Characterization of the 4D5Flu single-chain antibody with a stimulus-responsive elastin-like peptide linker: A potential reporter of peptide linker conformation

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Abstract

Single-chain antibodies (scFvs) are comprised of IgG variable light and variable heavy domains tethered together by a peptide linker whose length and sequence can affect antigen binding properties. The ability to modulate antigen binding affinity through the use of environmental triggers would be of great interest for many biotechnological applications. We have characterized the antigen binding properties of an anti-fluorescein scFv, 4D5Flu, containing stimulus-responsive short elastin-like peptide linkers and nonresponsive flexible linkers. Comparison of length-matched flexible and short elastin-like peptide linkers indicates that a stimulus-responsive linker can confer stimulus-responsive control of fluorescein binding. A linker length of either six or 10 amino acids proved to have the largest thermally induced response. Similar differences in binding free energy changes indicate a common underlying mechanism of thermal responsiveness. Contrary to the thermal behavior, the effect of salt, another elastin β-turn-inducing stimulus, stabilized antigen binding in the six- and 10-amino-acid linkers such that elastin-like linkers became less stimulus-responsive as compared with flexible linkers. Again, the thermodynamic analysis indicates a common mechanism of salt responsiveness. Characterization of the room-temperature binding affinities and evidence indicating a dimeric state of the scFvs concomitantly suggest the major contribution to the stimulus-responsive behavior derives from the perturbation of interdomain associations, rather than the linker-constrained disruption of the intramolecular association. The ability to use stimulus-responsive peptide modules to exert a novel control over protein function will likely find application in the creation of allosteric antibodies and scFv-based biosensors, and as a platform to enable the evolution of new stimulus-responsive peptides.

Keywords: single-chain antibody; stimulus-responsive peptides; elastin-like peptides; sELP; peptide conformational change; diabody; modular assembly

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Nature frequently employs combinations of protein domains to create control elements for the mediation of complex cellular interactions (Cho and Stahelin 2005; Pawson and Linding 2005; Bhattacharyya et al. 2006). Mimicking this approach, protein engineers have had success in combining existing and designed domain architectures to create unique control mechanisms for rationally designed protein regulation (Beerli and Barbas 2002; Ostermeier 2005; Chin 2006). Incorporation of stimulus-responsive peptide modules into existing protein constructs offer one such way to control protein function through the introduction of environmental perturbations (Dueber et al. 2003; Buskirk and Liu 2005; Dattelbaum et al. 2005; Skretas and Wood 2005; Lim and Franklin

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2006; Banta et al. 2007), but determining a suitable location for domain or module insertion into an existing protein construct is not trivial. For example, a structure-based approach to the creation of an allosteric ubiquitin–barnase fusion, controlled by the thermodynamics of mutually exclusive folding, depended on a large difference between N- and C-terminal distances for barnase and ubiquitin (Radley et al. 2003). And, the design of a β-lactamase controlled by the concentration of sucrose required a directed evolution approach involving circular permutation (Guntas et al. 2005).

Single-chain antibodies (scFvs) are constructed by fusing the genes for the variable light (V_L) and variable heavy (V_H) domains by a short artificial peptide linker, thus reconstituting a single binding site (Bird et al. 1988). The length and structure of the peptide linker are known to affect scFv ligand binding and expression levels (Essig et al. 1993; Griffiths et al. 1993; Alfthan et al. 1995; Turner et al. 1997; Arndt et al. 1998). Therefore, this system is ideal for the exploration of the use of stimulus-responsive peptides for the modulation of protein function, as the insertion site for the peptides is clearly defined. Generally, protein engineers aim to construct scFvs that are unaffected by the peptide linker, and so commonly used linkers are designed to be inert and flexible, often comprised of three to six repeats of GGGGS (Arndt et al. 1998).

Functional peptide domains are attractive for the incorporation of allosteric control into a target protein because their decreased size reduces the likelihood of completely disrupting protein function upon insertion. For example, a calcium-sensitive β-roll peptide has been used to modulate bioactive mesh size in a streptavidin protein array (Ringler and Schulz 2003). pH-responsive self-assembling peptide modules have been fused to fluorescent proteins, which enables the formation of bioactive hydrogels (Wheeldon et al. 2007). Leucine zipper function was regulated using light following the cross-linking of an azobeneze group between two engineered cysteine residues (Kumita et al. 2003). And a short elastin-like peptide has been used to introduce thermal responsiveness to protein A binding (Reiersen and Rees 1999).

Elastin-like polypeptides (ELPs) are concatenations of the naturally derived elastin repeat sequence, commonly VPGVG, of over 100 repeats (Urry et al. 1985). These polypeptides have been extensively studied for their various properties, including the inverse temperature transition, where the largely unstructured elastin repeats gain β-turn structure at temperatures greater than the transition temperature (T_t) (Urry 1999). A similar effect has also been observed with increasing salt concentrations (Urry 1993). The major driving force behind this conformational change is entropic due to the movement of water of hydrophobic hydration to the bulk (Urry 1999). Short elastin-like peptides (sELPs) of six to 18 amino acids in length exhibit similar changes in secondary structure in response to increased temperature. However, these sELPs do not undergo the hallmark precipitation that characterizes the longer ELPs (Reiersen et al. 1998). sELPs have been used to modulate the binding of a Protein A minidomain to IgG in response to changes in temperature (Reiersen and Rees 1999). A similar system utilized salt to modulate binding of an elastin-like peptide-linked Protein A minidomain (Reiersen and Rees 2000).

A recent study investigated the potential of using an sELP linker in the anti-fluorescein scFv, 4D5Flu, to control the binding affinity of the fluorescein antigen (Megeed et al. 2006). The performance of scFvs with elastin-like linkers of various lengths (six, 12, and 15 amino acids) were compared with an scFv with a flexible linker 15 amino acids long. It was demonstrated that when the temperature was raised from 25°C to 55°C, there was a greater release of bound fluorescein for 4D5Flu with an elastin-like peptide linker six amino acids long compared with the release of 4D5Flu with a flexible 15-amino-acid linker. This finding represents an important "proof of principle" that a stimulus-responsive peptide linker can be used to confer stimulus-responsive control over scFv antigen binding affinity.

Since sELPs respond to increases in temperature and salt by gaining β-turn structure, we reasoned that this conformational change was responsible for the stimulus-responsive behavior observed by Megeed et al. (2006) and hypothesized that the scFv may be useful as a universal reporter or sensor of the conformational state of an attached peptide linker. A more detailed analysis, however, is required before this technique can be utilized in practice. In this paper, we demonstrate that the thermally responsive behavior is retained for sELP linkers of six and 10 amino acids when compared with their length-matched flexible counterparts. Other lengths studied did not exhibit thermally responsive behavior. Salt-triggered elastin β-turn formation resulted in the stabilization of sELP linkers, resulting in decreased responsiveness when compared with length-matched flexible linker mutants. It is likely that diabody formation and small-scale perturbations of diabody intermolecular association play an important role in the underlying mechanism producing the stimulus-responsive behavior. These results taken together stress the importance of calibrating the linker length and the choice of the environmental stimulus when using scFvs as peptide conformational change sensors.

Results

Purification of 4D5Flu mutants

4D5Flu mutants with flexible or sELP-based linkers (Fig. 1) were purified by immobilized metal affinity

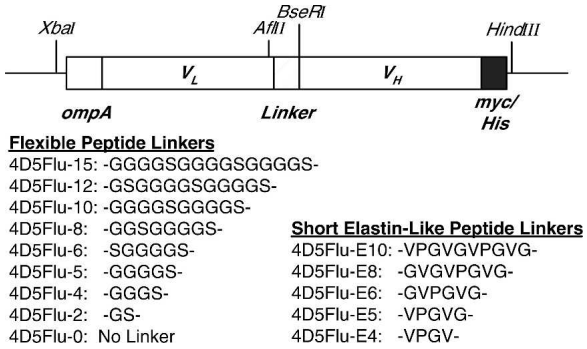

Figure 1. 4D5Flu linker mutants. 4D5Fu mutants are created with flexible peptide linkers varying in length from zero to 15 amino acids and short elastin-like peptide (sELP) linkers varying in length from four to 10 amino acids. Variable light (V_L) and variable heavy (V_H) domains are unaltered. The secretion signal (ompA) is cleaved during periplasmic localization. All 4D5Flu mutants are tagged with c-myc and polyhistidine (myc/His).

chromatography (IMAC) and subsequently by size-exclusion chromatography. Instead of finishing the purification with cation exchange (Jung and Pluckthun 1997; Megeed et al. 2006), we used gel filtration. This allowed us to investigate the effect that diabodies and other multimers may have on our system. Gel filtration chromatograms of IMAC-purified 4D5Flu-15 (dashed line) and 4D5Flu-10 (solid line) are shown in Figure 2. For 4D5Flu-15, two peaks (Fig. 2A,B) possessed active scFv, while only one peak for 4D5Flu-10 (Fig. 2B) possessed significant activity. Active fractions were pooled, and yields were typically ~1 mg/L of culture as determined by spectrophotometric analysis, consistent with previous reports (Jung and Pluckthun 1997; Megeed et al. 2006). The apparent purity of scFv samples was >99% as determined by SDS-PAGE (Fig. 3). In Figure 3, lane B contains crude lysate, lane C contains IMAC-purified sample, and lanes D and E contain active size-exclusion-purified 4D5Flu-15. Samples from lanes D and E eluted as separate peaks on the gel filtration column: The sample in lane D eluted with a shorter retention time than the sample in lane E. However, on a denaturing SDS-PAGE gel, samples D and E migrated similarly. The retention times of active 4D5Flu-15 peaks (Fig. 2A,B) are consistent with dimer (56 kDa) and monomer (28 kDa) forms of the scFv. Similar results were observed during the purification of the 4D5Flu-12 mutant. For all other linker lengths examined, the only fractions with significant activity eluted with retention times characteristic of scFv dimers.

Equilibrium dissociation constant unaffected by linker length

The responsive nature of scFv antigen binding was measured by the change in the equilibrium dissociation constants (K_Ds) under various conditions. K_Ds were determined by titration of a known amount of fluorescein with purified scFv at 25°C and fitting to the equation presented in the Methods section (Jung and Pluckthun 1997). A least-squares analysis of this equation is sensitive to the concentration of antibody, which was determined by spectrophotometric analysis, and therefore is sensitive to sample purity. All samples were >99% pure as assessed by SDS-PAGE (see Fig. 3, for example). Figure 4 shows the K_D values, which ranged from 20 ± 2.6 nM to 58 ± 3.1 nM (tabulated data available in Supplemental Table 1), which is in good agreement with the range of previously reported values (22 nM to 43.8 nM) for flexible GGGGS-type linkers (Jung and Pluckthun 1997; Megeed et al. 2006). When the flexible linker length was incrementally reduced, the K_D values remained relatively unaltered, maintaining nanomolar affinity even when the two domains were directly fused together without a peptide linker (K_D = 58 ± 3.1 nM). While pairwise t-tests for length-matched sELP and flexible-linker mutants indicated a statistically significant difference in K_D for linker lengths of five, six, and 10 amino acids, K_D values for all scFv mutants investigated are still close to the previously reported values for 4D5Flu.

It has been widely reported that as the peptide linker length is decreased, scFvs readily form diabodies and other multimers that may be capable of reconstituting a monomeric-like binding pocket with monomeric-like binding affinity (MacKenzie et al. 1996). Diabodies can form when the V_H and V_L of two different antibody molecules associate to form an active binding pocket. The remaining V_L and V_H would likewise associate to form another active binding pocket. Thus, diabody formation

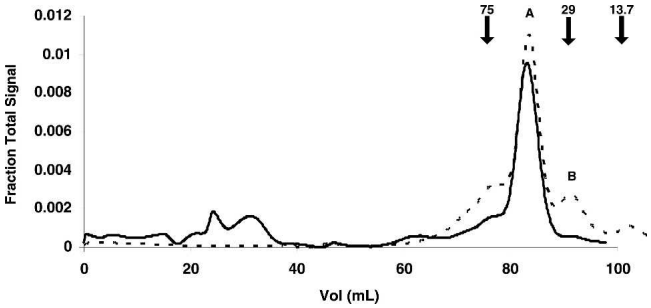

Figure 2. Gel filtration purification of 4D5Flu-15 and 4D5Flu-10. Gel filtration was performed on a Hi Load 16/60 Superdex 200-pg Gel Filtration column (GE Healthcare). (Dashed curve) 4D5Flu-15 chromatogram, (solid curve) 4D5Flu-10. (Arrows at *top*) Elution volumes of three standard proteins: conalbumin (75 kDa), carbonic anhydrase (29 kDa), and lysozyme (13.7 kDa). Gel filtration was the final purification step and also provided insight into the physical state of the scFv. The majority of the scFv eluted in peak A, with a retention time indicative of a molecular weight consistent with the size of diabodies. Peak B eluted with a retention time indicative of a molecular weight consistent with the size of the monomeric scFv. All scFvs with linkers shorter than 10 amino acids eluted predominantly as dimers.

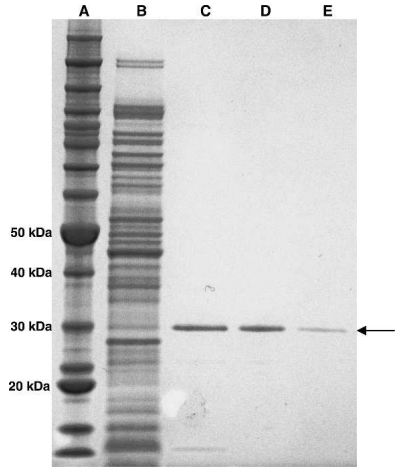

Figure 3. SDS-PAGE analysis of purification and purity of 4D5Flu-15. All samples were run on a 4%–20% SDS-PAGE under denaturing conditions. (Lane *A*) Standard proteins (Invitrogen), (lane *B*) crude cell lysate, (lane *C*) IMAC-purified sample, (lanes *D*,*E*) samples with significant activity from two different peaks following size-exclusion chromatography. Lane *D* contains active 4D5Flu-15 that eluted with a shorter retention time than the sample in lane *E*, indicating a larger apparent molecular weight. Taken together with the data from Fig. 2, the SDS-PAGE results suggest that the sample in lane *D* contains dimeric scFv and the sample in lane *E* likely contains monomeric scFv.

may mask the effect of a prohibitively short linker. Gel filtration chromatography (Fig. 2) confirmed the presence of diabodies for scFvs of all lengths.

The effect of length on the thermal responsive release of fluorescein

The thermal response of 4D5Flu mutants was analyzed by measuring the concentration of fluorescein released as the temperature was slowly (0.5°C/min) increased from 25°C to 55°C. Cycling was repeated twice, and the release of fluorescein was found to be reversible within 5% for each cycle, with the exception of the case where there is a zero-amino-acid linker (data not shown), which is consistent with previous findings (Megeed et al. 2006). Thermally induced release data were collected for all mutants. Figure 5 shows the thermally induced release of fluorescein for the six-, eight-, and 10-amino-acid linker mutants. The six- and 10-amino-acid linker lengths exhibited the best separation between flexible and sELP linkers out of all the lengths examined. For the six-amino-acid linkers, Figure 5A shows that at lower temperatures, both flexible and sELP linkers have similar responses. However, at 48°C, the difference between flexible and sELP linkers became statistically significant, and the separation between the two continued to increase. Similar results were obtained for the 10-amino-acid linkers (Fig. 5C). The temperature at which the difference became significant was 53°C. However, there was no statistical difference between the sELP and flexible linkers eight amino acids in length (Fig. 5B).

The largest measured difference in fluorescein released occurred at 55°C, which was the highest measured temperature. The thermally induced release of fluorescein was calculated as the difference between the initial and final concentrations of free fluorescein (100 nM fluorescein total). Figure 6A shows the cumulative release of fluorescein for scFvs with a flexible and sELP linker of various lengths upon raising the temperature from 25°C to 55°C. For flexible linkers greater than zero, the amount of fluorescein released ranged from 28 nM to 41 nM and was reversible upon thermocycling. The scFv with a linker length of zero did not show reversible binding and released almost 60 nM fluorescein, larger than any other scFv fluorescein release. A weak trend was observed that as the linker length is shortened the release of fluorescein increased. However, a few data points lie contrary to the observed trend. A single-factor ANOVA analysis of thermally induced release data of all mutants with flexible linkers greater than zero indicated a statistically significant difference in K_D with respect to linker length. Furthermore, the trend may more accurately be described as step-like, with the longest linker lengths (12 and 15) releasing the least, intermediate lengths (six, eight, and 10) releasing more fluorescein, and shortest lengths (two, four, and five) releasing the most fluorescein.

The amount of fluorescein released by the sELP linker-containing mutants ranged from 29 nM to 44 nM (Fig. 6A). A weak trend, similar to the flexible linker data, was observed: As the linker length is shortened, the release of fluorescein increases. However, once again, a few data points lie contrary to the observed trend. A single-factor ANOVA analysis of all elastin-like linker thermally induced release data indicated a statistically significant

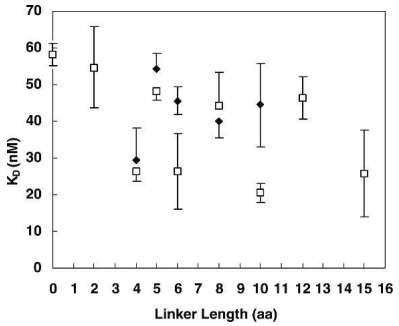

Figure 4. Solution-based assay of fluorescein binding. The equilibrium dissociation constant (K_D) was determined by titrating fluorescein with varying amounts of antibody at 25°C. (□) K_D values for flexible linkers, (◆) K_D values for sELP linkers. Error bars represent the standard deviation of individual experiments. Where the error bars overlap, only the *upper* and *lower* error bars are shown for clarity. Each titration was performed in at least triplicate ($n \geq 3$).

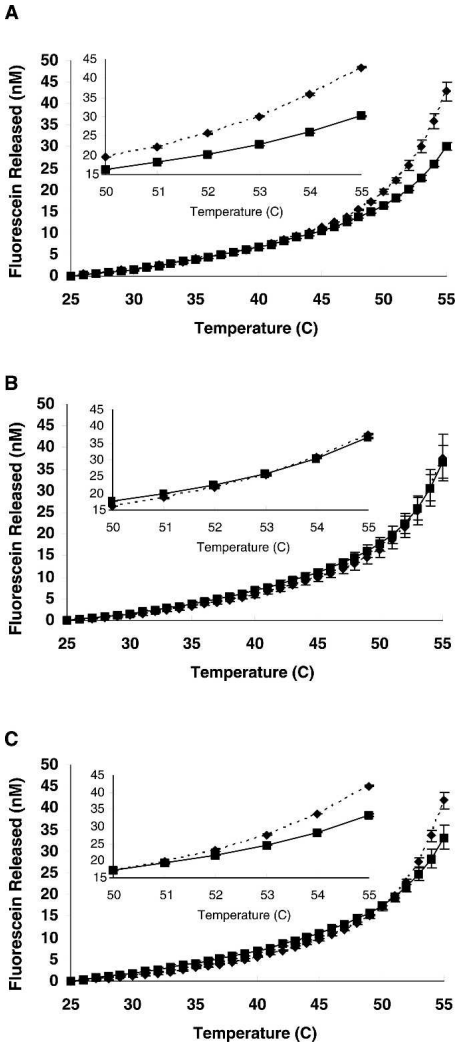

Figure 5. Increase in thermal response due to elastin-like peptide linker. Temperature-corrected free fluorescein concentrations for flexible (■) and sELP (◆) linker mutants were measured on a iCycler (BioRad) and plotted as a function of temperature. Data for linkers of six amino acids (*A*), eight amino acids (*B*), and 10 amino acids (*C*) are presented. The thermal scanning rate was 0.5°C/min. Comparing linkers of equal length, sELP linkers exhibit an enhanced release of fluorescein as temperature increases for a linker of six amino acids (*A*) and 10 amino acids (*C*). For linkers eight amino acids in length (*B*), there is no difference between the flexible and the sELP linker. (*Inset*) The higher temperature data. Error bars represent the standard deviation of individual experiments. Each experiment was performed in at least triplicate (n ≥ 3).

difference in release of fluorescein with respect to linker length. Using Tukey's post-hoc analysis on the single-factor ANOVA data, only 4D5Flu-E8 exhibited a thermal response that was statistically different from the other sELP linker-containing mutants analyzed. The 4D5Flu-E8 mutant released only 36 nM fluorescein, while the other mutants analyzed released 42–44 nM fluorescein (Supplemental Table S1).

Comparing the measured length-matched data for flexible and sELP mutants and performing a two-way ANOVA indicated there is not only a statistically significant difference between the flexible and sELP linker scFv thermal release characteristics, but there is also an interaction between the effect of linker length and composition. This interaction can best be described through an examination of the release behaviors for flexible and elastin-like mutants. For all linker lengths analyzed, the sELP linker-containing mutants released either more or the same amount of fluorescein as compared with their length-matched flexible counterparts. At 55°C, the K_Ds for flexible and sELP linker antibodies were 400–1000 nM, increased from 20–50 nM (Fig. 6; tabulated data in Supplemental Table 1). There is a significant difference in release behavior between sELP and flexible-linker-containing scFvs that are both six and 10 amino acids in length. At 25°C, the binding affinities of the flexible and sELP mutants six amino acids in length are statistically different; however, their difference is quite small and within the range of values for longer flexible-linker

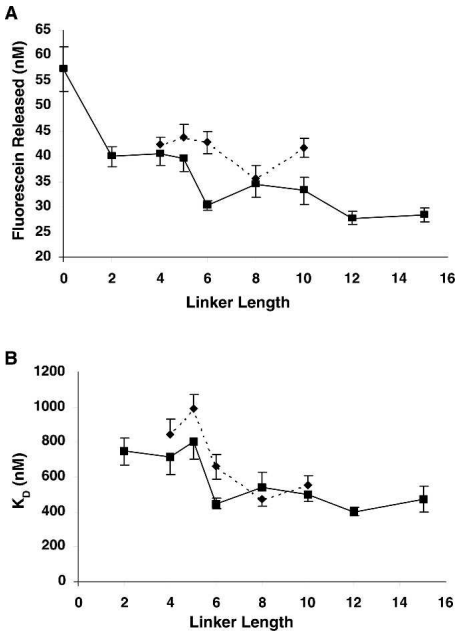

Figure 6. Thermally induced release of fluorescein. (*A*) Fluorescein released by scFv mutants with flexible linkers (■) and with sELP linkers (◆) at 55°C was measured as the temperature-corrected difference between free fluorescein concentration at 25°C and 55°C. The thermal scanning rate was 0.5°C/min in an iCycler (BioRad) with fluorescence measured using the green channel. The largest differences in fluorescein release between length-matched scFvs occur with linker lengths of six and 10 amino acids. (*B*) Calculated equilibrium dissociation constants at 55°C for flexible linkers (■) and sELP linkers (◆). Error bars represent the standard deviation. Where the error bars overlap, only the *upper* and *lower* error bars are shown for clarity. Each experiment was performed in at least triplicate (n ≥ 3).

mutants. On the other hand, at 55°C, the 4D5Flu-E6 had a K_D over 200 nM larger (47% increase) than 4D5Flu-6. A smaller difference is noted for five amino acids, while no difference is observed for linkers of four or eight amino acids.

Salt-responsive release of fluorescein is different than thermal-responsive release

Large ELPs are known to be responsive to changes in salt concentration in the same manner in which they are temperature-responsive. Increased salt concentrations cause ELPs to form β-turn structures (Urry 1993). Samples containing 500 nM purified scFv and 100 nM fluorescein were incubated in PBS with varying amounts of NaCl. The six- and 10-amino-acid linker-length mutants were used in the salt study since they performed best in the temperature-responsive study. Figure 7A shows the fluorescein released due to the addition of NaCl for the pair of six-amino-acid linker-length mutants. As the concentration of salt increases, there is an increase in the concentration of fluorescein released from the scFvs. Interestingly, however, the flexible-linker mutant always released more fluorescein than the sELP linker mutant of equal length. The maximum separation between flexible and elastin linker occurs when 0.7 M NaCl is added. Figure 7B shows the fluorescence release due to the addition of NaCl for 10-amino-acid linker mutant scFvs. The response characteristics observed for 10-amino-acid linkers were similar to those observed for six-amino-acid linkers, including the increase in release of fluorescein and the maximum release at 0.7 M NaCl.

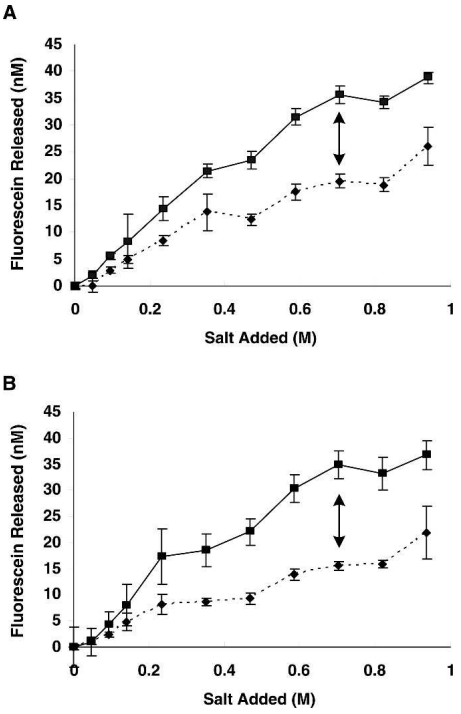

Figure 7. Decrease in responsiveness with increased ionic strength. Comparing linkers of equal length, flexible peptide linkers exhibit an enhanced release of fluorescein as temperature increases. (*A*) Temperature-corrected free fluorescein concentration for 4D5Flu-6 (■) and 4D5Flu-E6 (◆) are plotted as a function of NaCl added. (*B*) Temperature-corrected free fluorescein concentration for 4D5Flu-10 (■) and 4D5Flu-E10 (◆) are plotted as a function of NaCl added. (Arrows) Salt concentration that gives rise to the largest difference in fluorescein release (0.7 M). Error bars represent the standard deviation of individual experiments. Each experiment was performed in at least triplicate ($n \geq 3$).

When comparing the responsiveness of the scFv to either temperature or salt, it is convenient to discuss the behavior in terms of changes in the free energy of binding. Naturally, there is an increase in the free energy of antigen antibody complex upon increasing the temperature (25°C to 55°C) or adding salt (0–0.7 M), which is denoted as ΔG (Fig. 8). It is the difference between the binding free energy of the responsive (sELP) and non-responsve (flexible) mutants, Δ(ΔG), that drives the separation of stimulus-responsive peptide-containing scFvs in bioaffinity-based selection experiments. The difference in Δ(ΔG) is similar for the temperature experiments using either the six- or 10-amino-acid linkers. Likewise, the difference is similar for the salt experiments using either linker; however, it is in the opposite direction compared with the temperature experiment.

Discussion

Room-temperature binding affinities of 4D5Flu mutants were relatively unaffected by linker length. The presence of diabodies at all linker lengths masks the effect of prohibitively short linkers. Regardless, our experiments confirm and clarify the phenomena described by Megeed et al. (2006). Their comparison of sELP linker to a longer flexible linker demonstrated a stimulus-responsive difference in binding affinity. However, in a directed evolution experiment, where scFv libraries with randomized peptide linker sequences are selected for stimulus-responsive behavior, it is essential to compare linkers of equal length. Our work demonstrated that there is a notable difference in thermal responsiveness for flexible and sELP linkers of equal length for six and 10 amino acids. Interestingly, no significant thermal responsiveness was observed for a sELP linker of eight amino acids (GVGVPGVG). It is possible that the two GVG sequences that flank the proline residue confer too much flexibility to the linker. Megeed's study noted that no responsive behavior was seen when two flexible GGGGS sequences flanked a single sELP sequence (Megeed et al. 2006).

Next, we set out to determine the optimal linker length of 4D5Flu scFv such that the difference in the binding affinity between length-matched, nonresponsive flexible and stimulus-responsive elastin-like linker mutants was

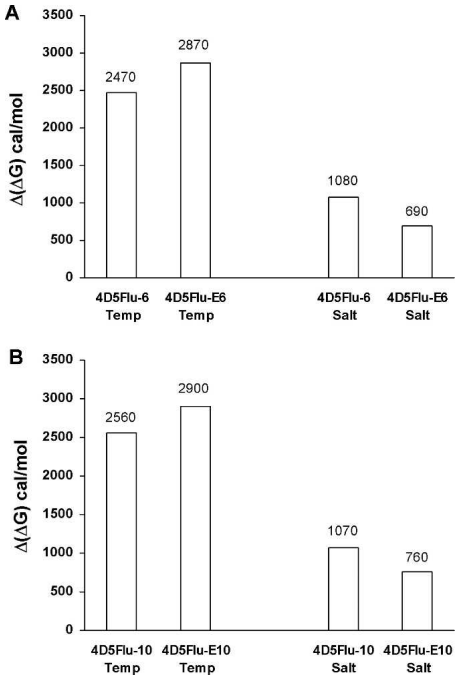

Figure 8. Stimulus-responsive change in binding free energy. The difference between the free energy of binding at the final and initiation conditions (condition of maximum difference, 0.855 M NaCl added for salt experiments or 55°C for thermal experiments, and the ambient condition 0.155 M NaCl and 25°C) for elastin and flexible linkers of either six amino acids (*A*) or 10 amino acids (*B*) is shown for both temperature and salt. For thermal-responsive experiments, flexible linkers appear to confer greater stability compared with sELP linkers as temperature is increased. However, for the salt-responsive scFv experiments, the sELP linkers confer the greater stability under conditions of higher salt concentration.

maximized. For the mutants we analyzed, a linker of six amino acids is the optimal length for thermally induced release of fluorescein (10 amino acids also provides a significant thermally induced response). Reiersen and coworkers were successful in using an elastin peptide (GVPGVG) to make a thermally responsive Protein A minidomain. They report that circular dichroism (CD) spectra were consistent with the formation of a type I β-turn as the temperature is increased (Reiersen and Rees 1999). The β-turn CD signal correlated with the change in binding affinity for IgG. In their experiments, the temperature was raised only to 37°C, a temperature not sufficiently high to affect the helices in the minidomain. The scFv was subjected to two rounds of temperature cycling, and the room temperature quenching was unaffected, indicating the release of fluorescein is not due to irreversible thermal denaturation of the antibody fragments. The only mutant that did not show reversible binding was 4D5Flu-0, indicating that the scFv can tolerate linker lengths as low as two amino acids. The upper limit measured was 15 amino acids, the longest linker we studied.

The reversibility demonstrated for 4D5Flu suggests that the general structure of the antibody domains remains unaltered at 55°C. In further support of this idea, the 4D5 antibody was engineered to have high thermodynamic stability (Jung and Pluckthun 1997). As the temperature is increased, the binding affinity decreased as a result of the increased off rate associated with the higher temperature, and by the effect of the responsive linker. Our analysis of sized-matched scFv linker mutants was meant to demonstrate solely the effects of the linker on scFv stimulus-responsive behavior. It seems likely that the increased temperature only significantly affects the peptide-tethered light and heavy domain interaction and the peptide linker. Thus, the stimulus-responsive behavior of the scFv is in fact due to the stimulus-responsive nature of the peptide linker and not to some other effect (such as thermal denaturation). Our findings suggest that the major contribution to the stimulus-responsive behavior is the perturbation of interdomain associations (diabodies) rather than the linker-constrained disruption of intramolecular (monomer) association forming the fluorescein binding pocket. While intramolecular dissociation may be occurring for the small population of monomer, the diabody species dominates the ensemble stimulus-responsive behavior.

A more detailed look at the individual fluorescein release profiles provides a richer analysis of the thermal responsiveness conferred by the sELP peptide linkers. The amount of fluorescein released by 4D5Flu-E6 and 4D5Flu-6 mutants was not statistically different until 48°C. For the 4D4Flu-E10 and 4D5Flu-10 mutants, the difference in fluorescein released was not statistically different until 53°C was reached. It may be possible to characterize this as the transition temperature of the elastin peptide β-turn formation; however, secondary structure data will be needed to confirm this. These transition temperatures are higher than expected based on the work of Reieresen, where at pH 7, GVGVPGVG has a transition temperature of 41°C. This can be explained by either the contribution of the charged and hydrophobic residues flanking the elastin peptide (Reiersen et al. 1998), or by the thermodynamic opposition to structural fluctuations leading to β-turn formation due to the bound ligand (Sagawa et al. 2007). These results suggest that a single-chain antibody or similar construct has potential to be used as a nonspectroscopic means of sensing peptide conformational change in peptide linkers.

Since both increasing temperature and increasing salt concentration cause elastin β-turn formation, we initially expected similar results for the temperature and salt experiments. However, the elastin linker mutants were less responsive to an increase in salt concentration than were the flexible mutants. In one report, the salt-responsive behavior of a sELP linker in a Protein A minidomain

was studied (Reiersen and Rees 2000). Sodium sulfate salt was used to induce a type I β-turn in the sELP linker, but since it is a kosmotrope, it may also have increased the α-helical content of the region responsible for IgG binding, contributing to the observed salt-responsive behavior. Sodium chloride, on the other hand, should shield electrostatic effects and enhance the hydrophobic nature of the residues in the protein. It has been suggested that agents enhancing the hydrophobic effect can alter fluorescein binding affinity through increased domain dissociation (Muller et al. 1994). However, it was also demonstrated that increased ionic strength stabilizes diabody formation (Arndt et al. 1998). While there is no crystal structure available for the 4D5Flu scFv, several hypotheses can be proposed to explain these phenomena, based on the crystal structure of the 4D5 scFv and the 4–4-20 scFv from which 4D5Flu was engineered. It is possible that the structure formed by the elastin β-turn, which has a shorter end-to-end distance than the length-matched flexible linker, tethers the two domains closer together, thereby inhibiting their dissociation. It is also possible that the effect of salt caused the linker to be buried in a salt-shielded hydrophilic patch on the antibody surface. This would explain why the elastin mutants are more thermally responsive when compared with flexible mutants and more stable in the presence of salt.

A comparison of elastin and flexible linkers of the same length provides a more realistic indication of how these scFv conformational change sensors would perform in biopanning experiments aimed at the directed evolution of new stimulus-responsive peptides. Similar changes in $\Delta(\Delta G)$ of both the six- and 10-amino-acid linkers suggest that a similar phenomenon is responsible for the thermal responsive behavior observed. Salt-induced differences in $\Delta(\Delta G)$ for six- and 10-amino-acid linkers are also similar to one another, again suggesting a common underlying mechanism. The difference in $\Delta(\Delta G)$ for the temperature experiments was ~0.4 kcal/mol, similar to several long-range electrostatic interactions (Loewenthal et al. 1993). Similarly, this change in energy can also be equated to the loss of ~16 $Å^2$ of hydrophobic surface area (Chothia 1974).

This work highlights the importance of the choice of stimulus and calibration of the linker length and sequence for the design of a stimulus-responsive scFv. This knowledge and insight into the mechanism creating the stimulus-responsive behavior will aid in the development of novel stimulus-responsive scFvs. One potential application of this work is the creation of stimulus-responsive antibodies where elastin or other short stimulus-responsive peptides are used to control antigen binding. This strategy could be applied to control therapeutic or diagnostic scFvs. Using molecular evolution, one could select linkers that confer responsive behavior to novel physiologically relevant stimuli. While there is certainly a global penalty for using temperature or salt to alter scFv binding affinity, molecular evolution may be used with a stimulus that does not levy as high a thermodynamic penalty or suffer from interference with antigen binding to scFv with a nonresponsive peptide linker. Similarly, scFvs may potentially be used as a nonspectroscopic reporter of peptide linker conformational state. Development of this protein-based tool, compatible with common molecular evolution techniques, could quickly identify the conformational state of a peptide under a given set of conditions and speed the development of novel stimulus-responsive peptides (Chockalingam et al. 2007). As biotechnology continues to create novel proteins and peptides with potentially important applications, development of short nondisruptive peptide control mechanisms may be of great importance in making current technologies ''smarter'' and creating a toolbox of peptide regulatory elements that can be combined with other protein and peptide modules.

Materials and Methods

Materials

QuikChange mutagenesis kits were purchased from Stratagene. All other enzymes were purchased from New England Biolabs. All chemicals were purchased from Sigma-Aldrich, unless otherwise noted. Oligonucleotides were purchased from either Sigma Genosys or Integrated DNA Technologies.

Construction of plasmids for single-chain antibody mutants

The 4D5Flu-15 plasmid was a generous gift of Andreas Plückthun at the University of Zurich (Jung and Pluckthun 1997). BseRI and AflII restriction enzyme sites were used for cassette mutagenesis. The 4D5Flu-10 mutant containing a $(GGGGS)_2$ linker was generated by cassette mutagenesis using 5′-TTAAGCGTGCTGGAGGTGGTGGTTCTGGAGGTGGTGGT TCTGA-3′ and 5′-AGAACCACCACCTCCAGAACCACCACCT CCAGCACGC-3′. The 4D5Flu-5 mutant containing five amino acids (GGGGS) was likewise created by cassette mutagenesis using 5′-TTAAGCGTGCTGGAGGTGGTGGTTCTGA-3′ and 5′-AG AACCACCACCTCCAGCACGC-3′. Ligations were performed using T4 DNA Ligase for 16 h at 16°C. Starting with the 4D5Flu-10 mutant, QuikChange mutagenesis was used to create the 4D5Flu-12 mutant $GS(GGGGS)_2$. The mutagenic oligonucleotides used were 5′-GTACTAAAGTTGAACTTAAGCGTGCTGG TTCTGGAGGTGGTGGTTCTGGAGGTGGTGG-3′ and its compliment. The 4D5Flu-5 mutant was used a template for the creation of the 4D5Flu-6 mutant S(GGGGS). The mutagenic oligonucleotides used were 5′-CTAAAGTTGAACTTAAGCGTGCTTCCGG AGGTGGTGGTTCTGAAGTACAGC-3′ and its compliment. The 4D5Flu-6 mutant was used to make the 4D5Flu-8 mutant GGS (GGGGS). The mutagenic oligonucleotides used were 5′-GTTGA ACTTAAGCGTGCTGGAGGTTCCGGAGGTGGTGGTTCTGAA-3′ and its compliment. The 4D5Flu-5 mutant was also the template for the 4D5Flu-4 mutant (GGGS). The mutagenic oligonucleotides

used were 5′-GAACTTAAGCGTGCTGGTGGTGGTTCTAAG-3′ and its compliment. From this mutant, the 4D5Flu-2 mutant (GS) was created using 5′-TAAAGTTGAACTTAAGCGTGCTGGTT CTGAAGTACAGCTGGTAGAAAGCG-3′ and its compliment. Finally, the 4D5Flu-0 mutant (no linker) was made using 5′-CTAAAGTTGAACTTAAGCGTGCTGAAGTCACACTGGTAGA AAGCGG-3′ and its compliment.

The 4D5Flu-E6 mutant containing an elastin-like polypeptide (ELP) linker, G(VPGVG), was a generous gift of Martin Yarmush at Harvard Medical School (Megeed et al. 2006) and was used as a template for the creation of the 4D5Flu-E8 mutant GVG(VPGVG). The mutagenic oligonucleotides used were 5′-GTTGAACTTAACGTGCTGGAGTAGGTGTTCCGGGCGTAG GTGAAGTAC-3′ and its compliment. This was then used as a template for the creation of the 4D5Flu-E10 $(VPGVG)_2$ mutant. The mutagenic oligonucleotides used were 5′-CTAAAGTTGAA CTTAAGCGTGCTGTTCCGGGAGTAGGTGTTCCGGGCGTAG GTGAAG-3′ and its compliment. The 4D5Flu-E6 mutant was also used as a template for the 4D5Flu-E5 mutant (VPGVG) using 5′-GTTGAACTTAAGCGCGCTGTTCCGGGCGTAGGTGAAG-3′ and its compliment. The 4D5Flu-E5 mutant was also used as a template for the 4D5Flu-E4 mutant (VPGV) using 5′-GCTGTTC CGGGCGTAGAAGTACAGCTGGTAG-3′ and its compliment. A schematic describing the mutants can be found in Figure 1.

Single-chain antibody expression and purification

Expression and purification protocols were similar to those reported previously (Jung and Pluckthun 1997; Megeed et al. 2006) with small modifications. Briefly, 4D5Flu mutants were expressed in JM83 *Escherichia coli* cells. Ten milliliters of an overnight culture was used to inoculate 1 L of LB media. Cultures were grown to $OD_{550} = 0.5$ and induced with IPTG to a final concentration of 1 mM. Cultures were grown for 3 h before harvesting at 6000*g*. Cell pellets were resuspended in 20 mM HEPES, 500 mM NaCl, pH 6.9 and sonicated. Clarified lysate was purified using HisTrap IMAC on an AKTA-FPLC (GE Healthcare). Nonspecifically bound protein was removed with 50 mM immidizole. Fractions eluted with 250 mM immidizole were concentrated on Centriplus 10-kDa filter devices (Millipore) and further purified on a HiLoad 16/60 Superdex 200-pg gel filtration column (GE Healthcare). Gel filtration experiments were performed in PBS with a 1 mL/min flow rate. Apparent molecular weights for gel filtration experiments were determined measuring the elution volume of lysozyme, carbonic anhydrase, and conalbum protein standards (GE Healthcare) under experimental operating conditions. Apparent purity was >99% as determined by SDS-PAGE (Fig. 3). Active fractions were pooled and either used immediately or aliquoted and frozen (−20°C) for use within 1 mo.

Fluorescein binding assay

Fluorescein binding experiments were preformed as described previously (Jung and Pluckthun 1997) with small modifications. Protein concentrations were determined spectrophotometrically using an extinction coefficient calculated as described previously (Gill and von Hippel 1989). Protein concentrations used in the assay ranged from micromolar to low nanomolar. Titration of fluorescein with antibody was performed in black, round-bottomed 96-well plates (Costar). Fluorescein was excited at 483 nm, and the emission was measured at 515 nm on a SpectraMax M2 plate reader (Molecular Devices). Fluorescence data were fit to Equation (1) (Jung and Pluckthun 1997) using a least-squares analysis.

$$F = F_0 + (F_\infty - F_0) \times \frac{\frac{[Ab_{tot}]+[Ag_{tot}]+K_D}{2} - \sqrt{\left(\frac{[Ab_{tot}]+[Ag_{tot}]+K_D}{2}\right)^2 - [Ab_{tot}][Ag_{tot}]}}{[Ag_{tot}]} \quad [1]$$

The dependent variable F is the fluorescence intensity of fluorescein measured at a concentration of scFv $[Ab_{tot}]$ as the independent variable. F_0 and F_∞ are the fluorescence intensities in the absence of scFv and in the presence of saturating scFv, respectively. $[Ag_{tot}]$ is the total concentration of fluorescein used in each well. The parameters determined from the least-squares fit were K_D and F_∞. Each binding assay was performed in at least triplicate.

Temperature modulation of fluorescein binding

Samples of 4D5Flu mutants (500 nM) were incubated in fluorescein (100 nM) for 10 min at 25°C. The temperature was modulated from 25°C to 55°C (+0.5°C/min), and data were collected using an iCycle Real-Time PCR thermocycler (Bio-Rad). Experiments were performed in at least triplicate, and data were corrected for temperature effects on fluorescence.

Salt modulation of fluorescein binding

Samples of 4D5Flu mutants (500 nM) were incubated in fluorescein (100 nM) with varying salt concentrations (0.155–1.315 M) for 30 min at 25°C. Experiments were performed in black, round-bottomed 96-well plates (Costar). Fluorescein was excited at 483 nm, and the emission was measured at 515 nm on a SpectraMax M2 plate reader (Molecular Devices). Experiments were performed in at least triplicate.

Statistics

Pairwise statistical significance was determined using Student's t-test, where $\alpha = 0.05$. Two-way ANOVA was performed using the Microsoft Excel Statistics package. Tukey's post hoc analysis was used to determine statistically significant differences using two-way ANOVA data. Errors bars represent the standard deviations.

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