FOR THE RECORD

# Designed heterodimerizing leucine zippers with a range of pIs and stabilities up to $10^{-15}$ M

JONATHAN R. MOLL,[1] SERGEI B. RUVINOV,[2] IRA PASTAN,[3] AND CHARLES VINSON[1]

[1]Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA
[2]Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA
[3]Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA

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**Abstract**

We have designed a heterodimerizing leucine zipper system to target a radionuclide to prelocalized noninternalizing tumor-specific antibodies. The modular nature of the leucine zipper allows us to iteratively use design rules to achieve specific homodimer and heterodimer affinities. We present circular-dichroism thermal denaturation measurements on four pairs of heterodimerizing leucine zippers. These peptides are 47 amino acids long and contain four or five pairs of electrostatically attractive $\mathbf{g} \leftrightarrow \mathbf{e}'$ (i, i′ +5) interhelical heterodimeric interactions. The most stable heterodimer consists of an acidic leucine zipper and a basic leucine zipper that melt as homodimers in the micro ($T_m$ = 28°C) or nanomolar ($T_m$ = 40°C) range, respectively, but heterodimerize with a $T_m$ >90°C, calculated to represent femtamolar affinities. Modifications to this pair of acidic and basic zippers, designed to destabilize homodimerization, resulted in peptides that are unstructured monomers at 4 μM and 6°C but that heterodimerize with a $T_m$ = 74°C or $K_{d(37)} = 1.1 \times 10^{-11}$ M. A third heterodimerizing pair was designed to have a more neutral isoelectric focusing point (pI) and formed a heterodimer with $T_m$ = 73°C. We can tailor this heterodimerizing system to achieve pharmacokinetics aimed at optimizing targeted killing of cancer cells.

**Keywords:** Leucine zipper; dimerization; electrostatic interactions; radioimmunotherapy; salt bridges; heterodimer

Leucine zipper–containing proteins have been adapted for a variety of purposes, but in the natural setting they are only known to function within cells. Heterodimerizing leucine zippers have been used previously to bring chimeric proteins together in solution (Scott et al. 1996), within cells (Katz et al. 1998), and extracellularly (Behncken et al. 2000). We intend to use designed heterodimerizing leucine zippers to deliver radionuclides to the surface of cancer cells. This allows a pretargeting approach in which a tumor-specific antibody appended to one leucine zipper monomer is first bound to the cancer cells. After removal of nonspecifically bound and circulating antibody, the complementary zipper containing a radionuclide can be introduced.

Presently, investigators are using the streptavidin/biotin system to introduce a radionuclide to a prelocalized cancer antibody (Axworthy et al. 2000; Breitz et al. 2000; Weiden et al. 2000). In this three-step procedure, streptavidin-conjugated antibody first is injected and allowed to accumulate on the tumor surface. After peak tumor uptake has occurred, the unlocalized antibody is removed from the circulation with a clearing agent. Finally, a small molecular weight biotin conjugate, radiolabeled to high specific activity, is injected. The radiolabeled biotin either binds to streptavidin on the antibody or is rapidly excreted in the urine, thereby minimizing its toxic effects. Although this method has

Reprint requests to: Charles Vinson, Building 37, Room 4D06, Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA; e-mail: Vinsonc@dc37a.nci.nih.gov; fax: (301) 496-8419.

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shown promise in clinical trials, it has several disadvantages. First, biotin occurs naturally in human plasma thus blocking streptavidin sites on the conjugated antibody. Second, both avidin and streptavidin are immunogenic.

We present a heterodimerizing leucine zipper system that overcomes both limitations with the streptavidin/biotin system and offers potential advantages. First, heterodimeric leucine zippers derived from human B-ZIP proteins are unlikely to be immunogenic. Second, there are no known leucine zippers circulating in the blood to complicate the function of a heterodimerizing leucine zipper system. Third, as the dimerization affinity of both homo- and heterodimers can be experimentally manipulated, we have the ability to select heterodimerizing leucine zipper pairs with the optimal pharmacokinetics.

The structural rules used in our design (Alber 1992; Baxevanis and Vinson 1993; Hodges 1996; Lupas 1996) are derived from studies of troponin C (Zhou et al. 1994), the yeast GCN4 leucine zipper (Harbury et al. 1993), and vertebrate leucine zippers (O'Shea et al. 1992; Krylov et al. 1998;). These heterodimerizing zippers are variants of VBP, a B-ZIP protein extensively studied in this laboratory. The VBP homodimer contains four pairs of attractive $\mathbf{g} \leftrightarrow \mathbf{e}'$ interhelical electrostatic interactions. Previously, we mutated the last two heptads of VBP to produce either an acidic zipper, $EE_{34}$ ($T_m$ = 22°C, $K_{d(37)} = 8.1 \times 10^{-4}$ M) or a basic zipper, $RR_{34}$ ($T_m$ = 27°C, $K_{d(37)} = 3.1 \times 10^{-5}$ M). These leucine zippers have two pairs of attractive and two pairs of repulsive $\mathbf{g} \leftrightarrow \mathbf{e}'$ salt bridges in the homodimer, but the heterodimer has four pairs of attractive $\mathbf{g} \leftrightarrow \mathbf{e}'$ salt bridges and similar stability to the natural VBP homodimer ($K_d$ = ~$10^{-8}$ M). Mutating all four salt bridges to produce $EE_{1234}$ and $RR_{1234}$ maintains four pairs of salt bridges in the heterodimer, but homodimers have four pairs of repulsive interactions. Consistent with this, homodimers are unstructured monomers at 6°C but heterodimers have a $T_m$ of 51°C, identical to the original VBP homodimer (O'Shea et al. 1992; Krylov et al. 1998).

We used $EE_{1234}$ and $RR_{1234}$ as a starting point for the leucine zipper protein designs presented here. By altering residues in the leucine zipper **a**, **d**, **g**, and **e** positions, we have designed heterodimerizing pairs with varying pIs and with stabilities in the femta- to nanomolar range. Designing heterodimers with a range of thermodynamic properties will allow us to select pairs with the appropriate pharmacokinetics for the delivery of radionuclides to antigen-specific antibodies prelocalized to cancer cells.

## Results

### *Stabilizing the heterodimer ($T_m$ > 90°C): $EE_{1234}$L and $RR_{1234}$L*

To increase heterodimer stability of $EE_{1234}/RR_{1234}$ from $T_m$ = 51.5°C, $\Delta G = -11.6$ kcal/mole ($K_{d(37)} = 7 \times 10^{-9}$ M), we altered the hydrophobic interface by mutating the first and fifth **d** positions to leucine, replacing an isoleucine and cysteine, respectively. Previous work indicates that a cysteine to leucine change in the fifth **d** position of the VBP leucine zipper contributes 7.2 kcal/mole/dimer to stability. An isoleucine to leucine change contributes 6.0 kcal/mole/dimer to stability (Moitra et al. 1997). Several amino acids in the **b**, **c**, and **f** positions also were altered to eliminate charged residues that may interfere with attractive $\mathbf{g} \leftrightarrow \mathbf{e}'$ salt bridges (see Fig. 1A for schematic). All lysine residues, except those at the end of the N-terminal or C-terminal extensions, were replaced to allow specific attachment of the radionuclide (or antibody) at the termini via lysine chemistry. We denote these new peptides $EE_{1234}$L and $RR_{1234}$L with the L indicating the stabilized hydrophobic interface.

The $EE_{1234}$L and $RR_{1234}$L peptides have increased stability as either homodimers or heterodimers (Fig. 2A; Table 1). Homodimer stability was increased from unstructured at 6°C to $T_m$ = 28°C for $EE_{1234}$L and $T_m$ = 40°C for $RR_{1234}$L. We have shown that a R ↔ R salt bridge is 0.47 kcal/mole more stabilizing than a E ↔ E salt bridge (Krylov et al. 1998). Thus, we calculate that $RR_{1234}$L should be 3.76 kcal/mole more stable than $EE_{1234}$L (Krylov et al. 1994), in

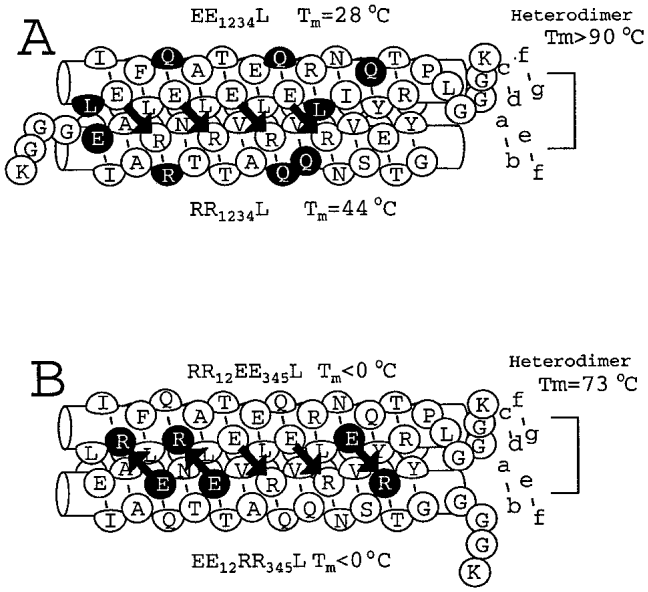


**Fig. 1.** Schematic of two heterodimerizing leucine zipper pairs. The coiled coil region is presented, without the supercoiling, with the relative placement of the seven amino acids in the coiled coil heptad presented by circles. To the right is the identification of the **a**, **b**, **c**, **d**, **e**, **f**, and **g** positions of the coiled coil. (Solid arrows) The potential electrostatic interactions between the **g** position of one helix and the following **e**′ position of the opposite helix (we refer to this interaction as the $\mathbf{g} \leftrightarrow \mathbf{e}'$ or the i, i′ +5 interaction). (Black circles with white lettering) Changes from the previous construct in the series. (*A*) Heterodimer of $EE_{1234}$L and $RR_{1234}$L. These constructs are altered from $EE_{1234}$ and $RR_{1234}$. (*B*) Heterodimer of $RR_{12}EE_{345}$ and $EE_{12}RR_{345}$. These are derived from $EE_{1234}$L and $RR_{1234}$L.

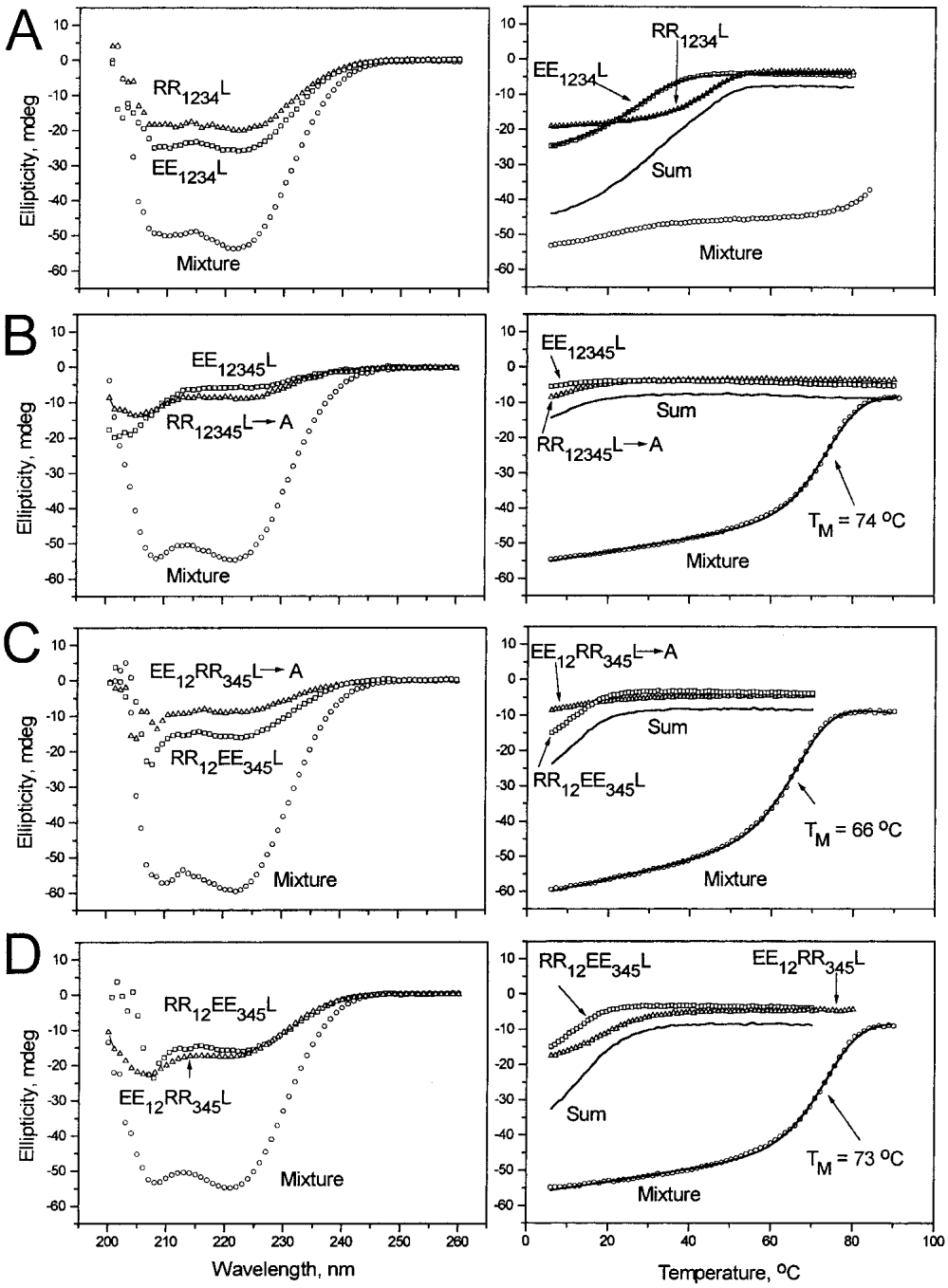


**Fig. 2.** CD spectral data (*left*) and thermal denaturation curves (*right*) for heterodimerizing leucine zippers. (Circles) Heterodimer; (squares) acidic monomer/homodimer; (triangles) basic peptide. CD denaturation curves are recorded at 222 nm. (Solid line) Expected thermal denaturation if the two proteins do not interact. The line through each denaturation curve is a fitted curve for a two-state transition as described in Materials and Methods. (*A*) $EE_{1234}L/RR_{1234}L$; (*B*) $EE_{12345}L/RR_{12345}L \rightarrow A$; (*C*) $EE_{12}RR_{345}L \rightarrow A/RR_{12}EE_{345}L$; (*D*) $EE_{12}RR_{345}L/RR_{12}EE_{345}L$.

good agreement with the 3.2 kcal/mole observed difference in stability.

The $EE_{1234}L/RR_{1234}L$ heterodimer denaturation is incomplete, melting above 90°C, which prevents determination of its thermodynamic parameters from these data. The $EE_{1234}L/RR_{1234}L$ heterodimer has four E ↔ R and four R ↔ E salt bridges compared with eight R ↔ R salt bridges found in the $RR_{1234}L$ homodimer. We have shown that E ↔ R is 1.2 kcal/mole more stable than R ↔ R and R ↔ E is 1.5 kcal/mole more stable than R ↔ R (Krylov et al. 1998). Thus, we calculate that the heterodimer should be 10.8 kcal/mole more stable than $RR_{1234}L$, giving an ex-

**Table 1.** *Thermodynamic parameters of heterodimerizing zippers*

| Protein (homodimer)<br>Θ, $T_m$(ΔG, ΔH)$K_{d(37)}$ | Protein (homodimer)<br>Θ, $T_m$(ΔG, ΔH)$K_{d(37)}$ | Protein (heterodimer)<br>Θ, $T_m$(ΔG, ΔH)$K_{d(37)}$ | Heterodimer MW (kD)<br>calculated (experimental) |
|---|---|---|---|
| $EE_{1234}L$ (pI = 3.7)<br>−25, 28 (−6.2, −45) $4.4 \times 10^{-5}$ | $RR_{1234}L$ (pI = 12.4)<br>−19, 44(−9.4, −66) $2.5 \times 10^{-7}$ | $EE_{1234}L/RR_{1234}L$<br>−53, >90, N.D., N.D., ~$10^{-15}$[a] | 10,870 (11.8 ± 0.8) |
| $EE_{12345}L$ (pI = 3.6)<br>$T_m < 0$ | $RR_{12345}L \rightarrow A$ (pI = 12.7)<br>$T_m < 0$ | $EE_{12345}L/RR_{12345}L \rightarrow A$<br>−55, 74 (−15.5, −75) $1.1 \times 10^{-11}$ | 10,844 (11.6 ± 1.0) |
| $RR_{12}EE_{345}L$ (pI = 4.7)<br>$T_m < 0$ | $EE_{12}RR_{345}L \rightarrow A$ (pI = 11.0)<br>$T_m < 0$ | $RR_{12}EE_{345}L/EE_{12}RR_{345}L \rightarrow A$<br>−59, 66 (−14.6, −81) $4.7 \times 10^{-11}$ | N.D. |
| $RR_{12}EE_{345}L$ (pI = 4.7)<br>$T_m < 0$ | $EE_{12}RR_{345}L$ (pI = 10.9)<br>$T_m < 0$ | $RR_{12}EE_{345}L/EE_{12}RR_{345}L$<br>−57, 73 (−15.4, −74) $1.3 \times 10^{-11}$ | 10,886 (11.1 ± 1.2) |

The table presents ellipticity at 6°C (Θ), the melting temperature ($T_m$, °C), ΔG (in kcal/mole), ΔH (kcal/mole), and $K_d$ (molar) at 37°C in circular dichroism thermal melts for homodimer and heterodimer samples, and molecular weights of mixtures at 6°C as determined by analytical ultracentrifugation (Krylov et al. 1994).

[a] Calculated from previously derived thermodynamic data (Krylov et al. 1998) as described in the text.

(MW) molecular weight; (N.D.) not determined.

pected $K_{d(37)}$ of $5.1 \times 10^{-15}$ M. A similar calculation relative to the $EE_{1234}L$ homodimer gives a $K_{d(37)}$ of $1.4 \times 10^{-15}$ M, numbers consistent with the heterodimer denaturation curve. These values are similar to the dissociation constant for steptavidin/biotin that has $K_{d(37)} = 1 \times 10^{-15}$ M (Axworthy et al. 2000).

Sedimentation equilibrium experiments confirm that the $EE_{1234}L$ and $RR_{1234}L$ mixture is dimeric (Fig. 3; Table 1), making this the most stable dimeric leucine zipper described to date.

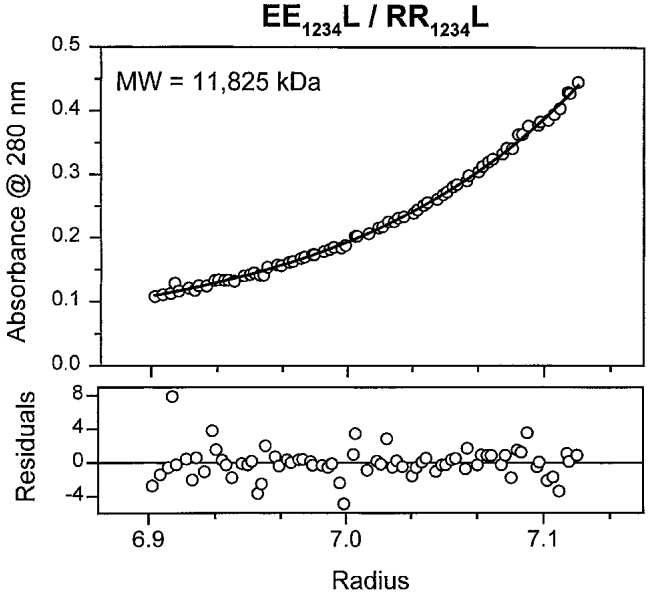


**Fig. 3.** Molecular weight (MW) determination by sedimentation equilibrium of $EE_{1234}L/RR_{1234}L$ at 6°C. The sample was in 12.5 mM potassium phosphate at pH 7.4, 150 mM KCl, 0.25 mM EDTA. (Solid line) Theoretical curve for dimer molecular weight; (circles) plotted actual data. (*Bottom panel*) The residuals for fitting the experimental data to a dimer model. No systematic error was observed.

*Destabilizing the homodimers: $EE_{12345}L$ and $RR_{12345}L \leftrightarrow A$*

Although we had achieved exceptional heterodimer stability, we were concerned that homodimerization in vivo would interfere with heterodimer formation. To decrease homodimer stability, we introduced an additional repulsive electrostatic $\mathbf{g} \leftrightarrow \mathbf{e}'$ pair in both zippers. In $EE_{1234}L$, we changed the fifth $\mathbf{g} \leftrightarrow \mathbf{e}'$ pair from I ↔ E to E ↔ E to form $EE_{12345}L$. Similarly, in $RR_{1234}L$, we changed I ↔ E to R ↔ R to form $RR_{12345}L$.

To further decrease the stability of $RR_{1234}L$, the more stable homodimer, we mutated the fourth **d** position from leucine to alanine to create $RR_{12345}L \rightarrow A$. Previous work indicates that this should decrease stability of the homodimer by 9.2 kcal/mole (Moitra et al. 1997). Both homodimers show the beginning of a transition at 6°C in the 4-μm sample indicating a $T_m$ below 6°C (Fig. 2B). The $EE_{12345}L/RR_{12345}L \rightarrow A$ mixture is a dimer with a $T_m$ of 74°C, corresponding to ΔG = −15.5 kcal/mole and a dissociation constant of $K_{d(37)} = 1.1 \times 10^{-11}$ M (Table 1). The low stability of the homodimers prevents us from determining heterodimer specificity. However, in previous work we determined that the heterodimer between $EE_{34}$ and $RR_{34}$ is 6.8 kcal/mole more stable than $EE_{34}$ homodimer. This corresponds to a ratio of $5.7 \times 10^4$ for $K_{d(37)}$ homodimer/$K_{d(37)}$ heterodimer, or a 240-fold difference in $K_d$/heptad. For the $RR_{34}$ homodimer, heterodimerization with $EE_{34}$ is favored 50-fold/heptad (Krylov et al. 1994). Assuming five pairs of independent attractive interhelical salt bridges, we calculate that $EE_{12345}L$ prefers to heterodimerize with $RR_{12345}L \rightarrow A$ by $50^5$- or $3 \times 10^8$-fold. Likewise, $RR_{12345}L \rightarrow A$ prefers to heterodimerize with $EE_{12345}L$ by $240^5$- or $8 \times 10^{11}$-fold.

*Attaining a more neutral pI: $EE_{12}RR_{345}L \rightarrow A$ and $RR_{12}EE_{345}L$*

A potential clinical problem is the extreme acidity of $EE_{12345}L$ and the extreme basicity of $RR_{12345}L \rightarrow A$. Dis-

counting the lysine at the termini that will be chemically modified in the chimeric proteins, $EE_{12345}L$ has 12 glutamates and 2 arginines with a calculated pI of 3.6. $RR_{12345}L \rightarrow A$ has 13 arginines and 2 glutamates (pI = 12.7). These highly charged peptides may participate in nonspecific interactions in vivo. To make a peptide with more neutral overall charge, we changed the four amino acids in the first two **g** and **e** positions from glutamate to arginine to create $RR_{12}EE_{345}L$. $RR_{12}EE_{345}L$ now has eight glutamates and six arginines with a pI = 4.7. Similarly, four amino acids were changed from arginine to glutamate in the first two salt bridges of $RR_{12345}L \rightarrow A$ to create $EE_{12}RR_{345}L \rightarrow A$. This protein has six glutamates and nine arginines (pI = 11.0).

Figure 2C and Table 1 present thermal denaturation data for these peptides monitored at 222 nm by circular-dichroism (CD) spectroscopy. The homodimers were unstructured at 6°C whereas the heterodimer between $EE_{12}RR_{345}L \rightarrow A$ and $RR_{12}EE_{345}L$ has a $T_m$ of 66°C with a $\Delta G$ of −14.6 kcal/mole ($K_{d(37)} = 4.7 \times 10^{-11}$ M), which is 0.9 kcal/mole less stable than $EE_{12345}L/RR_{12345}L \rightarrow A$. This decrease in stability is presumably because of repulsive interactions between the second and third heptads. There will be one $E \leftrightarrow E$ and one $R \leftrightarrow R$ $\mathbf{e} \leftrightarrow \mathbf{g}'$ or i, i′ +2 repulsive interaction in the $EE_{12}RR_{345}L \rightarrow A$ and $RR_{12}EE_{345}L$ heterodimer that may account for the 0.9-kcal/mole decrease in stability. A similar decrease in stability has been observed in the C/EBP leucine zipper when a salt bridge is reversed (Moll et al. 2000). The sharpness of the melting transition reflected in the high enthalpies ($\Delta H$) suggests that the $EE_{12}RR_{345}L \rightarrow A/RR_{12}EE_{345}L$ heterodimer is a homogeneous sample, able to populate only a limited number of protein conformations. There was some concern that the monomer would fold onto itself producing intramolecular electrostatic interactions that may be observed in a less cooperative thermal denaturation, but this does not appear to be the case.

To restabilize the heterodimer, we changed $EE_{12}RR_{345}L \rightarrow A$ to $EE_{12}RR_{345}L$ by changing the fourth **d** position from alanine to leucine. We also moved the lysine linker from the N terminus to the C terminus to facilitate the chemical linking of this peptide to the antibody (Fig. 1B). The $EE_{12}RR_{345}L$ and $RR_{12}EE_{345}L$ mixture is a dimer with a $T_m$ of 73°C, an increase of 7°C (Fig. 2D; Table 1). Because of a decrease in the measured $\Delta H$, we calculate an increase in stability of only 0.8 kcal/mole. From previous studies, we would expect an $L \leftrightarrow L$ **d** position interaction to be >4.6 kcal/mole more stable than an $L \leftrightarrow A$ interaction. This discrepancy may reflect moving the lysine linker from the N terminus to the C terminus.

## Discussion

We designed a series of heterodimerizing leucine zipper peptides that have a greatly expanded range of homo- and heterodimer stabilities and pIs. This system could be widely applicable in bringing together a diverse array of molecules. Our aim is to use these heterodimerizing leucine zippers as an alternative to the streptavidin/biotin system that has been used in human cancer patients to deliver a radionuclide to prelocalized tumor antibodies (Axworthy et al. 2000; Breitz et al. 2000; Weiden et al. 2000). These heterodimerizing leucine zippers may overcome some of the experimental problems associated with the avidin/biotin system.

Previous work has shown that heterodimerizing leucine zippers can be designed by having one amphipathic α helix with glutamates in the **e** and **g** positions and a second amphipathic α helix with either lysines or arginines in the **e** and **g** positions. The homodimer is destabilized by repulsive interhelical $\mathbf{g} \leftrightarrow \mathbf{e}'$ interactions whereas the heterodimer is stabilized by attractive interhelical $\mathbf{g} \leftrightarrow \mathbf{e}'$ interactions. The stability of leucine zippers also can be regulated by changes in the **a** and **d** positions of the hydrophobic core (Moitra et al. 1997; Wagschal et al. 1999; Tripet et al. 2000). These two simple design rules have been used to successfully build four pairs of heterodimerizing peptides with the desired properties. The experimental versatility of this system will allow us to screen a series of heterodimerizing leucine zippers with a range of homo- and heterodimer stabilities for a pair that has the best pharmacokinetics.

Two experimental concerns were addressed in these designs. We wanted to attach the radionuclide to the acidic peptide because acidic peptides are rapidly excreted by the kidney, thus limiting toxicity resulting from circulating radionuclide. We also wanted the zipper bound to the antibody to be unstable. We expect that the zipper–antibody chimera will be constrained by diffusion on the cell surface after binding of the antibody, potentially leading to homodimerization at micromolar $K_d$ and preventing heterodimerization.

In the first phase, $EE_{1234}L$ and $RR_{1234}L$ were designed to increase heterodimer stability as compared with $EE_{1234}$ and $RR_{1234}$ by placing leucine residues in the hydrophobic interface. The heterodimer was extremely stable with a calculated dissociation constant of $\sim 1 \times 10^{-15}$, which is similar to that reported for the streptavidin/biotin system. The homodimers, however, had stabilities in the micro- to nanomolar range, which could kinetically preclude heterodimer formation in vivo.

In the second phase, we increased the number of attractive salt bridges in the heterodimer and repulsive interactions in the homodimers by adding an additional heptad of salt bridge interactions to produce $RR_{12345}L$ and $EE_{12345}L$. We thereby maximized $\Delta\Delta G$ between hetero- and homodimers and increased the range of affinities attainable. Introduction of a destabilizing alanine residue into the fourth **d** position to create $RR_{12345}L \rightarrow A$ made this homodimer unstable even at 6°C, and only moderately destabilized the heterodimer.

The first two design phases comprise an acid-base delivery system that may be useful in facilitating excretion of unincorporated radionuclide. We were concerned, however, that proteins with such extremely basic and acidic pIs may bind nonspecifically to host molecules. In the third design, we addressed this potential problem by altering the order of the $\mathbf{g} \leftrightarrow \mathbf{e}'$ salt bridges to make peptides with more neutral overall charge. The 0.9-kcal/mole decrease in stability in the heterodimer of the more neutral peptide $EE_{12}RR_{345}L \rightarrow A/RR_{12}EE_{345}L$ (compared with $EE_{12345}L/RR_{12345}L \rightarrow A$) is likely due to repulsive i, i′ +2 interactions between the second and third heptads in the heterodimer (Kohn et al. 1995; Moll et al. 2000). To once again increase heterodimer stability, we reintroduced leucine in the fourth **d** position to produce $EE_{12}RR_{345}$ and $RR_{12}EE_{345}$. This change increased the stability 7°C.

We have a range of possibilities to further increase heterodimer stability, if necessary. The **a** positions are $A_1N_2V_3V_4V_5$. The first **a** position alanine could be changed to valine or leucine to increase stability without affecting dimerization. The asparagine in the second **a** position is critical for two reasons. It limits oligomerization to dimers (Harbury et al. 1993), and it regulates the parallel orientation of the monomer, preventing the peptides with mixed salt bridges from homodimerizing in an antiparallel manner (Oakley and Kim 1998). We may, however, be able to replace the **a** position valines with leucine to further increase both homo- and heterodimer stability. We also can elevate heterodimer stability by increasing the length of the leucine zippers to incorporate more hydrophobic interface and attractive $\mathbf{g} \leftrightarrow \mathbf{e}'$ salt bridges.

Radioimmunotherapy is a promising method to fight cancer. The advantage of the heterodimerizing leucine zippers is that their peptide sequences can be manipulated to produce homo- and heterodimers of any desired stability to optimize the pharmacokinetic properties of the antibody–leucine zipper–radionuclide chimeras.

## Materials and methods

### *Proteins*

The name and then the protein sequence of the peptides, grouped into g,a,b,c,d,e,f heptads are as follows:

| | *def* | *g a b c d e f* | *g a b c d e f* | *g a b c d e f* | *g a b c d e f* | *g a b c d e f* | *g a b c d* |
|---|---|---|---|---|---|---|---|
| $EE_{1234}L$: | LEI | EAAFLEQ | ENTALET | EVAELEQ | EVQRLEN | IVSQYET | RYGPLGGGK |
| $RR_{1234}L$: | KGGGLEI | RAAFLRR | RNTALRT | RVAELRQ | RVQRLRN | IVSQYET | RYGPL |
| $EE_{12345}L$: | LEI | EAAFLEQ | ENTALET | EVAELEQ | EVQRLEN | EVSQYET | RYGPLGGGK |
| $RR_{12345}L \rightarrow A$: | KGGGLEI | RAAFLRR | RNTALRT | RVAELRQ | RVQRARN | RVSQYRT | RYGPL |
| $RR_{12}EE_{345}L$: | LEI | RAAFLRQ | RNTALRT | EVAELEQ | EVQRLEN | EVSQYET | RYGPLGGGK |
| $EE_{12}RR_{345}L \rightarrow A$: | KGGGLEI | EAAFLER | ENTALET | RVAELRQ | RVQRARN | RVSQYRT | RYGPL |
| $EE_{12}RR_{345}L$: | LEI | EAAFLER | ENTALET | RVAELRQ | RVQRLRN | RVSQYRT | RYGPLGGGK |

The $RR_{1234}L$ and $EE_{1234}L$ peptides were made using the Impact T7 system from New England Biolabs (Chong et al. 1997). The genes encoding these peptides were inserted into the multicloning site (*Nde*I/*Sap*I) of the pTYB1 vector to create an in-frame fusion between their C terminus and the N terminus of the gene encoding intein, a protein splicing element from *Saccharomyces cerevesiae*. At the C terminus of the intein gene is DNA encoding a chitin-binding domain. After T7 overexpression, the crude extract is bound to a chitin column. The chimera is induced to undergo an intein-mediated self-cleavage by overnight incubation at 4°C in the presence of 30 mM DTT, causing the specific elution of the leucine zipper. The remaining peptides were generated by peptide synthesis from Genosys. The molar concentrations were calculated using the molar extinction at 230 nm of the peptide bond (300/bond) and the contribution of tyrosine (4980/residue) at 230 nm (Cantor and Schimmel 1980).

### *Circular dichroism*

CD studies were performed using a Jasco J-720 spectropolarimeter with a 5-mm rectangular CD cuvette and heated as described previously (Krylov et al. 1994). All protein stock solutions were in 12.5 mM potassium phosphate at pH 7.4, 150 mM KCl, 0.25 mM EDTA.

### *Thermodynamic calculations*

Melting temperature (Tm) and enthalpy ($\Delta H$) values were determined from denaturation curves assuming a two-state equilibrium dissociation of α-helical dimers into unfolded monomers using $\Delta Cp$ of $-1.2$ kcal/mole$^{-1}$ (Krylov et al. 1994). $\Delta G$ values are reported at 37°C.

### *Equilibrium sedimentation*

Equilibrium sedimentation measurements were performed using a Beckman XL-A Optima Analytical Ultracentrifuge equipped with absorbance optics and a Beckman An-60Ti rotor. Samples were loaded at three concentrations, 20, 40, and 60 μM into a six-hole centerpiece and spun at 28,000 rpm for 28 h. Nine data sets for three concentrations were jointly fit for a singular molecular weight. Compositional partial specific volumes for the proteins were calculated according to Zamyatnin (1984). All scans were performed at 6°C.

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