-8-naphthalene sulfonate (ANS) binding as described (Michaelson et al., 2009). Transition mid-points (Tm values) from the thermogram data were determined using the non-two-state model within the Origin 7 software provided by the manufacturer. The temperature-dependent ANS fluorescence curves were uniformly fit to a single two-state unfolding model (Kuhlman et al., 1998) for derivation of scFv Tm values using the non-linear curve fitting routine in KaleidaGraph™ assuming ΔCp is independent of temperature and proportional to the difference in solvent exposed surface area between the folded and unfolded states (Haynie and Freire, 1993; Myers et al., 1995).

Affinity measurements for BHA10 binding to LTBR-Fc fusion protein were performed using surface plasmon resonance (SPR) on a Biacore 3000 instrument (Biacore Inc., Piscataway, NJ) as described (Michaelson et al., 2009). All experiments were performed in HBS-EP buffer, pH 7.4. Twenty micrograms per milliliter biotinylated PENTA-His antibody (Qiagen) were immobilized onto a streptavidin-coated CM5 chip at a flow rate of 10 μl/min for ~1 min. About 0.1 μM solutions of wild-type and engineered BHA10 scFvs were injected over the chip at a flow rate of 5 μl/min for 10 min. A series of solutions ranging from 1 to 200 nM LTBR-Fc were subsequently injected at a flow rate of 30 μl/min onto the scFv-coated surface. Data were corrected by double-referencing against a control flow cell containing no PENTA-His antibody and against the flow cell used for measuring specific scFv: LTBR-binding events by injecting a series of buffer solutions. Sensorgram curves were analyzed using the BiaEval 3.0 manufacturer’s software. Kd values were calculated by fitting kinetic association and dissociation curves to a 1:1 Langmuir-binding model.

Construction, production and characterization of BsAb

TRAIL-R2 x LTBR BsAb was constructed as a carboxyl-terminal tetravalent IgG-like BsAb (Supplementary Fig. S1) using the stability-engineered BHA10 V H S16E + V L S46L scFv as previously described (Michaelson et al., 2009). Briefly, PCR was used to amplify BHA10 V H S16E + V L S46L scFv gene fragments using the oligonucleotide primers: 5'-GGGGTGATCCGGTGGAGGGGGCTCCG GCGGTGCGGGGTCCCCAGTCCACCTGTCGGTCGATCTG-3' and 5'-GTAAACGATCCTTATGGATCCTACCACTTGG-3'. The mutant BHA10 scFv gene fragment was gel isolated, digested with PpuM I and Kpn I restriction endonucleases, and ligated into plasmid pN5KGI harboring the chimeric anti-TRAIL R2 IgG (chi14A2) predigested with the same restriction endonucleases resulting in a fusion product of the stabilized BHA10 V H S16E + V L S46L scFv to the carboxyl terminus of the chi14A2 antibody CH3 domain through a 16 amino acid Ser (Gly) 3 linker. Correct sequence was confirmed by DNA sequence analysis. Plasmid DNA was used to transform DHFR-deficient CHO DG44 cells for stable production of antibody protein. Transfected cells were grown in alpha minus MEM medium containing 2 mM glutamine supplemented with 10% dialyzed fetal bovine serum (Invitrogen Corporation) and enriched as a stable bulk culture pool using fluorescently labeled antibodies and reiterative fluorescent-activated cell sorting (FACS) (Brezinsky et al., 2003). FACS was also used to generate individual cell lines. Cell pools or cell lines were adapted to serum-free conditions and scaled for antibody production in a WAVE bioreactor. Supernatant was harvested, precleared by ultrafiltration and BsAb titer determined by ELISA (Michaelson et al., 2009). The BsAb was purified by Protein A chromatography followed by anion exchange chromatography. Purity and molecular mass of the BsAb was assessed by non-reduced and reduced SDS-PAGE and analytical size-exclusion chromatography with in-line static light scattering as described (Michaelson et al., 2009). Preparation of wild-type TRAIL-R2 x LTBR BsAb has been described (Michaelson et al., 2009).
Solution Cell Proliferation Assay (Promega Corporation, Madison, WI). Plates were read in a micotiter plate reader at 490 nm (Spectromax Plus, Molecular Devices, Sunnyvale, CA).

Results

Focused library design

Small focused libraries of $\sim 10^4$–$10^5$ mutant BHA10 scFvs were designed and expression libraries screened for binding to LTβR following thermal challenge. Positions for mutagenesis were identified by statistical analyses (residue frequency analysis/consensus scoring and covariation analysis) and structure-based design.

Residue frequency data for identifying BHA10 $V_H$ and $V_L$ mutations to include in the scFv library design are supplied in Supplementary Table SI. The criteria for using residue frequency data to identify positions for mutagenesis are described in the Materials and Methods section. The amino acid residues listed to the right of the residue frequency calculations in Table I are those most commonly found in human sequences at the targeted position and were included as candidate stabilizing substitutions (Chothia et al., 1998).

The complementarity determining regions (CDRs) of the BHA10 $V_H$ and $V_L$ were not considered for stability optimization by this technique due to potential interruption of the interaction with the LTβR antigen.

Covariation analysis was used for deriving additional potentially stabilizing BHA10 mutations (Wang et al., 2009). Here, BHA10 scFv $V_H$ and $V_L$ residues that violate evolutionarily conserved covarying residues observed in antibody $V_H$ and $V_L$ domains were identified. The violating residues were then substituted with amino acid residues that enhanced or restored the identified covarying networks. For example, substituting Ser at position 46 to Leu (S46L; Kabat numbering) restored the identified covarying networks. For example, substituting Ser at position 46 to Leu (S46L; Kabat numbering) for the scFv, that is, the temperature where 50% binding of wild-type BHA10 scFv to LTβR is observed following thermal challenge. The primary screen yielded 14 unique clones that produced scFvs with improved thermal stability in accordance with the predetermined library screening temperature. Five clones had unique substitutions in the BHA10 $V_L$ domain—one clone contained a $V_L$S46L mutation and the remaining four clones all consisted of substitutions at $V_L$Q3—Q3A, Q3G, Q3S, Q3V and Q3D. Nine clones had single substitutions in the BHA10 $V_H$ domain at six different positions. Three of these nine clones contained unique $V_H$ stabilizing substitutions—$V_H$20I, $V_H$55G and P101D. The remaining six clones comprised two alternate stabilizing mutations at three positions within $V_H$: S16 (S16E and S16Q), M48 (M48G and M48I), and V67 (V67I and V67L). No mutations at positions $V_L$ 49 or 50 were identified that significantly improved scFv thermal stability.

Stability screening of scFv library

The library screening temperature, set at 50°C, was predetermined to be +2–3°C higher than the $T_{50}$ value of BHA10 scFv, that is, the temperature where 50% binding of wild-type BHA10 scFv to LTβR is observed following thermal challenge. The primary screen yielded 14 unique clones that produced scFvs with improved thermal stability in accordance with the predetermined library screening temperature. Five clones had unique substitutions in the BHA10 $V_L$ domain—one clone contained a $V_L$S46L mutation and the remaining four clones all consisted of substitutions at $V_L$Q3—Q3A, Q3G, Q3S, Q3V and Q3D. Nine clones had single substitutions in the BHA10 $V_H$ domain at six different positions. Three of these nine clones contained unique $V_H$ stabilizing substitutions—$V_H$20I, $V_H$55G and P101D. The remaining six clones comprised two alternate stabilizing mutations at three positions within $V_H$: S16 (S16E and S16Q), M48 (M48G and M48I), and V67 (V67I and V67L). No mutations at positions $V_L$ 49 or 50 were identified that significantly improved scFv thermal stability.

### Table I. Oligonucleotides used for stability engineering the BHA10 scFv

<table>
<thead>
<tr>
<th>BHA10 library position</th>
<th>Sequence*</th>
<th>Design method(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_L$ 3</td>
<td>5’-GGTGGTAGTGACATTVNS GACCCAGTCTCCTTAGC3’</td>
<td>Residue frequency</td>
</tr>
<tr>
<td></td>
<td>AT</td>
<td></td>
</tr>
<tr>
<td>$V_L$ 46</td>
<td>5’-GGGAAGGCTCTAATAATTA GTATTCTTGGGCC-3’</td>
<td>Residue frequency, computation, covariation</td>
</tr>
<tr>
<td>$V_L$ 49 and 50</td>
<td>5’-GTCGCGGTAGGAGGCMN RMNATCGAGTTTGGG3’</td>
<td>Residue frequency</td>
</tr>
<tr>
<td>$V_H$ 16</td>
<td>5’-GGGACACCTTCAGTGACBN GGTCATGCTCAGTACAATG3’</td>
<td>Residue frequency, computation, covariation</td>
</tr>
<tr>
<td>$V_H$ 20</td>
<td>5’-GGGGCTCAGTGAAGWTR TCTGGAAAGGCTTCTG5’</td>
<td>Residue frequency</td>
</tr>
<tr>
<td>$V_H$ 48</td>
<td>5’-CAGGGGACTTGAGGVCXK GGATGATTTATCCAC3’</td>
<td>Residue frequency</td>
</tr>
<tr>
<td>$V_H$ 55</td>
<td>5’-GGATGGATTTATCCCTGAAGAATGTCAGCTCAGTACATG AG3’</td>
<td>Covariation</td>
</tr>
<tr>
<td>$V_H$ 67</td>
<td>5’-GAAGTTCAAGGGGACAGNYC AACAATCAGTGACAG-3’</td>
<td>Residue frequency</td>
</tr>
<tr>
<td>$V_H$ 101</td>
<td>5’-GATCCTGGGAAGGTTTGGAC TACTGGGGCCAAGGGAC3’</td>
<td>Residue frequency, $V_H$–$V_L$ interface</td>
</tr>
</tbody>
</table>

*Positions targeted for mutagenesis are indicated by underline. Ambiguous bases are abbreviated as follows: W = A or T, V = A or C or G, Y = C or T, S = C or G, M = A or C, N = A or C or G or T, R = A or G, K = G or T, B = C or G or T.
Mutant BHA10 scFvs scoring positive in the primary screen were retested for binding to LTβR in the thermal challenge assay across a range of temperatures. Here the binding activities of wild-type and mutant BHA10 scFvs were quantitatively compared as $T_{50}$ values (°C). Fits within the $T_{50}$ assay were not created to model thermodynamic unfolding or kinetic aggregation phenomena but rather the $T_{50}$ values provide a relative stability measurement that can be used to rank order the contributions of different mutations towards the thermal stability of the scFv. We grouped the mutant scFvs into two categories. The first, exemplified by mutations at $V_H$ 3 (Q3A, Q3G, Q3S, Q3V and Q3D), $V_H$ 48 (M48I and M48G), $V_H$ 67 (V67I and V67L), $V_H$ 20 (V20I) and $V_H$ 16 (S16Q), exhibited modest increases in thermal stability relative to the wild-type BHA10 scFv (Table II). The second category comprised mutations at $V_H$ 16 (S16E), $V_H$ 55 (V55G), $V_H$ 101 (P101D), and $V_L$ 46 (S46L) had more profound effects on the behavior of the mutant scFvs in this assay. One stabilizing mutation ($V_L$K13E) serendipitously arose from a PCR error (data not shown).

**Enhanced thermal stability by combining mutations**

To further improve scFv thermal stability, select combinations of individual BHA10 scFv stabilizing mutations were constructed by site-directed mutagenesis and examined for binding to LTβR in the thermal challenge assay (Table II). Four combinations resulted in enhanced stability. The double mutants - $V_H$ S16E + $V_L$ S46L and $V_H$ S16Q + $V_L$ S46L yielded $T_{50}$ values ($T_{50}$ = 71°C and 68°C, respectively) that were very similar to what would be expected if the mutations had independent and additive effects on the thermal stability of the scFvs. Addition of the heavy chain stabilizing mutation V55G to $V_H$ S16E + $V_L$ S46L increased the $T_{50}$ value to 75°C. Combining all four of the most significant stabilizing mutations- $V_H$ S16E, V55G, P101D and $V_L$ S46L further enhanced the thermal stability to $T_{50}$ = 82°C, a + 33°C increase in thermal stability relative to the wild-type BHA10 scFv in this assay format.

**Biophysical characterization of stabilized scFvs**

Wild-type and stabilized BHA10 scFvs were produced in *E. coli* and soluble scFv purified from total cellular lysates. scFvs were first purified by immobilized metal ion affinity chromatography and then further purified to homogeneity by Protein L chromatography. Yields ranged from 0.6 to 2.8 mg purified scFv per liter starting culture volume. Each scFv was found to be predominantly monomeric and none of the purified scFvs had detectable levels of oligomers larger than dimer.

The thermostability of each scFv was assessed using both DSC and temperature dependent ANS-fluorescence. The thermostability measurements for all the scFvs derived from DSC or ANS-binding experiments were fairly comparable and showed similar trends (Table III). DSC scans of the most stabilizing single mutations, $V_H$ S16E, $V_L$ S46L, $V_H$ V55G and $V_H$ P101D are shown in Fig. 1A. We found unfolding of each scFv to be irreversible under the conditions tested and therefore made no attempt to calculate scFv free energies of unfolding or any other thermodynamic parameters. Instead, the $T_m$ values were used to rank-order the stability enhancements afforded by each individual or combined mutations. The DSC experiments readily discriminated between the $V_H$ and $V_L$ unfolding transitions in most cases. The ANS-binding experiments were not capable of discriminating between separate unfolding transitions (i.e. $V_H$ vs. $V_L$ unfolding); thus, only the apparent $T_m$ was provided for the ANS-binding experiments. The apparent $T_m$ observed by ANS binding appeared to correlate well with the $T_m$ of the first domain to unfold, either $V_H$ or $V_L$ depending on the mutations, as determined by DSC (Table III). Additionally, the intrinsic ANS fluorescence measured at room temperature decreased as the thermal stability of the scFvs increased (Fig. 1B).

Examination of the DSC values in Table III revealed that most mutations affected only their individual V-domains stability, while others simultaneously increased the stability of both domains. Mutations at positions 16 and 55 of $V_H$ increased the $T_m$ of the $V_H$ domain by +2 (S16E) or +3°C (S16E) and +11°C (V55G), respectively, with little or no effect on the $T_m$ of $V_L$. In contrast, the $V_H$ P101D and $V_L$ S46L mutations increased the $T_m$ of both variable domains. In the case of $V_L$ S46L, the $T_m$ of $V_H$ and $V_L$ each melted independently at a temperature roughly +7°C higher than the wild-type domains. In contrast, the $V_H$ P101D mutant

### Table II. $T_{50}$ values for BHA10 scFvs

<table>
<thead>
<tr>
<th>Mutation</th>
<th>$T_{50}$ (°C)</th>
<th>Mutation</th>
<th>$T_{50}$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (wt scFv)</td>
<td>51</td>
<td>VL Q3V</td>
<td>53</td>
</tr>
<tr>
<td>$V_L$ S46L</td>
<td>61</td>
<td>VL Q3D</td>
<td>54</td>
</tr>
<tr>
<td>$V_H$ S16E</td>
<td>60</td>
<td>VL Q3G</td>
<td>54</td>
</tr>
<tr>
<td>$V_H$ S16Q</td>
<td>56</td>
<td>$V_H$ V67I</td>
<td>55</td>
</tr>
<tr>
<td>$V_H$ P101D</td>
<td>67</td>
<td>$V_H$ V67L</td>
<td>58</td>
</tr>
<tr>
<td>$V_H$ V20L</td>
<td>56</td>
<td>$V_H$ V55G</td>
<td>64</td>
</tr>
<tr>
<td>$V_H$ M48G</td>
<td>54</td>
<td>$V_H$ S16E + $V_L$ S46L</td>
<td>71</td>
</tr>
<tr>
<td>$V_L$ M48I</td>
<td>53</td>
<td>$V_H$ S16Q + $V_L$ S46L</td>
<td>68</td>
</tr>
<tr>
<td>$V_L$ Q3A</td>
<td>53</td>
<td>$V_H$ S16E, V55G + $V_L$ S46L</td>
<td>75</td>
</tr>
<tr>
<td>$V_L$ Q3S</td>
<td>53</td>
<td>$V_H$ S16E, V55G, P101D + $V_L$ S46L</td>
<td>82</td>
</tr>
</tbody>
</table>

The $T_{50}$ value for the wild-type (wt) and mutant scFvs was determined as described in the Materials and Methods section.

### Table III. Biophysical properties of stability-engineered scFvs

<table>
<thead>
<tr>
<th>Construct</th>
<th>$V_H$ $T_m$ (°C)</th>
<th>$V_L$ $T_m$ (°C)</th>
<th>scFv $T_m$ (°C)</th>
<th>$V_H$ $K_D$ (nM)</th>
<th>$V_L$ $K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type scFv</td>
<td>57.7</td>
<td>67.2</td>
<td>67.0</td>
<td>2.1</td>
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<tr>
<td>$V_H$ S16E</td>
<td>60.7</td>
<td>68.1</td>
<td>66.5</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>$V_H$ S16Q</td>
<td>59.4</td>
<td>68.4</td>
<td>69$^a$</td>
<td>1.9</td>
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</tr>
<tr>
<td>$V_H$ S46L</td>
<td>65.6</td>
<td>74.2</td>
<td>71.9</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>$V_H$ V55G</td>
<td>–</td>
<td>68.4</td>
<td>74.6$^a$</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>$V_H$ P101D</td>
<td>–</td>
<td>71.9</td>
<td>75.3$^a$</td>
<td>5.2</td>
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</tr>
<tr>
<td>$V_H$ S16E, $V_L$ S46L</td>
<td>71.0</td>
<td>74.6</td>
<td>75.8$^a$</td>
<td>3.4</td>
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<tr>
<td>$V_H$ S16Q, $V_L$ S46L</td>
<td>67.3</td>
<td>74.9</td>
<td>75.4$^a$</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>$V_H$ S16E, $V_L$</td>
<td>–</td>
<td>77.7</td>
<td>79.3$^a$</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>V55G, $V_L$ S46L</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>$V_H$ S16E, $V_H$</td>
<td>84.1</td>
<td>77.0</td>
<td>81.3$^a$</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Unable to discriminate $V_H$ vs. $V_L$. One apparent transition.

The $T_m$ values of the wild-type and select mutant BHA10 scFvs were determined by both DSC and ANS binding as described in the Materials and Methods section. As indicated, in some cases it was impossible to assign thermal transitions to either $V_H$ or $V_L$. In these cases, the lower temperature transitions were arbitrarily ascribed to $V_H$. Calculated $K_D$ values for each of the scFvs towards immobilized LTβR were determined by Biacore.
increases in the $V_H$ $T_m$: approximately +14°C for the $V_H$ S16E + $V_L$ S46L double mutant BHA10 scFv and +26°C for the $V_H$ S16E, V55G, P101D + $V_L$ S46L quadruple mutant. A good correlation ($R^2 = 0.9$) was found to exist between the $T_m$'s as determined by DSC and the $T_{50}$ values observed in the screen (Fig. 1). As shown in Table III and Supplementary Fig. S2, all of the mutant scFvs had similar binding constants for LT$\beta$R, ranging from 1 to 2.5-fold when compared with the parental BHA10 scFv.

**Construction and production of stability-engineered BsAb**

Previously reported DSC analyses of 17 proprietary antibodies suggested that Fab $T_m$ values greater than ~65°C were associated with favorable IgG biophysical behavior (Garber and Demarest, 2007). For that reason, a stability-engineered BHA10 scFv also possessing a $T_m \geq 65°C$ was selected for generating a BsAb. The stabilized $V_H$ S16E + $V_L$ S46L BHA10 scFv with a 72°C $T_m$ was used to build an IgG-like TRAIL-R2 x LT$\beta$R BsAb by fusing the scFv to the carboxyl end of an anti-TRAIL-R2 IgG. A DHFR-deficient CHO cell line stably transfected with plasmid DNA encoding the stability-engineered TRAIL-R2 x LT$\beta$R BsAb was adapted to serum-free conditions and scaled for BsAb production. Culture supernatants readily expressed titers up to 21.5 mg/l of BsAb.

We first compared the SEC elution profile of protein A purified stability-engineered TRAIL-R2 x LT$\beta$R BsAb to that of the previously described wild-type (unstabilized) TRAIL-R2 x LT$\beta$R BsAb (Michaelson et al., 2009) to examine for the presence of soluble aggregates by analytical size exclusion chromatography (Fig. 2A). The chromatogram profile of wild-type TRAIL-R2 x LT$\beta$R BsAb showed ~40% soluble aggregates. In contrast, the BsAb with the stabilized $V_H$ S16E + $V_L$ S46L BHA10 scFv significantly reduced aggregates to ~7%, levels comparable to that observed with standard IgGs and identical to what was observed for the TRAIL-R2 IgG (data not shown). The BsAb was further purified by anion exchange chromatography and analyzed by reducing and non-reducing SDS-PAGE (Fig. 2B). The reduced lanes show the expected sizes of the heavy and light chain proteins. Analytical SEC was used to assess the purity of the stability-engineered TRAIL-R2 x LT$\beta$R BsAb. The elution profile of the stability-engineered TRAIL-R2 x LT$\beta$R BsAb showed >99% pure, monomeric BsAb essentially free of higher order molecular weight species. Importantly, there was no significant detection of degradation byproducts that often was observed with the wild-type TRAIL-R2 x LT$\beta$R BsAb (data not shown).

**In vitro activity of stability-engineered TRAIL-R2 x LT$\beta$R BsAb**

The stability-engineered BsAb was tested for dual-binding activity to TRAIL-R2 and LT$\beta$R by SPR. BsAb kinetic association/dissociation curves were generated to immobilized TRAIL-R2 followed by a secondary association/dissociation phase with varying concentrations of soluble LT$\beta$R (Fig. 3). These data indicate that the BsAb is capable of simultaneously binding to both ligands.

We next tested inhibition of tumor cell growth in an *in vitro* proliferation assay. Figure 4A shows that the control 14A2 IgG antibody had subnanomolar potency (IC$_{50}$ = 191 pM) at inhibiting the growth of WiDr tumor
cells, a colon carcinoma cell line. The control BHA10 IgG was approximately 15-fold more potent than 14A2 IgG in this assay albeit with slightly reduced cell killing efficacy (79% vs. 95%). The combination of the two antibodies exhibited anti-tumor cell potency (EC_50 = 23 pM) and cell killing efficacy similar to BHA10 and 14A2 IgGs, respectively. The stability-engineered TRAIL-R2 x LTβR BsAb was slightly more potent (EC_50 = 14 pM) than the combination of antibodies in this assay, similar to that previously reported (Michaelson et al., 2009). Treatment of the breast carcinoma cell line MDA231 with 14A2 IgG and BHA10 IgG antibodies resulted in negligible anti-tumor activity (EC_50 > 4500 pM) as single agents as well as when used in combination (Fig. 4B). The stability-engineered TRAIL-R2 x LTβR BsAb, however, showed greater tumor cell killing (EC_50 = 64 pM) of the MDA231 cells, again comparable to the previously described BsAb (Michaelson et al., 2009).

Discussion

We previously reported that selective introduction of a disulfide bond between the V_H and V_L domains of BHA10 scFv enabled high-level production of stable IgG-like BsAbs as either N- or C-terminal fusion proteins (Michaelson et al., 2009). In subsequent studies, though, we discovered that scFv fragments derived from a number of different antibodies demonstrated great variability in their ability to tolerate disulfide bonds positioned between the V_H and V_L domains as manifested by lower expression and protein degradation. Accordingly, we set out to develop a more general approach for producing stable IgG-like BsAbs.
Here we used a combination of statistical analyses and structure-based methods to design small, focused libraries of mutant scFvs and screened the domains in a high-throughput thermal challenge assay. Thermally stable scFvs were identified and by combining appropriately chosen mutations, thermodynamically stable scFv proteins that retained full antigen-binding affinity were generated. Introduction of the stability-engineered scFv as part of an IgG-like BsAb enabled scalable production and purification of a functionally active, stable BsAb. We anticipate that methods for the production of high-quality IgG-like BsAbs will find important application in exploring the utility of this second-generation class of therapeutic antibodies.

All single mutations picked from the library screens: $V_H$ S16E, S16Q, V55G, P101D and $V_L$ S46L significantly stabilized the $V_H$ domain and in some instances, the $V_L$ domain (Table II). The rationale behind testing these positions for stability enhancements often came from multiple forms of analysis. While not shown explicitly in this report, collectively, the three stability prediction methodologies generated libraries where roughly one in eight designs results in scFvs exhibiting improved thermostability. Consensus methods predicted that all of the mutations $V_H$ S16E, S16Q, V55G and $V_L$ S46L would stabilize the BHA10 scFv (Table II). Because the $V_H$ V55G residue was within CDR2 it was initially ignored as potentially disruptive toward ligand binding. However, the subsequent introduction of the covariation analysis also strongly suggested that $V_H$ V55G mutation would be a stabilizing substitution. In fact, the two most stabilizing mutations identified $V_H$ V55G and P101D were both found to reside within CDRs. Structural analyses of the wild-type and mutant BHA10 Fabs revealed that $V_H$ V55 possesses unfavorable phi–psi angles, and substitution of a glycine at this position relieved strain in the CDR2 loop (Jordan et al., 2009). Consistent with previous reports, these data indicate that CDR sequences can have strong effects on the thermodynamic stability of scFvs (Honegger et al., 2009).

While the $V_L$ S46L mutation was identified via all predictive methods described here, its selection by modeling was primarily as a residue which would increase the stability of the $V_H$–$V_L$ interface. Non-interface $V_H$ mutations S16E, S16Q and V55G increased the apparent $T_m$ of the BHA10 $V_H$ by +3, +2 and +11°C, respectively, with little apparent effect on $V_L$. The $V_H$ P101D was also determined to lie near the $V_H$–$V_L$ interface and was found to have a profound effect on the thermal stability of both V domains. This mutation in particular led to an apparently cooperative unfolding of the $V_H$ and $V_L$ domains resulting in a single melting transition for this molecule. Structural analyses of the $V_H$ P101D and $V_L$ S46L mutations show an altered electrostatic arrangement by introducing a salt bridge between $V_H$ P101D and $V_H$ R98. The bulky leucine residue resulting from the $V_L$ S46L mutation increased van der Waals contacts and caused $V_L$ Y55 to adopt a favorable alternate conformation improving solvation energy (Jordan et al., 2009). We propose that mutations at the $V_H$–$V_L$ interface may provide the most effective means of forming a stabilized Fv region and provide a rationale for prioritizing stability designs. We did observe that incorporation of additional $V_H$ mutations eventually led to a loss of cooperativity of $V_H$–$V_L$ unfolding of the BHA10 scFvs (Table III). As the $V_H$ $T_m$ increased above 75°C the $T_m$ of the $V_L$ began to decrease. This suggests that hyperstabilization of the $V_H$ domain may lead to decreased folding cooperativity and a weakened interaction between the two domains.

Increasing the stability of the scFv decreased the intrinsic binding of the hydrophobic, fluorescent dye ANS at ambient temperature (15°C, Fig. 1B). The wild-type scFv binds weakly to ANS suggesting that the protein may permanently or transiently expose hydrophobic surface area to solvent. Stabilized scFvs appeared to completely lose the ability to bind ANS under ambient conditions—the fluorescence of ANS in the presence of the most stabilized scFvs was greater than that of the ANS alone in the solvent. Reduction of ANS binding by scFv stabilization may indicate that less hydrophobic surface area gets exposed to solvent suggesting that stabilized scFvs may have a lower intrinsic propensity for aggregation.

When incorporated into a BsAb, the stabilized scFv led to a dramatic improvement in the biophysical properties of the molecule, as shown in Fig. 2. We chose the double mutant $V_H$ S16E plus $V_L$ S46L as this scFv stabilizes both domains, has an affinity very similar to the wild-type molecule, and displayed a $T_m$ that was similar to well-behaved Fab domains (Garber and Demarest, 2007). Introduction of these stabilizing mutations into the TRAIL-R2-LTBR BsAb enabled production of this molecule, greatly reducing the levels of aggregated protein. As in our previous report, the stabilized IgG-like BsAb displays in vitro activity that is greater than the individual antibodies alone or in combination as shown in Fig. 4 (Michaelson et al., 2009).

We have used the screening technology described in this manuscript to successfully stabilize a number of different scFvs with a representative example shown in Supplementary Fig. S3. In this particular case, the wild-type scFv derived from the anti-CD23 antibody IDEC152 (Nakamura et al., 2000) was found to have a $T_{50}$ value of 38°C. The thermal stability screen for the anti-CD23 scFv identified 19 mutations at 12 positions, which individually stabilized this molecule, and a combination of six of these mutations raised the $T_{50}$ of this scFv to 62°C (Supplementary Fig. 3). These data indicate that the approach used in this report is not specific to the BHA10 scFv, and may be a general method for stability-engineering scFvs.

Here we show that a combination of predictive statistical and computational tools can be used to select mutations that confer improved thermal stability of a scFv without grossly perturbing affinity of the molecule for its cognate antigen. While the methodology employed in this manuscript does not allow for the degree of sequence diversity achievable by other techniques, the targeted nature of the library design reduces the number of candidate mutations to a set that can be easily screened. The addition of covariation analysis to residue frequency analysis was found to expand the potential repertoire of substitution candidates, and suggested changes to CDR residues that otherwise may not have been chosen. Once individual mutations are identified and ranked, combinations of mutations can be easily and rapidly constructed and screened in an iterative process until a molecule with the desired $T_m$ is obtained. Such stabilized scFvs can then be used as building blocks enabling the production of bispecific and multivalent antibodies with favorable biophysical property.
Supplementary data
Supplementary data are available at PEDS online.

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References