

*Chapter 4*CELL-BINDING DOMAIN CONTEXT AFFECTS CELL BEHAVIOR ON
ENGINEERED PROTEINS**4.1 Abstract**

A family of artificial extracellular matrix proteins developed for application in small-diameter vascular grafts is used to examine the importance of cell-binding domain context on cell adhesion and spreading. The engineered protein sequences are derived from the naturally occurring extracellular matrix proteins elastin and fibronectin. While each engineered protein contains identical CS5 cell-binding domain sequences, the lysine residues that serve as crosslinking sites are either (i) within the elastin cassettes or (ii) confined to the ends of the protein. Endothelial cells adhere specifically to the CS5 sequence in both of these proteins, but cell adhesion and spreading are more robust on proteins in which the lysine residues are confined to the terminal regions of the chain. These results may be due to altered protein conformations that affect either the accessibility of the CS5 sequence or its affinity for the $\alpha_4\beta_1$ integrin receptor on the endothelial cell surface. Amino acid choice outside the cell-binding domain can thus have a significant impact on the behavior of cells cultured on artificial extracellular matrix proteins.

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4.2 Introduction

A common goal in biomaterials design is the engineering of cell-adhesive materials. The identification within native proteins of numerous cell-binding domains that mediate cell-matrix interactions,¹⁻⁷ most notably the tripeptide sequence RGD,⁸⁻¹⁰ has greatly aided the pursuit of this goal. Immobilization of RGD and other adhesion peptides onto a variety of polymeric substrates has been utilized to enhance cell adhesion.¹¹ Although many of these peptides have been reduced to their "minimal binding sequences," the identity of flanking amino acid residues has been shown to alter activity.^{10,12} A variety of experiments, including site-directed mutagenesis and phage display, have been used to determine neighboring sequences that enhance cell adhesion.^{10,12-15} For example, replacing the serine residue in the wild-type peptide GRGDSPC with asparagine yields a peptide that is six times more effective at inhibiting cell attachment to fibronectin.¹² Furthermore, RGD sequences flanked by cysteines that form disulfide bridges and cause the peptide to cyclize exhibit stronger cell adhesion through the $\alpha_{IIb}\beta_3$ integrin than linear RGD sequences, perhaps because the cyclic peptide mimics the conformation of the native ligand.^{13,16}

While engineered peptides grafted onto synthetic polymer substrates yield cell-adhesive materials, an alternative approach involves the design and synthesis of more complex polypeptides that mimic some of the essential properties of the extracellular matrix (ECM). Genetic engineering allows highly specialized artificial proteins to be obtained in good yield with high fidelity,¹⁷⁻¹⁹ and the modular nature of recombinant DNA methodology allows facile synthesis of engineered proteins containing structural sequences and cell-binding domains derived from naturally occurring proteins or created

by rational design. This approach has yielded cell-adhesive biomaterials containing cadherin-like domains, RGD sequences, and CS5 sequences derived from the alternatively spliced IIIICS fragment of fibronectin.²⁰⁻²⁹

The protein engineering approach to biomaterials design allows incorporation of full-length cell-binding domains, as opposed to the minimal binding sequences typically used to modulate cell adhesion to synthetic biomaterials. It is reasonable to anticipate that full-length cell-binding domains may elicit more authentic responses. For example, engineered elastin-like proteins containing the full CS5 sequence were shown to promote cell adhesion,²¹ while those incorporating the minimal binding sequence REDV were not adhesive.³⁰ Furthermore, protein engineering offers the prospect of additional levels of control in that the complete primary sequence can (and must) be specified, not just the sequences integral and proximal to the cell-binding domains. To what extent do more remote elements of amino acid sequence affect cell-adhesion properties? We address this question here by characterizing cell response to engineered proteins that present the CS5 cell-binding domain within two different elastin-like contexts.

The elastin-like proteins examined in this work were originally designed for application in small-diameter vascular grafts.^{22,24,25} Cardiovascular disease afflicts more than 61 million Americans³¹ and causes 4 million deaths in Europe every year.³² Although large-diameter grafts in regions of high blood flow remain patent for many years, replacement of small- and medium-diameter vessels has met with limited success.³³⁻³⁵ Unopposed proliferation of myofibroblasts leads to stenosis of such grafts and subsequent thrombosis. It is believed that (i) the inability of the graft material to support the development of an endothelial cell monolayer and (ii) the compliance

mismatch between the prosthetic graft and the host tissue both contribute to graft failure. Therefore, our initial design criteria in engineering materials for small-diameter vascular grafts were (i) enhancing endothelial cell adhesion and (ii) tuning the elastic modulus of the material to match that of the affected artery. To meet these goals, CS5 cell-binding domains were used to enhance endothelial cell adhesion and elastin-like repeats were included to confer elastomeric behavior. Past studies have shown that these engineered proteins can support adhesion of endothelial cells in physiologically relevant fluid flows.²⁴ Lysine residues were incorporated into the sequences as specific crosslinking sites, allowing formation of free-standing films with tensile moduli similar to those of native elastins.^{22,25,36,37}

In this study, we examine the importance of amino acid context in affecting the response of human umbilical vein endothelial cells (HUVECs) to the CS5 cell-binding domain. The engineered protein sequences are listed in Figure 4.1. **Ki** contains lysine residues (**K**) at intervals of 25 amino acids internal (**i**) to the elastin-like domain. The CS5 cell-binding domain and elastin-like domain are repeated three times within the protein. **Ki*** is a similar protein except for the fact that the minimal binding sequence of the CS5 domain has been scrambled to provide a negative control. Comparison of HUVEC adhesion to **Ki** and **Ki***, in addition to peptide inhibition studies, has shown that cell adhesion to the CS5 cell-binding domain in **Ki** is sequence-specific.²⁶ **Kt** also includes three repeats of the CS5 cell-binding domain and the elastin-like cassette; however, the lysine residues are located only at the N- and C-termini (**t**). Peptide inhibition studies performed on a protein similar to **Kt**, containing five repeats of the CS5 and elastin-like domains, have demonstrated that HUVEC adhesion is primarily a

consequence of sequence-specific interactions with the CS5 cell-binding domain.²⁴ Although endothelial cells adhere specifically to the cell-binding domain in this family of engineered elastin-like proteins, the slight modification in primary sequence from **Ki** to **Kt** is shown here to significantly affect HUVEC behavior. These results show clearly that the context of the engineered protein, i.e., the identity of amino acids at sites distant from the cell-binding domain, can affect cell spreading and adhesion.

Protein Ki:

M-MASMTGGQQMG-HHHHHHH-DDDDK-(LD-GEEIQIGHIPREDVDYHLYP-G[(VPGIG)₂VPGKG(VPGIG)₂]₄VP)₃-LE
 T7 tag His tag Cleavage site CS5 binding domain Elastin-like domain

Protein Ki*:

M-MASMTGGQQMG-HHHHHHH-DDDDK-(LD-GEEIQIGHIPREVDDYHLYP-G[(VPGIG)₂VPGKG(VPGIG)₂]₄VP)₃-LE
 T7 tag His tag Cleavage site Scrambled CS5 binding domain Elastin-like domain

Protein Kt:

M-MASMTGGQQMG-RKTMG[LD-GEEIQIGHIPREDVDYHLYP-G(VPGIG)₂₅VP]₃-LEKAAKLE
 T7 tag CS5 binding domain Elastin-like domain

Figure 4.1 Amino acid sequences of the artificial extracellular matrix proteins. **Ki** has three cassette repeats with lysine residues internal to the elastin-like domain. **Ki*** is similar, but contains a scrambled CS5 binding domain as a negative control. **Kt** has three cassette repeats with lysine residues at the termini.

4.3 Materials and Methods

4.3.1 Protein Expression and Purification

Ki, **Ki***, and **Kt** were expressed in *E. coli* and purified as previously described.^{22,24,25} Purity was assessed by SDS-PAGE, mass spectrometry, and Western

blotting with anti-T7 tag-horseradish peroxidase conjugate antibody (Amersham). The molecular weights of the three proteins are 37,120; 37,120; and 42,974, respectively.

4.3.2 Protein Adsorption

To determine protein adsorption isotherms, 50 μL of each protein solution (0.05–8 mg/ml in PBS) was adsorbed onto a 96-well tissue culture polystyrene plate overnight at 4 $^{\circ}\text{C}$. The substrates were rinsed three times with 100 μL of PBS, and adsorbed protein was quantified via the bicinchoninic acid (BCA) method.^{38,39} Briefly, 50 μL aliquots of PBS was added to the wells containing adsorbed protein. To create a calibration curve, 50 μL of protein solutions of known concentrations were added to the 96-well plate. An equal volume of the working reagent containing an alkaline Cu^{2+} solution was added and incubated at 60 $^{\circ}\text{C}$ for 1 h. After the plates equilibrated to room temperature, the absorbance was read at 562 nm. The absorbance values observed for the protein standards were fit to a linear calibration curve, which was used to determine the amount of adsorbed protein in each sample.⁴⁰ Each concentration was tested in at least two independent experiments in triplicate. For cell culture experiments, solutions of engineered proteins (1 mg/ml in PBS unless otherwise noted) and fibronectin (10 $\mu\text{g}/\text{ml}$ in PBS) were adsorbed onto tissue culture polystyrene overnight at 4 $^{\circ}\text{C}$. The treated substrates were rinsed with PBS, blocked with 0.2% heat-inactivated bovine serum albumin (BSA fraction V, Sigma) for 30 min, and rinsed again with PBS.

4.3.3 *Cell Culture*

Human umbilical vein endothelial cells (HUVECs, Bio Whittaker) were maintained in a 37 °C, 5% CO₂ humidified environmental chamber. The cells were grown in Endothelial Growth Medium-2 (2% serum, Bio Whittaker), which was replaced every 2 days. Near confluent HUVEC cultures were passaged nonenzymatically by treatment with 0.61 mM EDTA (Gibco). Passages 2–10 were used.

4.3.4 *Cell Spreading—Scanning Electron Microscopy*

HUVECs were seeded on engineered proteins in serum-free Endothelial Basal Medium-2 (EBM-2, Bio Whittaker) and incubated for 30 min. Substrates were rinsed twice with PBS and fixed with formaldehyde. Fixed samples were subjected to critical point drying and gold/platinum sputter coating prior to imaging on a JOEL 6400 V scanning electron microscope. Two independent experiments were performed for each substrate.

4.3.5 *Cell Spreading—Phase Contrast Microscopy*

HUVECs in serum-free EBM-2 were allowed to spread on engineered proteins and imaged at 15 min intervals by using a 10× phase contrast objective on a Nikon Eclipse TE300 inverted microscope. Images were density-sliced to determine the number of well-spread (i.e., dark) versus non-spread (i.e., bright and refractive) cells using Scion Image for Windows.²⁶ Three independent experiments were performed. A one-tailed two-sample *t*-test that assumed equal variances was applied to determine statistical significance.

4.3.6 *Cell Resistance to Detachment*

Cell resistance to normal detachment forces was measured as previously described.²⁶ Briefly, cells were fluorescently labeled with calcein acetoxymethyl ester (Molecular Probes). Fluorescently labeled HUVECs were incubated on adsorbed protein substrates for 30 min. A solution of Percoll (21% w/w in PBS, Sigma) was added to each well, and the plates were centrifuged upright for 10 min at 1, 100, 1000, 2000, and 3000g. Nonadherent cells were removed and the remaining cells were quantified by their fluorescence (excitation at 485 nm and emission at 538 nm). A cell adhesion index (CAI) was calculated as the fluorescence reading of a test well divided by the fluorescence reading of HUVECs attached to fibronectin subjected to 1g (0.26 pN). Because Percoll has a higher density (1.123 g/ml) than the cells (~1.07 g/ml), a buoyant force is exerted on the cells. Using Archimedes' theorem,^{41,42} the range of detachment forces applied was estimated as 0.26–780 pN. At least three independent experiments with six replicates each were performed.

4.4 Results and Discussion

4.4.1 *Protein Synthesis and Characterization*

A typical wet cell mass from a 10 L batch fermentation was 200–250 g and expression yields for proteins **Ki**, **Ki***, and **Kt** were 10–20 mg/g wet cell mass. The expressed proteins were readily purified to provide multigram quantities of material. The

engineered proteins were adsorbed onto tissue-culture polystyrene, and their adsorption isotherms were determined (Figure 4.2) to ensure that the adsorbed films contained similar densities of cell-binding domains. At high (≥ 4 mg/ml) and low (≤ 0.05 mg/ml) solution concentrations, both lysine variants (**Ki** and **Kt**) adsorbed similarly to the polystyrene substrate. At intermediate concentrations (0.08–2 mg/ml), small but reproducible differences in adsorption levels were detected. **Ki** and **Ki*** exhibited indistinguishable isotherms (data not shown).

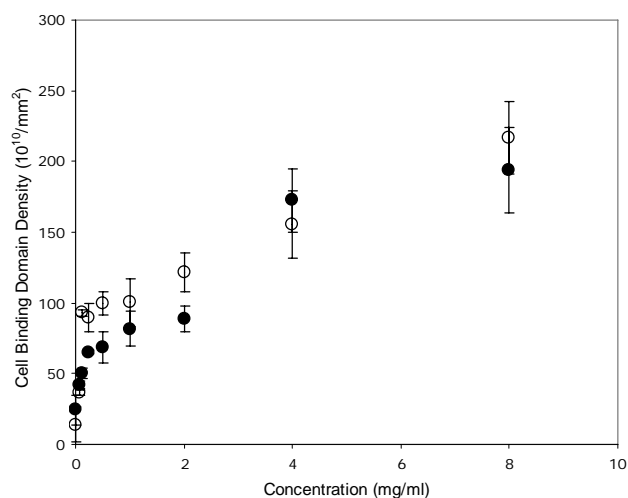


Figure 4.2 Adsorption isotherms of engineered proteins with terminal lysine residues, **Kt** (\circ), and with lysine residues internal to the elastin-like domain, **Ki** (\bullet), on tissue-culture polystyrene. Data are from one representative experiment performed in triplicate. Error bars represent one standard deviation.

4.4.2 HUVEC Resistance to Detachment Forces

Although previously published results showed that HUVEC adhesion to this family of engineered elastin-like proteins was primarily due to sequence-specific interactions with the CS5 cell-binding domain, we show here that amino acid choice within the elastin-like domains can significantly affect HUVEC resistance to detachment. To ensure that these effects were not due to differences in cell-binding domain density, we examined HUVEC adhesion over a range of protein concentrations (Figure 4.3). Assuming that each cell has 5.8×10^6 $\alpha_4\beta_1$ integrin receptors for the REDV sequence, of which one-half are available for surface interactions,⁴³ and a spread cell area of $150 \mu\text{m}^2$ after 30 min of incubation (see Figure 4.5b, below), the adsorbed protein surfaces displayed 10–100 times more cell-binding domains than available receptors per cell. Over this range of cell-binding domain densities, the cell adhesion indices (CAIs) on the terminal lysine protein (**Kt**) were consistently higher than those observed on the protein with lysine residues internal to the elastin domain (**Ki**). At high cell-binding domain densities, the CAI on **Kt** after a 10 min exposure to an estimated 260 pN detachment force, was nearly 100% (relative to HUVEC adhesion on fibronectin after a 10 min exposure to a 0.26 pN detachment force). In contrast, the CAI on **Ki** at these high cell-binding domain densities was ~20%. Thus **Kt** appears to be a more highly adhesive substrate than **Ki**, even though both engineered proteins include the same CS5 sequence.

The relationships between CAI and cell-binding domain density also differed for the two substrates. On **Kt**, cell adhesion increased with increasing protein concentration up to a cell-binding domain density of $40 \times 10^{10}/\text{mm}^2$, which corresponds to 20 cell-binding domains per available receptor, and then showed no further dependence on

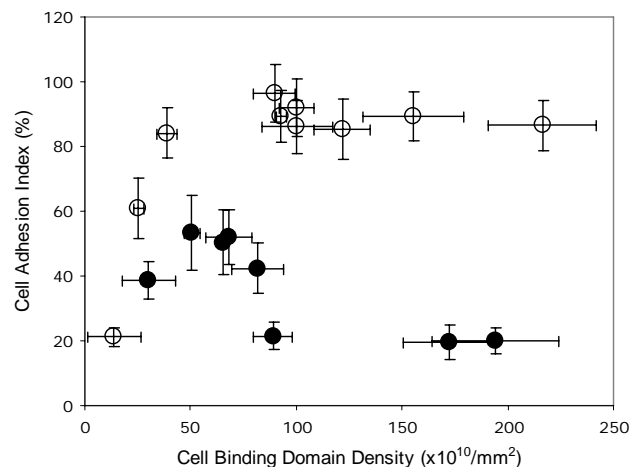


Figure 4.3 HUVEC resistance to a 260 pN normal detachment force after 30 min of incubation on tissue-culture polystyrene treated with varying amounts of **Ki** (●) or **Kt** (○) protein. Three independent experiments with six replicates were performed. Error bars represent one standard deviation.

protein concentration. Over a limited range of cell-binding domain densities ($35 \times 10^{10}/\text{mm}^2$ to $90 \times 10^{10}/\text{mm}^2$), the CAI on **Ki** was $\sim 50\%$; at higher densities, the CAI was reduced to $\sim 20\%$. The origin of this complex relationship between cell-binding domain density and CAI is unknown but may reflect a change in the conformation of the protein (or of the cell-binding domain) as a function of surface coverage. An alternative explanation might be cell loss due to substrate fracture at higher cell-binding domain densities; however, no evidence of protein delamination was observed by SEM (data not shown). Furthermore, no differences in cell viability were detected after 24 h between cells grown on either of the engineered protein substrates and those grown on fibronectin (data not shown). Cell viability was assessed by monitoring cleavage of the tetrazolium salt WST-1. Subsequent HUVEC studies were conducted on adsorbed protein surfaces

created from 1 mg/ml bulk solutions, which correspond to cell-binding domain densities of $80 \times 10^{10}/\text{mm}^2$ and $100 \times 10^{10}/\text{mm}^2$ for proteins **Ki** and **Kt**, respectively. This choice of bulk concentration for the adsorption solution allows us to compare cell behavior on **Ki** and **Kt** at cell-binding domain densities that elicit the highest cell adhesion indices on each substrate.

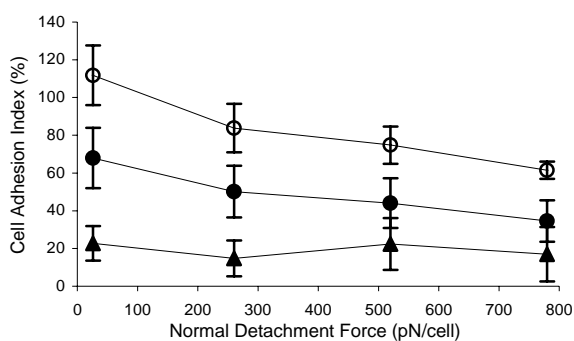


Figure 4.4 HUVEC resistance to normal detachment forces after 30 min of incubation on tissue-culture polystyrene treated with various proteins: **Kt** (○), **Ki** (●), and **Ki*** (▲). Three independent experiments with six replicates were performed. Error bars represent one standard deviation.

Over a range of detachment forces (26–780 pN), HUVECs cultured on **Kt** had consistently higher CAIs than those on **Ki** (Figure 4.4). As expected, the CAIs for both **Kt** and **Ki** decreased as higher detachment forces were applied. The CAI for HUVECs on **Ki***, the scrambled negative control protein, did not exceed 23% even at the lowest detachment forces examined in this work. These trends were also observed when the proteins were adsorbed on glass surfaces (see Supporting Information). Because there is a heptahistidine tag in **Ki** and **Ki*** but not in **Kt**, we verified that removal of the tag from

Ki does not affect the measured CAI at 26 pN (see Supporting Information). Thus, we believe that the change in context of the cell-binding domain, and not the supporting substrate or the heptahistidine tag, leads to a higher CAI on **Kt** as compared to **Ki**.

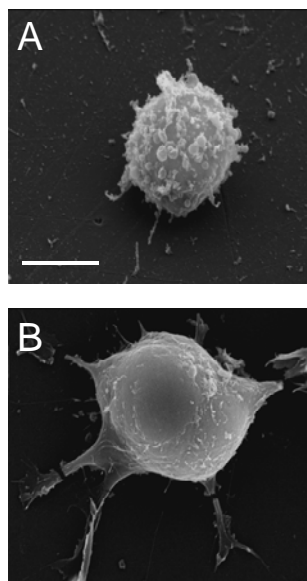


Figure 4.5 Scanning electron micrographs of HUVECs after 30 min of incubation on tissue-culture polystyrene treated with (a) **Ki** and (b) **Kt**. Two independent experiments were performed for each substrate. Both images are at the same magnification. Scale bar represents 7 μm .

4.4.3 HUVEC Spreading on Engineered Proteins

The amino acid context of the cell-binding domain was found to affect cell spreading as well as cell attachment. After 30 min of incubation, HUVECs on **Ki** remained small and rounded (Figure 4.5a) while those on **Kt** exhibited extended processes and had larger spread-cell areas (Figure 4.5b). When compared to cells spread on fibronectin, cells on **Ki** and **Kt** have smaller spread-cell areas. The SEM images in

Figure 4.5 are representative of typical HUVEC morphologies on **Ki** and **Kt**. To confirm these morphological observations, the percentages of well-spread cells were quantified by phase contrast microscopy. A larger percentage of HUVECs were well-spread on **Ki** ($\geq 20\%$) than on the sequence-scrambled negative control protein **Ki*** ($\leq 10\%$) after 60 min. At least twice as many HUVECs were well-spread on the terminal lysine protein (**Kt**) as on the internal lysine protein (**Ki**) after 15–60 min of incubation (Figure 4.6). Furthermore, **Kt** elicited more rapid cell spreading than **Ki**, although the rate of cell spreading was substantially lower than that observed on fibronectin.

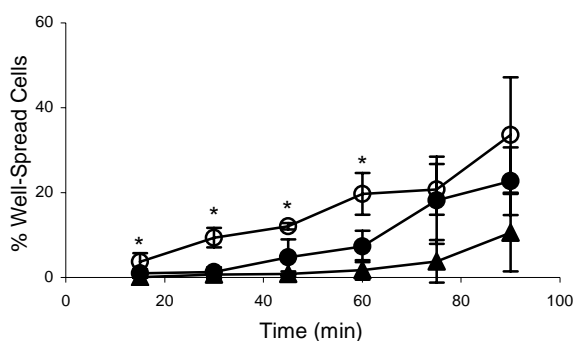


Figure 4.6 Percent of spread HUVECs on engineered proteins at various time points. Comparison of spread HUVECs on **Kt** (\circ), **Ki** (\bullet), and **Ki*** (\blacktriangle). Three independent experiments were performed. Asterisks indicate p -values ≤ 0.05 for HUVECs on **Kt** and **Ki**. Error bars represent one standard deviation.

4.5 Conclusion

The results presented here demonstrate that context, i.e., amino acid choice, can affect the activity of cell-binding domain sequences in engineered proteins. It has been

demonstrated previously that flanking amino acid residues can alter cell adhesion to binding sequences, but this work shows that amino acids located 15 or more residues away from the primary binding sequence can have a significant impact on HUVEC spreading and adhesion. Cell adhesion to this family of engineered proteins is primarily a consequence of sequence-specific recognition of the CS5 cell-binding domain regardless of context. Although all of the engineered proteins displayed similar adsorption isotherms, HUVEC morphology and adhesion to the adsorbed protein films were found to be context dependent. We suggest that changes in amino acid context may modify the protein conformation, which in turn may alter the accessibility or receptor affinity of the cell-binding domain.⁴⁴

4.6 Acknowledgments

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4.7 Supporting Information

Supporting figures include 1. Cell resistance to detachment forces on engineered proteins adsorbed to glass substrates, 2. Western analysis confirming complete cleavage of heptahistidine tag and T7 tag, and 3. Cell resistance to detachment forces on engineered proteins with heptahistidine tag and T7 tag removed.

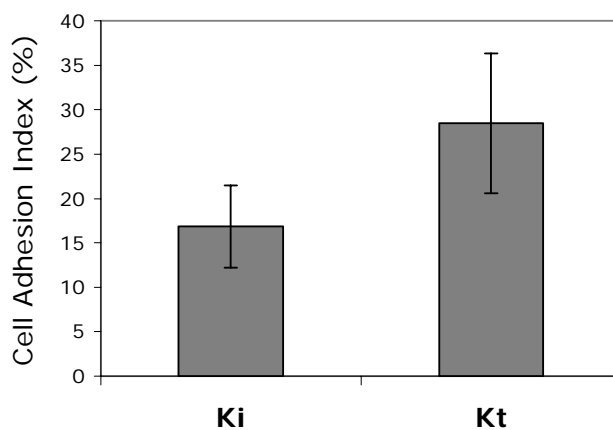


Figure 4.7 HUVEC resistance to a 26 pN normal detachment force after 30 min of incubation on glass adsorbed with **Ki** or **Kt** protein. Three independent experiments with six replicates were performed. Error bars represent one standard deviation.

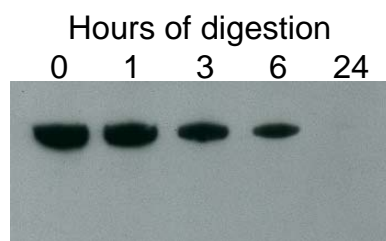


Figure 4.8 Western analysis confirming complete cleavage of heptahistidine tag and T7 tag from **Ki**. To remove the heptahistidine tag and T7 tag, the cleavage reaction was carried out at room temperature, 50 mM Tris, pH 8, 1 mg/ml protein, and 15 μ g/ml enterokinase (Roche) for 24 h. EKapture agarose (Novagen) was used to remove the enzyme. The peptide tag was removed via dialysis in pure water prior to lyophilization. No evidence of the T7 tag could be visualized on an over-exposed Western blot using an anti-T7 antibody. The yield for this entire sequence of steps including digestion, purification, dialysis, and lyophilization was approximately 60%.

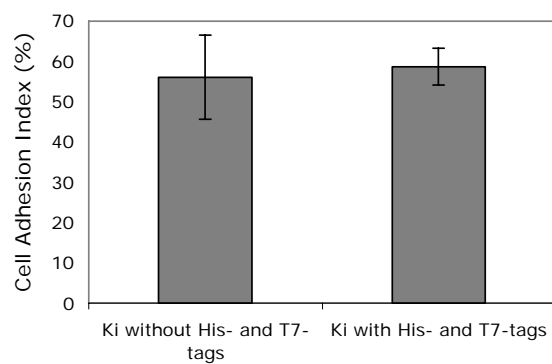


Figure 4.9 HUVEC resistance to a 26 pN normal detachment force after 30 min of incubation on tissue-culture polystyrene adsorbed with **Ki** protein prior to and after heptahistidine- and T7-tag removal. Three independent experiments in triplicate were performed. Error bars represent one standard deviation.

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44. One of the referees suggested that the observed differences in cell spreading and adhesion might be due to the difference in molar mass between **Ki** and **Kt** (molecular weights 37 and 43 kDa, respectively). We have performed experiments using **Kt** and a similar protein with a molar mass of 70 kDa (instead of three repeats of the CS5 cell-binding domain and elastin cassette, this protein has five repeats) and we have seen no differences in cellular response. Because a molecular weight difference of 27 kDa does not alter cellular response to the **Kt** sequence design, we do not expect that a molecular weight difference of 6 kDa contributes to the observed differences in cellular response to **Kt** and **Ki**.