Protein Engineering

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An Antibody with a Variable-Region Coiled-Coil "Knob" Domain**

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Abstract: *The X-ray crystal structure of a bovine antibody (BLV1H12) revealed a unique structure in its ultralong heavy chain complementarity determining region 3 (CDR3H) that folds into a solvent-exposed β-strand "stalk" fused to a disulfide crosslinked "knob" domain. We have substituted an antiparallel heterodimeric coiled-coil motif for the β-strand stalk in this antibody. The resulting antibody (Ab-coil) expresses in mammalian cells and has a stability similar to that of the parent bovine antibody. MS analysis of H–D exchange supports the coiled-coil structure of the substituted peptides. Substitution of the knob-domain of Ab-coil with bovine granulocyte colony-stimulating factor (bGCSF) results in a stably expressed chimeric antibody, which proliferates mouse NFS-60 cells with a potency comparable to that of bGCSF. This work demonstrates the utility of this novel coiled-coil CDR3 motif as a means for generating stable, potent antibody fusion proteins with useful pharmacological properties.*

Antibody complementarity determining regions (CDRs) typically consist of hypervariable loops of 8–16 residues that are involved in antigen recognition. Unlike most mammalian antibodies, a subgroup of bovine antibodies contain an ultralong heavy chain CDR3 (CDR3H) region with 40–67 residues.[1] A recently solved X-ray crystal structure of one such bovine antibody, BLV1H12, revealed a novel structural motif in its CDR3H region that folds into a solvent exposed, antiparallel β-strand "stalk" (20 Å in length) that terminates in a folded "knob" domain stabilized by three disulfide bonds (Figure 1 a).[2] This knob domain was shown to be involved in antigen binding by antibodies elicited to specific immunogens.

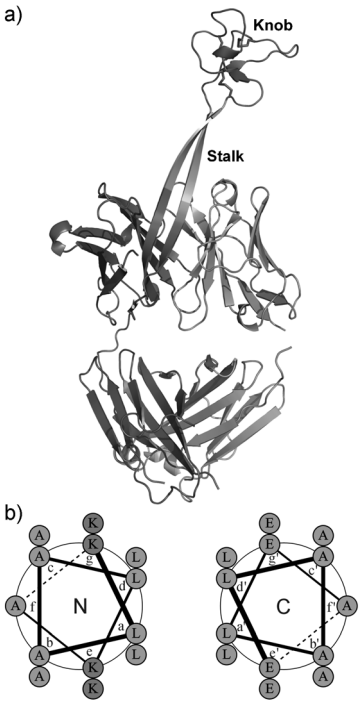

Figure 1. Substitution of the β-strand stalk in bovine antibody BLV1H12 with a coiled-coil motif. a) X-ray crystal structure of antibody BLV1H12 (PDB ID: 4K3D). b) Helical wheel representation of the antiparallel heterodimeric coiled-coil.[8] The sequence of the ascending peptide with linkers at each end is: H_2N-GGSGAKLAALKA-KLAALKGGGGS-COOH; the sequence of the descending peptide with linkers at each end is: H_2N-GGGGSELAALEAELAALEAGGSG-COOH.

We hypothesized that the rigid stalk region plays an important role in the folding of this unique antibody family by separating the folded polypeptide within the ultralong CDR3H from the immunoglobulin framework, thereby preventing misfolding of either domain. Indeed, we showed that the solvent-exposed, antiparallel β-strand stalk supports the substitution of the knob domain with functional polypeptides of various folds, which result in stably expressed antibody fusions with potent biological activities.[3]

The stalk region within the ultralong CDR3H of BLV1H12 is an unusual protein motif, as long solvent-exposed β-strands (seven residues on each strand) are rare. To date, partially solvent-exposed, antiparallel β-strands (less than five residues on each strand) have been found only in certain bacterial aminoacyl-tRNA synthetases.[4] It is likely that this novel structure is templated by geometrical constraints imposed on the ends of the β-sheet by the knob domain and variable-region interface. The β-sheet may be further stabilized by interstrand hydrogen bonds, side-chain

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hydrophobic interactions, and interactions with residues from adjacent CDR loops.

The question arises whether this unusual antiparallel β-strand motif is required for the unique stalk–knob structure of this ultralong bovine CDR3, or if can it be substituted with other rigid motifs to afford a stable antibody with a similar architecture. One candidate for a stalk replacement is the coiled-coil, a highly versatile structural motif that plays an important structural and functional role in a variety of proteins.[5] The coiled-coil is a superhelix consisting of two or more α-helices with a repeated pattern, referred to as the heptad repeat, of buried hydrophobic residues sandwiched by exposed hydrophilic residues.[6] Substitution of the β-strand motif in the bovine antibody with a coiled-coil may also generate a rigid stalk that effectively separates the functional knob domain from the main framework of antibody. Moreover, in comparison to the solvent exposed β-strands, our detailed understanding of those factors that affect coiled-coil structure and stability may allow us to further engineer the stalk to modulate the chemical, physical, and biological properties of the antibody fusions. Herein, we show that the substitution of a heterodimeric coiled-coil for the β-strand stalk in BLV1H12 results in an antibody (Ab-coil) whose CDR3H folds into an antiparallel coiled-coil structure and that has thermodynamic stability comparable to that of BLV1H12. This coiled-coil stalk also allows the generation of a functional fusion of antibody and bovine granulocyte colony-stimulating factor (bGCSF), which stimulates GCSF-dependent cell proliferation with a potency similar to that of bGCSF.

Most naturally occurring coiled-coil motifs have parallel strands, but substitution of the antiparallel β-strands in the bovine antibody stalk region requires an antiparallel coiled-coil motif. Fujii and coworkers have designed a synthetic antiparallel coiled-coil by connecting positively (basic) and negatively (acidic) charged helical peptides with a glycine-based linker.[7] Both peptides are characterized by heptad repeats with leucine residues at the *a* and *d* sites, and charged residues at the *e* and *g* sites, which promote and stabilize the coiled-coil structure (Figure 1b). These basic and acidic peptides were substituted for the ascending and descending β-strands of the stalk in BLV1H12, respectively, and are expected to adopt a heterodimeric coiled-coil structure when fused to the knob domain of BLV1H12. The substituted coiled-coil sequences contain 14 residues on each strand, which should give rise to a stalk approximately 21 Å in length, which is comparable to that of the β-strand stalk. To optimize the folding and stability of the resulting antibody, flexible GGSG and GGGGS linkers were placed at each end of the coiled-coil sequences.

We expressed the fragment antigen-binding (Fab) of the bovine coiled-coil CDR3 variant in order to characterize its folding and stability. The Ab-coil Fab and BLV1H12 Fab (Ab-beta) were expressed in freestyle HEK293 cells by transient transfection. Proteins were purified by Ni-NTA chromatography and analyzed by SDS-PAGE and mass spectrometry (Figure S1–S3). Under non-reducing conditions, the Ab-beta Fab migrates as a single band of 53 kDa and Ab-coil Fab migrates at 55 kDa. In the presence of 50 mM dithiothreitol (DTT), the light chains of Ab-beta and Ab-coil Fabs migrate at 23 kDa, whereas the heavy chains of the Ab-beta and Ab-coil Fabs migrate at 30 kDa and 32 kDa, respectively; this is consistent with the stalk–knob sequences. The final yield of the Ab-coil Fab is ca. 17 $mg\,L^{-1}$, which is similar to that of the Ab-beta Fab. Both proteins are stable in PBS (pH 7.4), and can be concentrated to over 10 $mg\,mL^{-1}$ without aggregation. We next measured the stabilities of the Ab-beta and Ab-coil Fabs using differential scanning fluorimetry (DSF) with SYPRO orange dye (Figure S4).[9] The experimental melting temperatures (T_m) are $74.6 \pm 0.3\,^{\circ}C$ for Ab-beta Fab and $74.1 \pm 0.3\,^{\circ}C$ for Ab-coil Fab. We previously found that the DSF melting temperatures for Fabs track closely with those determined by differential scanning calorimetry.[10] These data, together with the expression and solubility data, suggest that substitution of the β-strand stalk with the antiparallel coiled-coil does not significantly affect the stability of antibody BLV1H12.

To further investigate whether the substituted peptides adopt a coiled-coil structure when incorporated into the stalk region of BLV1H12, we carried out a study on the Ab-coil Fab using hydrogen–deuterium-exchange mass spectrometry (HDX-MS).[11] A previously characterized parallel heterodimeric coiled-coil consisting of acidic and basic peptides was used as a control (Figure S5).[12] Consistent with the previous study, circular dichroism (CD) spectral analysis revealed that either the purified acidic or basic peptide alone forms an unfolded, disordered structure in solution, whereas their mixture in a 1:1 molar ratio results in a stable helical structure (Figure S6).[12] Deuterium incorporation measurements revealed that the backbone amides of the base peptide alone exchange more than three times faster ($0.174 \pm 0.032\ s^{-1}$) than those in the acid–base complex ($0.052 \pm 0.011\ s^{-1}$; Figure 2a). After 10 s in an exchange buffer, the former has more than 80% deuterium incorporation, whereas the latter shows only 40% deuterium uptake. This result is consistent with the CD analysis, and indicates that, in the presence of the acid peptide, the base peptide forms an α-helical structure. The HDX curves show that the deuterium-exchange rates of the backbone amides within the coiled-coil regions of Ab-coil Fab ($0.024 \pm 0.009\ s^{-1}$ for the ascending coil and $0.027 \pm 0.007\ s^{-1}$ for the descending coil) are similar to those of the acid–base complex (Figure 2a) and also consistent with those of the α-helices in previous studies.[13] In addition, the average levels of deuterium incorporation into the coiled-coil regions are comparable to those of the acid-base complex, but are significantly lower than that of the base peptide alone (Figure 2b). These results, together with the previous T_m and expression data, suggest that the substituted sequences fold into an antiparallel coiled-coil structure when substituted for the solvent exposed β-strands in the CDR3H of BLV1H12.

Next we explored whether the Ab-coil structure allows the correct folding of the fused polypeptide and the generation of a functional antibody chimera in a similar fashion to BLV1H12. To test this notion, we first generated the full-length IgG forms of Ab-beta and Ab-coil. The resulting Ab-beta and Ab-coil IgGs were expressed, purified from mammalian cells by Protein A/G chromatography, and their

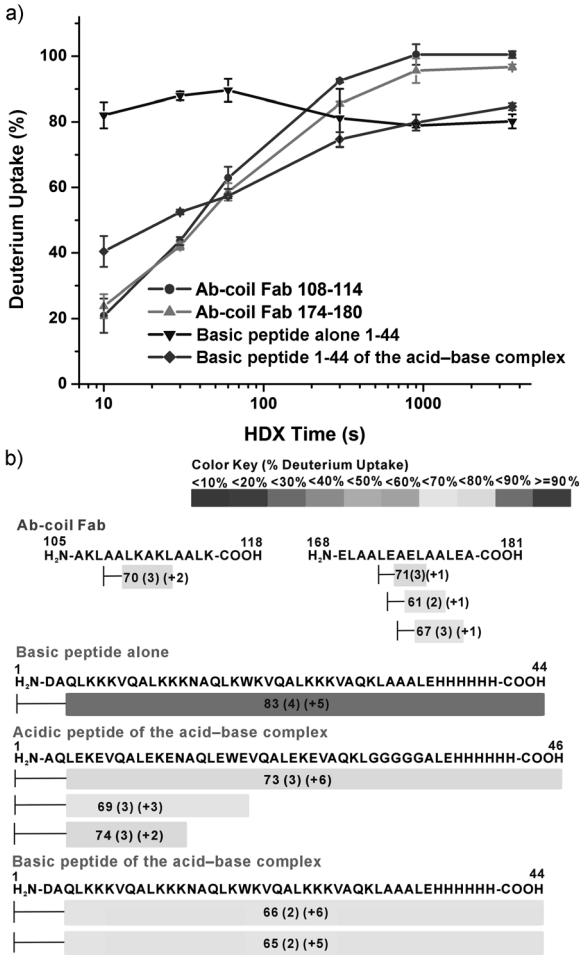

Figure 2. HDX-MS analysis of Ab-coil Fab, the basic peptide alone, and the acid–base complex. Antibody or peptide samples were diluted with D_2O-containing exchange buffer (50 mM HEPES, pH 8.0, 150 mM NaCl) and incubated at 4 °C for 10–3600 s. Measurements were repeated three times; all values were calculated based on experimental D_{max} values. a) Deuterium incorporation curves of the backbone amides within the coiled-coil regions of Ab-coil Fab and the basic peptide, as measured by liquid chromatography-mass spectrometry (LC-MS) at multiple time points. b) The average levels of deuterium incorporation for the coiled-coil regions of Ab-coil Fab, the basic peptide alone, and the acid–base complex. The value within each peptide is the avg.% of deuterium incorporation over six time points (10–3600 s). The numbers in parentheses are the standard deviation and the charge state of the analyzed peptide.

structures confirmed by SDS-PAGE and mass spectrometry (Figure S7–S9). Both antibodies expressed in similar yields and had comparable solubilities. We then generated Ab-beta–bGCSF and Ab-coil–bGCSF fusion proteins by replacing the knob domain with bGCSF using GGGGS linkers at each end of the bGCSF, as previously described.[3a] The resulting constructs were confirmed by SDS-PAGE and mass spectrometry (Figure S10–S15). The antibody–bGCSF fusion proteins expressed in mammalian cells afforded similar yields (ca. 17 $mg\,L^{-1}$) and solubilities as BLV1H12, which indicates that they are likely folded correctly.

We next examined the biological activity of the Ab-coil–bGCSF fusion protein using mouse NFS-60 cells that are

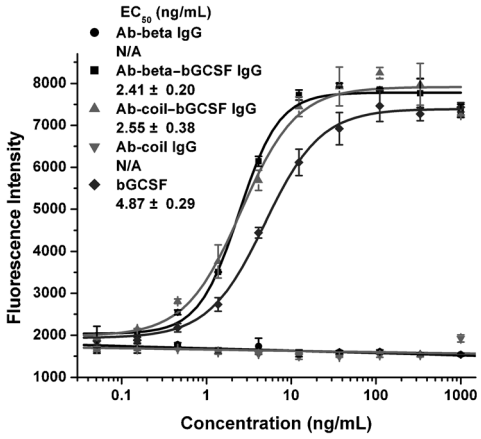

Figure 3. Ab-coil–bGCSF fusion protein stimulates proliferation of mouse NFS-60 cells in a dose-dependent manner. Cells cultured in RPMI-1640 medium with FBS (10%) and 2-mercapoethanol (0.05 mM) were treated with various concentrations of bGCSF, Ab-beta IgG, Ab-coil IgG, Ab-beta–bGCSF IgG, and Ab-coil–bGCSF IgG. Cell viability was quantified using an Alamar Blue (Invitrogen) assay; measurements were repeated three times. N/A = not applicable.

growth-dependent on GCSF.[14] Both Ab-beta–bGCSF and Ab-coil–bGCSF stimulate NFS-60 cell proliferation in a dose-dependent manner (Figure 3), whereas Ab-beta and Ab-coil by themselves have no proliferative activity, thus indicating that the observed activities of the antibody-bGCSF fusion proteins result from the fused bGCSF. The potencies of the antibody–bGCSF fusion proteins ($EC_{50} = 2.41 \pm 0.20\ ng\,mL^{-1}$ for Ab-beta–bGCSF, and $2.55 \pm 0.38\ ng\,mL^{-1}$ for Ab-coil–bGCSF) are comparable to that of bGCSF ($EC_{50} = 4.87 \pm 0.29\ ng\,mL^{-1}$). In addition, Ab-beta–bGCSF Fab and Ab-coil–bGCSF Fab stimulate NFS-60 cell proliferation in a dose-dependent fashion (Figure S16). The EC_{50} is $1.59 \pm 0.12\ ng\,mL^{-1}$ for Ab-beta–bGCSF Fab and $1.28 \pm 0.07\ ng\,mL^{-1}$ for Ab-coil–bGCSF Fab. Thus, grafting of bGCSF onto the substituted coiled-coil stalk in the CDR3H of the Ab-coil does not appear to affect the activity of this cytokine, which indicates that, as with the β-strand stalk, the antiparallel heterodimeric coiled-coil allows the creation of a functional antibody chimera by supporting the folding of the biologically active fused polypeptide.

In conclusion, we have shown that, by substituting coiled-coil sequences for the solvent-exposed antiparallel β-strands in the stalk region of bovine antibody BLV1H12, one can generate a novel extended CDR3H consisting of an antiparallel heterodimeric coiled-coil terminating in a folded domain. It is likely that this unique structure can be inserted into the CDRs of other antibodies as well, including human antibodies. Importantly, relative to the bovine-derived β-strand stalk, the coiled-coil motif provides a versatile structural module for further engineering the properties of functional antibody fusions. The β-strand and coiled-coil motifs may also allow the generation of novel antibody chimeras containing two or more polypeptides by fusing these motifs into other CDR loops, thus opening the door to engineered antibodies with dual activities. Future studies will include structural characterization of the coiled-coil antibody, and the

fusion of other cytokines, growth factors, and peptide hormones into the ultralong CDR3H region, as well as the generation of antibodies containing two or more CDR protein fusions.

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