Chapter IV. Cell-binding domain context affects cell behavior on engineered proteins

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 position of lysine residues that serve as crosslinking sites is either i) within the elastin cassettes or ii) confined to the terminal ends of the protein. This change in lysine positioning accounts for 3% of the total amino acid side chains and occurs more than 16 residues away from the CS5 cell-binding sequence. Endothelial cells specifically adhere to the CS5 cell-binding domain sequence in all of these proteins, but cell adhesion and spreading are more robust on proteins with only terminal lysines. These results indicate that amino acid choice outside of the cell-binding domain has a significant impact on cell behavior.

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1. 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 this goal. Immobilization of these peptide sequences onto a variety of polymeric substrates has been utilized to encourage 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.[10,12-14] 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 have stronger cell-adhesion interactions through the $\alpha_{IIb}\beta_3$ integrin than linear RGD sequences, perhaps because the cyclic peptide mimics the conformation of the native ligand.[13,15]

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,[16-18] 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 IIICS fragment of fibronectin.[19-26]

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. These full-length cell-binding domains may elicit stronger biological responses. For example, engineered elastin-like proteins containing the full CS5 sequence were shown to promote cell adhesion,[20] while those incorporating the minimal binding sequence REDV were not cell adhesive.[27] 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 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.[21,23,24] 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.[28-30] 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 pre-existing artery. To meet these goals, CS5 cell-binding domains were used to enhance endothelial cell adhesion and elastin-like repeats were included to provide mechanical integrity. Past studies have shown that these engineered proteins can support adhesion of endothelial cells in physiologically relevant fluid flows.[23] Lysine residues are incorporated into the sequences as specific crosslinking sites, allowing formation of freestanding films with tunable mechanical moduli similar to those of native elastins.[21,23,24,31]

In this study, we examine the importance of amino acid context in affecting the response of human umbilical vein endothelial cells (HUVEC) to the CS5 cell-binding

domain. The engineered protein sequences are listed in Figure 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 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.[25] **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 instead of three, have demonstrated that HUVEC adhesion is primarily a consequence of sequence-specific interactions with the CS5 cell-binding domain.[23] Although endothelial cells specifically adhere to the cell-binding domain in this family of engineered elastin-like proteins, the slight modification in primary sequence from **Ki** to **Kt** (a change of only 3% of the amino acid side chains) is shown 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, are shown to affect cell spreading and adhesion.

Protein **Ki**:

M-MASMTGGQQMG-HHHHHHH-DDDDK-{LD-GEEIQIGHIPREDVDYHLYP-G[(VPGIG)$_2$VPGKG(VPGIG)$_2$]$_4$VP}$_3$-LE

T7 tag | His tag | Cleavage site | CS5 binding domain | Elastin-like domain

Protein **Ki***:

M-MASMTGGQQMG-HHHHHHH-DDDDK-{LD-GEEIQIGHIPREVDDYHLYP-G[(VPGIG)$_2$VPGKG(VPGIG)$_2$]$_4$VP}$_3$-LE

T7 tag | His tag | Cleavage site | Scrambled CS5 binding domain | Elastin-like domain

Protein **Kt**:

M-MASMTGGQQMG-RKTMG[LD-GEEIQIGHIPREDVDYHLYP-G(VPGIG)$_{25}$VP]$_3$-LEKAAKLE

T7 tag | CS5 binding domain | Elastin-like domain

Figure 1. Amino acid sequences of the artificial extracellular matrix proteins. **Ki** has three cassette repeats with lysine residues inside 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.

2. Experimental section

2.1. Protein expression and purification

Ki, **Ki***, and **Kt** were expressed in *E. coli* and purified as previously described.[21,23,24] Purity was assessed by SDS-PAGE, mass spectrometry, and Western blotting with anti-T7 tag-horseradish peroxidase conjugate antibody (Amersham). Molecular weights of the four sequences are 37,120; 37,120; and 42,974 Da, respectively.

2.2. Protein adsorption

To determine protein adsorption isotherms, protein solutions (0.05 – 8 mg/ml in PBS) were adsorbed onto tissue culture polystyrene overnight at 4°C and rinsed three times with PBS. Adsorbed protein was quantified via the bicinchoninic acid (BCA) method.[32,33] 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 μg/ml in PBS) were adsorbed onto tissue culture polystyrene overnight at 4°C, rinsed with PBS, blocked with 0.2% heat-inactivated bovine serum albumin (BSA fraction V, Sigma) for 30 minutes, and rinsed with PBS.

2.3. Cell culture

Human umbilical vein endothelial cells (HUVEC, Bio Whittaker) were maintained in a 37°C, 5% CO_2 humidified environmental chamber. The cells were grown in Endothelial Growth Medium-2 (2% serum, Bio Whittaker), which was replaced every two days. Near confluent HUVEC cultures were passaged non-enzymatically by treatment with 0.61 mM EDTA (Gibco). Passages 2-10 were used.

2.4. Cell spreading – Scanning electron microscopy

HUVEC were seeded on engineered proteins for 30 min prior to fixation, critical point drying, and gold/platinum sputter coating. Samples were imaged using a JOEL 6400 V scanning electron microscope.

2.5. Cell spreading – Phase contrast microscopy

HUVEC in serum-free medium were allowed to spread on engineered proteins and imaged at 15 min intervals using a 10□ phase contrast objective on a Nikon Eclipse TE300 inverted microscope. Images were density-sliced to determine the number of spread (i.e., dark) versus non-spread (i.e., bright and refractive) cells using Scion Image for Windows.[25] Three independent experiments were performed. A one-tailed two-sample t-test that assumed equal variances was applied to determine statistical significance.

2.6. Cell resistance to detachment

Cell resistance to normal detachment forces was measured as previously described.[25] Briefly, cells were fluorescently labeled with calcein acetoxymethyl ester (Molecular Probes). Fluorescently labeled HUVEC were incubated on adsorbed protein substrates for 30 min. A solution of Percoll (Sigma) was added to each well and the plates were centrifuged upright for 10 minutes at 1, 100, 1000, 2000, and 3000g. Non-adherent cells were removed and the remaining cells were quantified by their fluorescence at an excitation of 485 nm and emission of 538 nm. A cell adhesion index (CAI) was calculated as the fluorescence reading of a test well divided by the fluorescence reading of HUVEC attached to fibronectin subjected to 1 *g*. Because Percoll has a higher density than the medium, a buoyant force is exerted upon the cells. Using Archimedes' theorem,[34,35] the range of detachment forces applied was estimated as 26-780 pN. At least three independent experiments with six replicates each were performed.

3. Results and discussion

3.1. 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. These protein sequences expressed well and were easily purified using thermal cycling to provide multi-gram quantities. The engineered proteins were physically adsorbed onto tissue-

culture polystyrene, and their adsorption isotherms were determined (Figure 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) bulk concentrations, both lysine variants (**Ki** and **Kt**) adsorbed similarly to the polystyrene substrate. At intermediate concentrations (0.08-2 mg/ml), small yet reproducible differences in adsorption levels were detected. **Ki** and **Ki*** exhibited indistinguishable isotherms (data not shown).

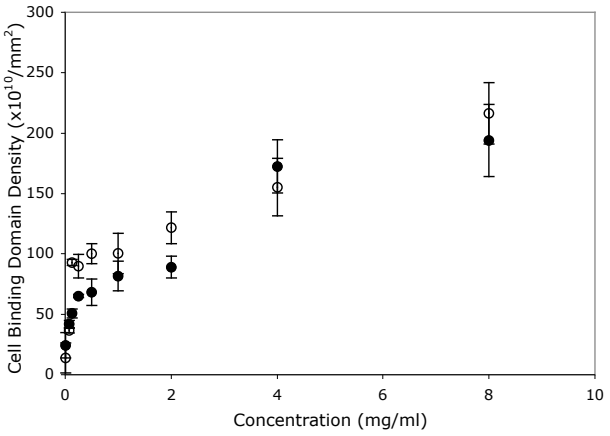

Figure 2. Adsorption isotherms of engineered proteins with terminal lysines, **Kt** (○), and with lysines internal to the elastin-like domain, **Ki** (●), on tissue-culture polystyrene. Data is from one representative experiment performed in triplicate. Error bars represent one standard deviation.

3.2. 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, the amino acid choice within the elastin-like domains was found to 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 3). Assuming that each cell has 5.8×10^6 receptors for the REDV sequence, of which half are available for surface interactions,[36] and

a spread cell area of 150 μm^2 after 30 minutes of incubation (see Figure 5b, below), the adsorbed protein surfaces displayed 10-115 times more cell-binding domains than available receptors per cell. Over this range of cell-binding domain densities, the CAI's on the terminal lysine protein (**Kt**) were consistently higher than those observed on the protein with lysines internal to the elastin domain (**Ki**); however, the relationship between CAI and cell-binding domain density was different for each substrate. Above a cell-binding domain density of $40 \times 10^{10}/mm^2$, which corresponds to 20 cell binding domains per available receptor, no differences in HUVEC resistance to a 260 pN detachment force were observed on **Kt**. Below a cell-binding domain density of $90 \times 10^{10}/mm^2$, the cell adhesion index (CAI) on **Ki** was ~50%; at higher densities, the CAI was reduced to ~20%. This relationship between cell-binding domain density and CAI are possibly attributed to differences in the conformation of cell-binding domain presentation at varying adsorption concentrations or differences in cell-binding domain spacing. An alternative explanation would 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 on either of the engineered protein substrates (data not shown). 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 and $100 \times 10^{10}/mm^2$ 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 range of cell adhesion indices observed on both substrates.

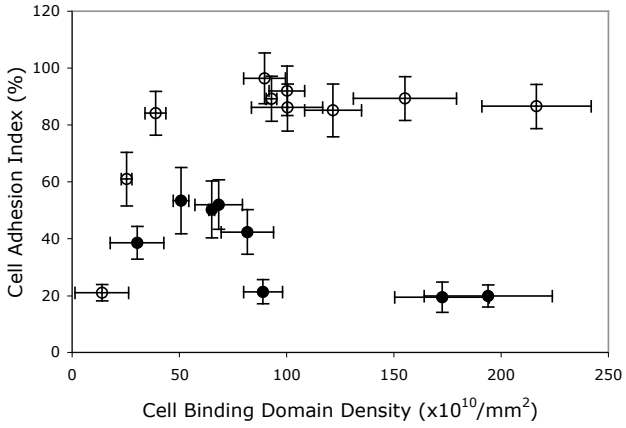

Figure 3: Cell resistance to detachment forces on various concentrations of engineered proteins adsorbed to tissue-culture polystyrene. HUVEC resistance to a 260 pN normal detachment force after 30 minutes of incubation on tissue-culture polystyrene adsorbed with varying amounts of **Ki** (●) or **Kt** (○) protein. Three independent experiments with six replicates were performed. Error bars represent one standard deviation.

Over a range of detachment forces (26-780 pN), HUVEC on **Kt** had consistently higher CAI's than those on **Ki** (Figure 4). HUVEC on **Ki***, the scrambled negative control protein, had the lowest CAI's at all detachment forces. These trends are qualitatively reproduced when the proteins are adsorbed onto glass surfaces (see supporting information). Because there is a histidine tag in the proteins with internal lysine residues but not the protein with terminal lysine residues, it was shown that removal of the histidine tag from **Ki** does not affect the measured CAI at 26 pN (see supporting information). Thus, we believe the change in cell-binding domain context, and not the supporting substrate or histidine tag, that leads to a higher CAI on **Kt** versus **Ki**.

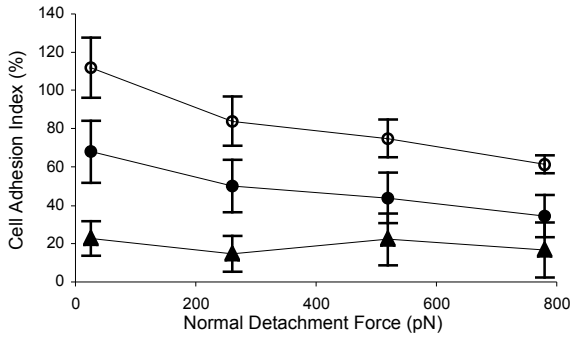

Figure 4. HUVEC resistance to normal detachment forces after 30 minutes of incubation on tissue-culture polystyrene adsorbed with various proteins: **Kt** (○), **Ki** (●), and **Ki*** (▲). Error bars represent one standard deviation.

3.3. HUVEC spreading on engineered proteins

In addition to exerting an influence on HUVEC resistance to detachment forces, the amino acid context was also found to vary cell spreading. After 30 minutes of incubation, HUVEC on **Ki** are small and rounded (Figure 5a) while those on **Kt** have extended processes and have a larger area in contact with the surface (Figure 5b). SEM images in Figure 5 are representative of typical HUVEC morphologies on **Ki** and **Kt**. To confirm these morphological observations, the percentages of well-spread cells were quantified using phase contrast microscopy. A larger percentage of HUVEC are well-spread on **Ki** than on the sequence-scrambled, negative control protein **Ki*** at 60 and 75 minutes. More HUVEC are well-spread on the terminal lysine protein (**Kt**) than on the internal lysine protein (**Ki)** at early time points, i.e., ≤60 minutes (Figure 6). These results suggest that changing the side chains of 3% of the amino acids in the protein without disrupting the CS5 cell-binding domain sequence alters cell spreading in response to the CS5 domain.

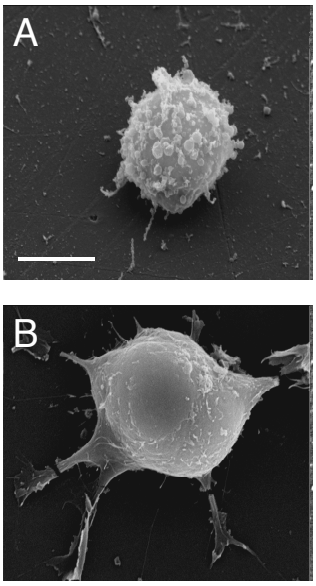

Figure 5. Scanning electron micrographs of HUVEC after 30 minutes of incubation on (a) **Ki** and (b) **Kt**. Both pictures are at the same magnification. Scale bar represents 7 μm.

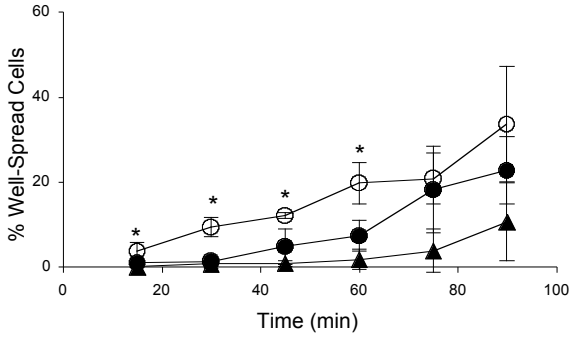

Figure 6. Percent of spread HUVEC on engineered proteins at various time points. Comparison of spread HUVEC on **Kt** (○), **Ki** (●), and **Ki*** (▲). Asterisks indicate p-values ≤0.05 for HUVEC on **Kt** and **Ki.** Error bars represent one standard deviation.

We believe that the observed differences in cell spreading and adhesion are directly affected by the location of the lysine residues in these proteins. These results demonstrate that the identity of amino acids located distant from the cell-binding domain can influence cellular response. Plausible explanations of these results are that the proteins containing

terminal lysines adopt an adsorbed conformation wherein either i) the three-dimensional structure of the CS5 cell-binding domain elicits a more robust spreading and adhesion response from cells or ii) the CS5 cell-binding domain is more accessible to the cells. Further experiments are needed to determine the validity of each hypothesis.

4. Conclusion

The results presented here extend the observation that context, i.e., amino acid choice, can affect the activity of cell-binding domain sequences. It has been previously demonstrated that flanking amino acid residues can alter cell adhesion to binding sequences, but this work shows that amino acids located 16 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. Changing the side chains of 3% of the amino acids affected both cell spreading and resistance to detachment forces. These results have implications for the field of protein engineering and peptide-grafted biomaterials by demonstrating that cell-material interactions can be modified by altering the context of the binding domain distal to the specific recognition sequence.

Acknowledgments

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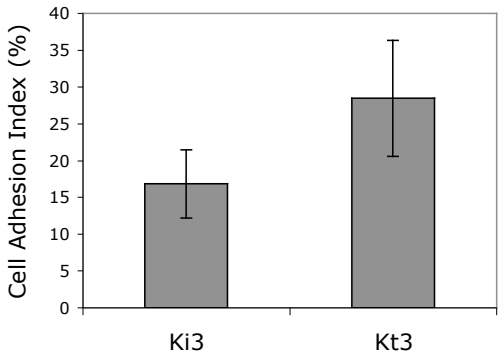
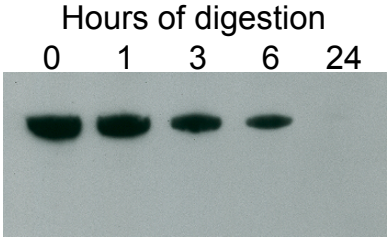
5. Supporting information

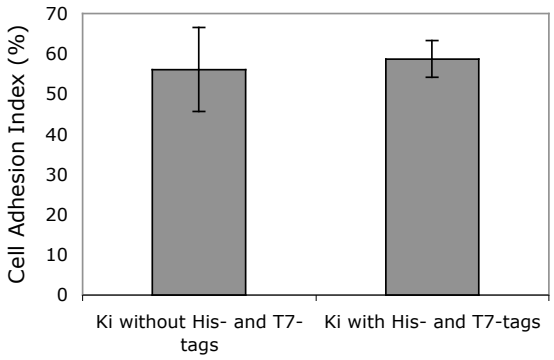
Supporting figures include 1. Cell resistance to detachment forces on engineered proteins adsorbed to glass substrates, 2. Western analysis confirming complete cleavage of histidine- and T7-tags, and 3. Cell resistance to detachment forces on engineered proteins with histidine- and T7-tags removed.



Supporting Information Figure 1: Cell resistance to detachment forces on engineered proteins adsorbed to glass substrates. HUVEC resistance to a 26 pN normal detachment force after 30 minutes of incubation on glass adsorbed with **Ki** or **Kt** protein. Three independent experiments with six replicates were performed. Error bars represent one standard deviation.



Supporting Information Figure 2: Western analysis confirming complete cleavage of histidine- and T7-tags from **Ki.** To remove the T7- and histidine-tags, the cleavage reaction was carried out at room temperature, 50mM Tris, pH 8, 1 mg/ml protein, and 15 □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 a T7-antibody. The yield for this entire sequence of steps including digestion, purification, dialysis, and lyophilization was approximately 60%.



Supporting Information Figure 3: Cell resistance to detachment forces on **Ki** with histidine- and T7-tags removed. HUVEC resistance to a 26 pN normal detachment force after 30 minutes of incubation on tissue-culture polystyrene adsorbed with **Ki** protein prior to and after histidine- and T7-tag removal. Three independent experiments in triplicate were performed. Error bars represent one standard deviation.

6. References

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