*Chapter V*

# PROGRESS TOWARD CLONING THE GENE OF AN RGD-CONTAINING PROTEIN FOR PHOTOPATTERNING*

## V.1 Abstract

We have previously described a new method for the production of cell-adhesive photolithographic protein patterns (Chapter 4) using the noncanonical amino acid *p*-azidophenylalanine (*p*$N_3$Phe). We demonstrated the procedure using an artificial extracellular matrix (aECM) protein [1] containing the CS5 cell-binding sequence derived from the IIICS region of fibronectin that is known to selectively adhere endothelial cells [2]. Previous work in our group has shown that endothelial cells attach both more quickly and strongly to an aECM protein containing an RGD-based sequence [3] from the CCBD of fibronectin [4]. We wished to create an expression strain harboring a plasmid encoding the artificial gene for an RGD-based sequence with phenylalanine residues within the elastin-like domain, which would permit photopatterning through the incorporation of *p*$N_3$Phe. The cloning strategy and progress toward the final construct will be described in this chapter.

**This work was performed with Stacey Maskarinec.*

## V.2 Introduction

As described in Chapter 4, we have recently demonstrated that incorporation of the noncanonical amino acid *p*-azidophenylalanine (*p*N3Phe) into a protein, dubbed artificial extracellular matrix protein with aryl azides (aECM-$N_3$), permits patterning of the protein on a surface using traditional photolithographic techniques. Further, we used this technique to create cell patterns through selective attachment of human umbilical vein endothelial cells to the aECM-$N_3$ protein, which contains a known endothelial cell-binding domain, when patterned on a non-adhesive background of poly(ethylene glycol) (PEG).

The sequence of the aECM-F protein we studied (Figure V-1) contained elastin-like repeats to provide structural integrity, regular Phe residues within the elastin-like regions as sites of crosslinking, and repeats of a cell-adhesive sequence (CS5) derived from the IIICS region of fibronectin that is known to adhere endothelial cells but not

CS5-F: MMASMTGGQQMGHHHHHHMG(LD-GEEIQIGHIPREDVDYHLY-PG((VPGVG)$_2$VPG**F**G(VPGVG)$_2$)$_5$VP)$_3$-LE

SC5-F: MASMTGGQQMGHHHHHHMKL{LDASFLD-GEEIQIGHIPREVDDYHLY-ASA[(VPGVG)$_2$VPG**F**G(VPGVG)$_2$]$_5$VP}$_3$LE

RGD-F: MASMTGGQQMGHHHHHHMKL{LDASFLD-YAVTGRGDSPASSKPIA-ASA[(VPGVG)$_2$VPG**F**G(VPGVG)$_2$]$_5$VP}$_3$LE

RDG-F: MASMTGGQQMGHHHHHHMKL{LDASFLD-YAVTGRDGSPASSKPIA-ASA[(VPGVG)$_2$VPG**F**G(VPGVG)$_2$]$_5$VP}$_3$LE

**Figure V-I.** Amino acid sequences of the artificial extracellular matrix proteins. The CS5-F protein was previously cloned by Nandita Sharma [1] and is discussed in Chapter 4. The target proteins for cloning are a construct containing a different cell-binding domain (RGD-F) and the negative control proteins for both cell-binding domains (RDG-F and SC5-F).

fibroblasts, platelets, or smooth muscle cells [2]. We will refer to this construct as CS5-F. Previous work in our laboratory on similar constructs (lacking Phe residues) [3] showed that endothelial cells form stronger attachments and form them more quickly when aECM proteins with repeats of an RGD-containing sequence derived from the CCBD region of fibronectin [4] are used. We wished to create an expression strain harboring a plasmid copy of the artificial gene for the RGD-containing version of the aECM protein we used previously. We also undertook to clone the appropriate negative control proteins for both the CS5-F and RGD-F sequences; these are designated SC5-F and RDG-F, respectively. The set of proteins is generically designated CBD-F for cell-binding domain construct with Phe residues. The sequences of the CBD-F (Figure V-1) proteins were designed based on CS5-F to contain repeats of the appropriate cell-binding domains as well as regular Phe residues within the elastin-like regions to permit photocrosslinking.

### *V.2.1 Cloning strategy*

The cloning strategy to obtain this sequence (Figure V-2) was based on one developed by Liu and Tirrell [5].

Synthetic DNA oligonucleotides encoding the desired CBDs (Figure V-3) were designed and ordered commercially. Phosphorylated oligonucleotides were ligated into the pUC19 vector at the *Eco*R I / *Bam*H I sites. The cell-binding domain regions were cut out of the pUC19-SC5, pUC19-RGD, or pUC19-RDG vectors with *Eco*R I / *Bam*H I

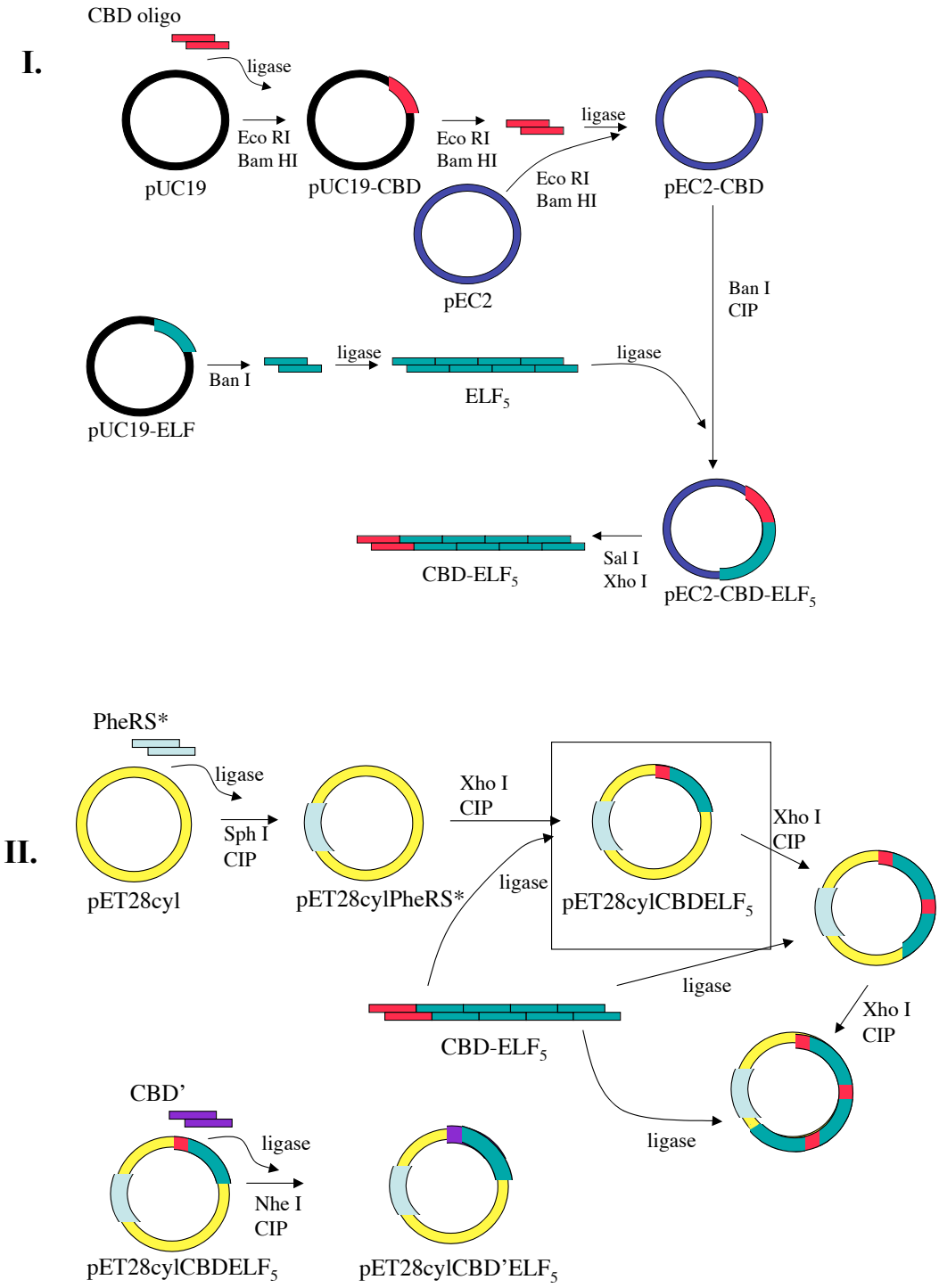


**Figure V-2.** The cloning strategy for CBD-F proteins.

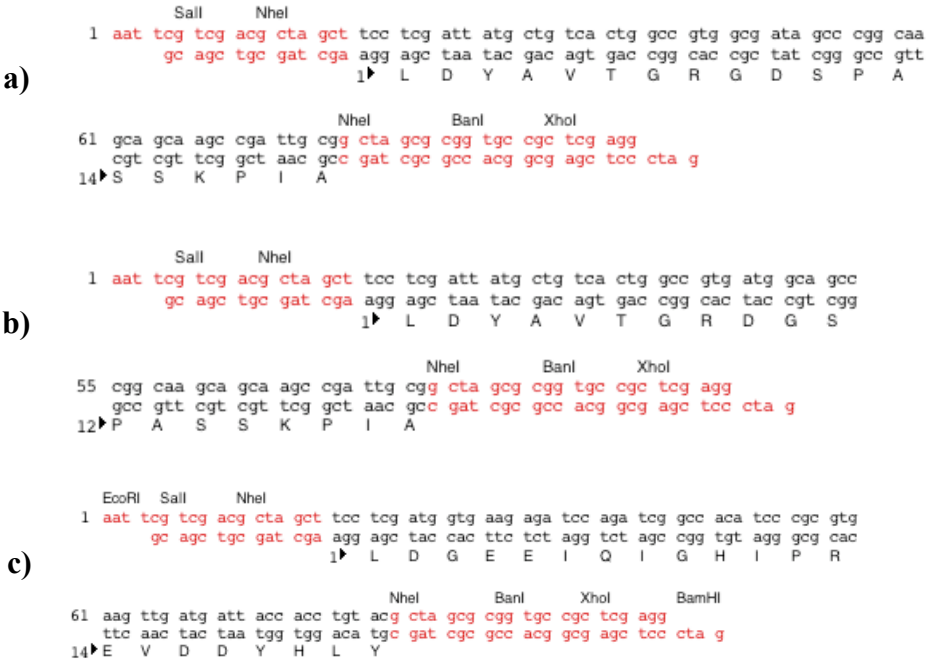


**Figure V-3.** DNA oligonucleotide sequences encoding **a)** the RGD cell-binding domain and **b)** the scrambled RDG and **c)** scrambled REVD negative control domains were designed and ordered commercially.

digestion and ligated into *Ban* I-linearized pEC2 vector [6] to produce pEC2-SC5, pEC2-RGD, and pEC2-RDG.

ELF monomer (Figure V-4) was obtained through *Ban* I digestion of pUC19-ELF [1]. The 75-bp fragment was multimerized through ligation with T4 DNA ligase, and the multimerization mixture was run on a 2% agarose gel. The band corresponding to pentamer (375 bp) was cut out and extracted from the gel. The pentamer DNA was ligated into pEC2-SC5 vector that had been digested with *Ban* I and dephosphorylated with CIP to yield pEC2-SC5-$ELF_5$.

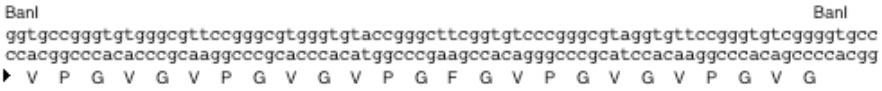


**Figure V-4.** DNA and protein sequence of the ELF monomer digested from pUC19-ELF.

Meanwhile, the gene encoding the A294G mutant PheRS (phes*) was cut from pUC19-FS [1] with *Sph* I. It was ligated into the *Sph* I site of pET28cyl [5] to create pET28cyl-phes*. The pEC2-SC5-ELF5 vector was digested with *Xho* I / *Spa* I, but attempts to ligate it into the pET28cyl-phes* failed. Sequencing revealed a second *Xho* I site on the pET28cyl-phes* between the *Sph* I ligation site and the start of the phes* gene. Future work will focus on removing this cut site through site-directed mutagenesis.

## V.3 Methods

### *V.3.1 pET28cyl-phes**

The phes* gene was isolated from pUC19-FS [1] through digestion with *Sph* I (NEB). It was ligated using T4 DNA ligase (NEB, room temperature, overnight) into pET28cyl [5] that had been linearized with *Sph* I (37°C, overnight) to produce pET28cyl-phes*. The A294G mutation was confirmed through DNA sequencing.

### *V.3.2 pEC2-RGD, RDG, and SC5*

The cell-binding domain oligonucleotides (Figure V-4) were ordered from Qiagen and resuspended at 5 μg/μL in 10 mM Tris buffer at pH 8.1. A 10-μL aliquot of each strand was added to 80 μL of annealing buffer (100 mM NaCl, 20 mM $MgCl_2$) and

heated to near boiling for 2 to 3 minutes. The solution was allowed to cool slowly back to room temperature and run on a 2 % agarose gel. The bands corresponding to the expected DNA (SC5 = 105 bp, RGD and RDG = 99 bp) were cut from the gel and extracted using a QIAquick Gel Extraction Kit (Qiagen).

The cell-binding domain fragments were phosphorylated using T4 polynucleotide kinase (NEB). Purified DNA was incubated at 37°C for 2 h with kinase in the supplied kinase buffer. The phosphorylated cell-binding domains were ligated (T4 DNA ligase [NEB], room temperature, 2 h) into pUC19 vector [1] that had been digested at 37°C overnight with *Eco*R I (NEB) and *Bam*H I (NEB). The pUC19-RGD, RDG, and SC5 vectors were transformed into XL-1 Blue competent cells (Stratagene).

The XL-1 Blue strains harboring the plasmids pUC19-RGD, RDG, and SC5 were grown in 5 mL overnight 2xYT cultures, and the plasmid DNA was isolated using a Miniprep Kit (Qiagen). The vectors were digested at 37°C overnight with *Eco*R I and *Bam*H I, the digestion mixtures were run on a 2 % agarose gel, and the appropriate cell-binding domain fragments were excised and extracted using a QIAquick Kit. Purified fragments were ligated into pEC2 plasmid [7] that had been digested with *Eco*R I and *Bam*H I to create pEC2-RGD, RDG, and SC5.

### *V.3.3 pEC2-SC5-ELF$_5$ and ELF$_6$*

The DNA fragment encoding one repeat of the desired elastin-like region with Phe sites, designated ELF (Figure V-5), was obtained through digestion of pUC19-ELF (Nandita Sharma). The plasmid DNA was isolated from a 500-mL overnight 2xYT

culture using a MaxiPrep Kit (Qiagen) and digested at 37°C overnight with *Ban* I (NEB). The digestion mixture was run on a 3 % agarose gel, the band corresponding to ELF monomer (75 bp) was excised, and the DNA was extracted with a Zymoclean Gel DNA Recovery Kit (Zymo Research). Two separate strategies were employed to multimerize the ELF monomer and insert it into the pEC2-SC5 plasmid. In the first scheme, the ELF monomer was self-ligated with T4 DNA ligase in the supplied ligase buffer at 0°C for <5 min before the ligation mixture was immediately loaded onto a 2 % agarose gel. The 375-bp band, corresponding to and ELF pentamer ($ELF_5$), was excised from the gel, and the DNA was extracted with a QIAquick Kit. The pEC2-SC5 plasmid was digested with *Ban* I (37°C, overnight) and dephosphorylated with calf intestine phosphatase (CIP, NEB, room temperature, 5 min) before being purified on a 2 % agarose gel. Linearized and dephosphorylated pEC2-SC5 was ligated for 3 h at room temperature with the purified ELF pentamer fragment, and the ligation mixtures were transformed into JM109 competent cells (Stratagene). Transformation colonies were test-digested with *Xho* I and *Sal* I; a band is expected at 468 bp if ELF pentamer successfully inserted into pEC2-SC5. A colony was isolated that carried the desired plasmid, designated pEC2-SC5-$ELF_5$.

In the second procedure attempted to produce pEC2-SC5-$ELF_5$, the linearized and dephosphorylated pEC2-SC5 plasmid was added directly to the ELF monomer ligation mixture 30 s after ligation was initiated by the addition of T4 DNA ligase. The ligation was allowed to proceed for 3 h before transformation of the mixture into JM109

competent cells. A colony was isolated that harbored a plasmid designated pEC2-SC5-ELF$_6$; the multimer that had been inserted was the hexamer of ELF.

## V.4 Results

Progress was made in the attempt to clone an artificial extracellular matrix protein containing the RGD cell-binding domain from fibronectin and regular Phe residues for incorporation of the analogue *p*-azidophenylalanine (RGD-F) along with negative control proteins RDG-F and SC5-F. The cloning strategy is depicted in Figure V-2.

A pET28cyl-phes* vector containing the A294G mutant phenylalanyl-tRNA synthetase was created and confirmed through sequencing. ELF monomer, isolated from the digestion of pUC19-ELF, was successfully multimerized with T4 ligase (Figure V-5).

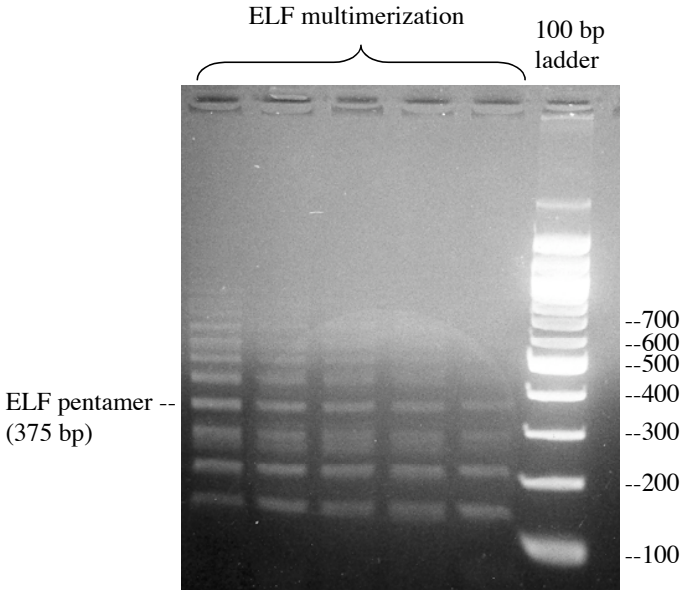


**Figure V-5.** Representative 3% agaraose gel of a multimerization reaction. ELF monomer DNA (with *Ban* I sticky ends) was incubated with T4 DNA ligase at 0°C for 3 min before being loaded onto the gel.

Excising the 375-bp band from the agarose gel and ligating the extracted DNA into a linearized pEC2-SC5 cloning vector resulted in a colony harboring a pEC2-SC5-$ELF_5$ (pentamer, Figure V-6). In a separate experiment, ELF monomer was multimerized in the presence of linearized pEC2-SC5 vector, followed by transformation of the crude multimerization mixture. This method produced a colony harboring pEC2-SC5-$ELF_6$ (hexamer, Figure V-6).

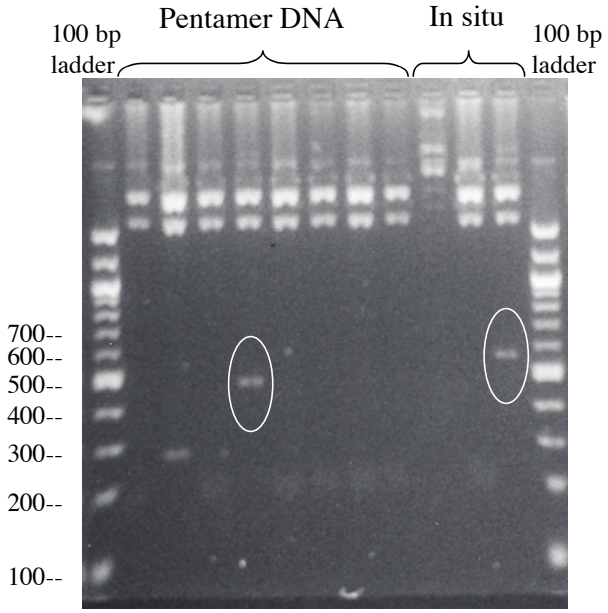


**Figure V-6.** Test digestions (with *Xho* I and *Sal* I) of colonies of cells transformed with ligation mixtures of pEC2-SC5/*Ban* I and ELF monomer with *Ban* I sticky ends. Transformants in lanes 2 to 9 resulted from ligations of pEC2-SC5/*Ban* I with ELF pentamer DNA that had been excised from a 3% agarose gel of the ELF multimerization. The circled band in lane 5 indicates presence of pEC2-SC5-$ELF_5$ (pentamer). Transformants in lanes 10 to 12 resulted from *in situ* ligations of pEC2-SC5/*Ban* I with ELF monomer. The circled band in lane 12 indicates the presence of pEC2-SC5-$ELF_6$ (hexamer).

## V.5 Conclusions

The progress made in cloning the desired artificial extracellular matrix protein constructs, especially in the difficult multimerization step, will facilitate future production of these proteins. The proteins, one containing the RGD cell-binding domain, and negative control proteins for both the RGD and CS5 cell-binding domains, will enable studies of cellular behavior on photocrosslinked substrates through the incorporation of the photoreactive amino acid *p*-azidophenylalanine.

## V.6 References

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