Chapter 1:

Introduction and overview

Diverse combinatorial protein libraries have the potential to generate molecules which can bind virtually any desirable target. The number of potential protein targets is enormous; including post translational modifications and alternative splicing, the human proteome contains millions of proteins (*1*). Including microbial proteins, the number of useful targets is even greater. The natural diversity represented by the mammalian immune system has long been exploited for the generation of novel affinity reagents (*2*). *In vitro* selection techniques such as phage display, ribosome display, and mRNA display have been developed for generation of novel protein affinity reagents as an alternative to animal immunization (*3*). With the advent of these techniques, synthetic and semi-synthetic immunoglobulin libraries have been developed (*4-6*). One drawback of using antibodies as affinity reagents, however, is that antibody immunoglobulin domains require intrachain disulfide bonds for stability (*7*). This requirement makes antibodies difficult to express in large quantities and also limits their utility for intracellular applications. In conjunction with the development of *in vitro* selection techniques, there has been interest in the generation of alternative protein scaffolds which, like antibodies, are able to tolerate sizable sequence diversity but, unlike antibodies, also express well in the intracellular environment (*3*, *8*, *9*).

There are many applications for the molecular recognition of natural proteins by novel protein affinity reagents (*10*). Recombinant antibodies are becoming increasingly common as therapeutics (*11*). Other applications include standard laboratory techniques such as immunoprecipitation, ELISA, western blot, and immunofluorescence microscopy. Applications for protein detection are expanding with the development of

ultra-sensitive nano-electonic devices (*12*) and the use of engineered antibodies in nuclear medicine (*13*).

Despite difficulties in the expression of antibody fragments, a number of applications for intracellular recognition of protein targets have been demonstrated by intracellular antibodies (intrabodies) (*14*). For example, protein affinity reagents can be used as tools for intracellular detection and visualization (*15*). Exogenous binders expressed as fluorescent protein fusions enable the visualization of dynamic proteins in live cells. This application also allows the detection of protein specific to particular conformational states or post translational modifications.

Novel protein affinity reagents may also be useful for functional genomics by the direct inhibition of protein activity inside the cell. This technique was first demonstrated in yeast by the expression of an antibody which limited the activity of alcohol dehydrogenase (*16*). Inhibitory proteins can be selected to target specific protein domains, conformations, or modifications, whereas both gene knock-out and RNAi techniques are not specific. Many intrabodies have been reported to inhibit proteins important in cancer development which are considered “undrugable” (*17*). However, the application for intrabodies as therapeutics is dependent on the progress of gene therapy technology.

One industrial application that does not face gene delivery obstacles is the generation of transgenic plants that express intrabodies. Intrabodies have been generated that confer resistance against disease (*18, 19*) and improve the metabolic properties of plants (*20*). In addition to these reports, there are also many examples of plant intrabodies that were developed for functional genomics experiments as an alternative to gene

silencing techniques (*21*). For example, an antibody that inhibits heat shock protein oligomerization *in vivo* was used to illustrate the functional importance of this protein where previous genetic techniques proved inconclusive (*22*).

These reports demonstrate the potential for using intrabodies to determine protein function. However, because many antibody fragments are not stable *in vivo*, the usable diversity of antibody libraries may only represent a fraction of the total diversity. In some cases, very few or even no antibody fragments are able to interact with the intended target *in vivo* (*18, 23*). In order to improve the probability of obtaining molecules functional *in vivo*, screens based on the two-hybrid method have been implemented after partial enrichment of complex antibody libraries (*23, 24*). Also, many intrabodies that are functional *in vivo* have the propensity to form aggregates (*25, 26*). The ability to bind and precipitate a protein target is desirable for certain therapeutic applications, but is not desirable for *in vivo* detection and functional genomics. While aggregation ensures inhibition by effectively removing the protein target from the cytosol, the aggregates may adversely affect the cell. For example, these types of aggregates have been shown to disrupt the ubiquitin dependent proteasomal degradation pathway and result in a propensity to induce apoptosis (*27, 28*).

There have been successful attempts to engineer antibody fragments that are more stable *in vivo* (*29-31*). Alternatively, non-immunoglobulin domains have been used as scaffolds for combinatorial libraries which may improve upon antibody libraries (*32*). One successful protein library is based on the ankyrin domain (*8, 33*). This library has been used in ribosome display selection experiments to generate novel affinity reagents that are functional in vivo (*34, 35*). One ribosome display selection yielded molecules

that were able to inhibit a tobacco etch virus proteinase *in vivo*, and stable expression of the ankyrin inhibitors in plants could lead to viral resistance (*36*).

The ankyrin library was created by consensus design where non-conserved residues were randomized throughout the protein surface (*8*). We took an alternative approach and utilized the fibronectin type III domain to create a combinatorial protein library with diversity localized within two adjacent randomized loops (*9*). The library scaffold was based on the 10^{th} fibronectin type III domain of human fibronectin, originally implemented by Koide et al. for phage display selections (*37*). This domain is topologically analogous to the immunoglobulin fold; however, unlike immunoglobulin variable domains, it does not contain disulfide bonds and is able to be expressed at high levels in bacteria. Chapter 2 describes the design and construction of our library as well as expression and stability analysis of representative library members. Over half of the molecules encoded in the library are able to be expressed in bacteria. The domain was also demonstrated to tolerate mutations at the 17 randomized loop positions as four representative variants were shown to be structurally stable. In addition to demonstrating the utility of this scaffold for selection techniques, this report illustrates the vast quantity of sequence space that is accessible for both natural and directed evolution of novel functions.

Our fibronectin-based combinatorial library was designed for *in vitro* selection by mRNA display (*38*). mRNA display technology is an elegant and simple method for the generation of novel high-affinity peptide and protein binders to specific protein targets (*39*). The utility of the technique lies in the ability to link a unique protein phenotype to its genotype via a covalent bond. This is achieved by splint-mediated ligation of an

oligonucleotide bearing a 3' puromycin to an mRNA pool synthesized by *in vitro* transcription of the DNA that encodes the combinatorial protein library (Figure 1.1, panel A). The mRNA library is then translated *in vitro* and fusion of the nascent peptide chain to the mRNA is catalyzed by the ribosome when the ribosome stalls at the poly-dA linker (Figure 1.1, panel B). This selection technique, which is entirely *in vitro*, has advantages over other selection techniques including phage display and yeast display in that higher complexity libraries, over 10 trillion unique molecules, are accessible. Also the mRNA display format is monovalent. Since a covalent bond links the fusions, this selection technique also has an advantage in that selections can be performed at any desired level of stringency.

One of the benefits for using *in vitro* selected protein affinity reagents for proteomics or functional genomics is the ability to generate molecules that bind in a domain, conformation, or post translational modification-specific manner. To highlight the utility of our fibronectin library, we sought to generate binders that modulate and detect one of the most important pathways in the cell, the NF-κB pathway. NF-κB proteins are ubiquitous transcription factors primarily involved in activation of pro-inflammatory genes (*40*). The NF-κB pathway also plays an important role in cell survival, as well as in neuronal signaling (*41*). Activation of the classical NF-κB pathway is controlled by ubiquitin dependent proteasomal degradation of three inhibitory proteins, IκBα, β, and ε (*42*). These proteins are phosphorylated by IKK at two serines within a conserved DSGXXS destruction motif that is recognized by the SCF-βTrCP E3 ligase when phosphorylated (*43*). Chapter 3 describes a selection for fibronectin molecules that specifically recognize the phosphorylated state of IκBα. We were able to evolve a

fibronectin molecule, labeled 10C17C25, that is able to discriminate between the phosphorylated and unphosphorylated states of IκBα with over 1000-fold specificity, measured by surface plasmon resonance. 10C17C25 was also able to recognize IκBα inside human kidney cells (293T) and inhibit its degradation. 10C17C25 was able to pull-down IκBα only in cells in which the NF-κB pathway was activated. We have also demonstrated for the first time the application of novel protein affinity reagents for use in FRET sensors of kinase activity. Our IKK FRET sensors are similar to previous sensors which rely on natural phospho-specific binding domains that detect kinase activity in cells reversibly in real-time without disruption of the pathway being detected (*44*, *45*).

With the growing threat of epidemics similar to the recent SARS outbreak, novel tools to detect and study viruses are needed. Many intrabodies have been described which modulate viral proteins (*17*). These experiments validate the potential of viral proteins as targets for antiviral therapies. Direct inhibition of viral proteins in cells infected with unmodified virus is a useful tool for neutralization of viral genes for functional analysis where gene knock-outs or RNAi techniques are not applicable (*25*, *46*). We sought to demonstrate the potential for using SARS nucleocapsid-binding fibronectins to detect and probe viral protein function in a domain-specific manner. Chapter 4 describes a selection which generated molecules that bind nucleocaspsid (N) protein with low nanomolar affinity after only 6 rounds of enrichment. The primary function of N is to package the RNA genome within the viral envelope. However, N is found in abundant levels in the serum of infected patients and represents a potential target for early detection of SARS infection. In addition to validating potential therapeutic strategies, these molecules may be used to better establish the many additional roles coronavirus N protein plays in viral

replication and in host-cell interactions (*47*). We were able to demonstrate over 1000-fold inhibition of viral production by intracellular expression of the most potent SARS-inhibiting fibronectin. Also, as we were able to obtain binders to non-overlapping epitopes, we were able to demonstrate the value of synergistic inhibition by targeting two domains within the same protein. Although most antiviral therapeutic targets are enzymes, we demonstrated the efficacy of inhibiting the protein interactions mediated by a virus structural protein.

Finally, Appendix A describes the vectors used for the expression of fibronectins. Vectors were created for evolving protein stability, expressing protein in both bacteria and mammalian cell culture, and for cloning selection products into a general FRET sensor vector. Appendix A illustrates the ORF of each vector used, describes the rationale for creating the vector, and describes the method for vector construction.

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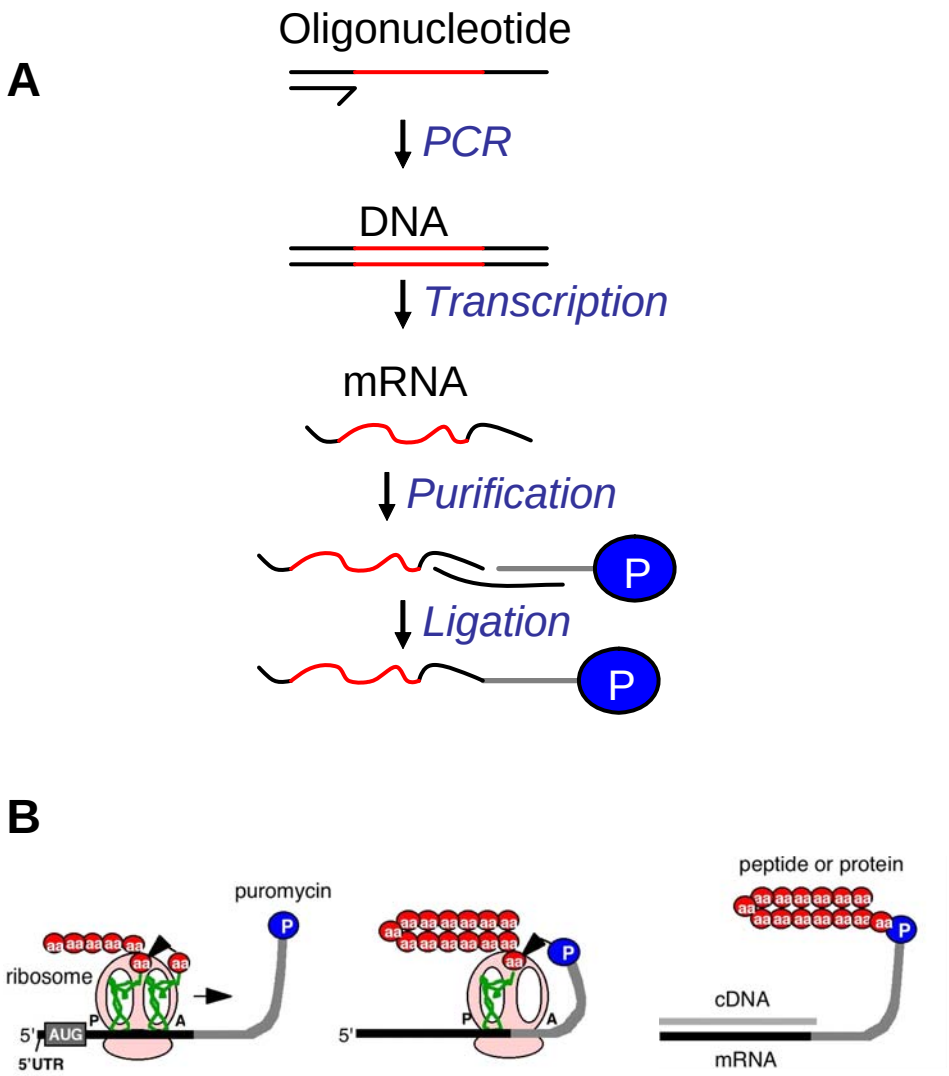

Figure 1.1 mRNA display library synthesis and fusion formation. A) Cartoon depicting synthesis of an mRNA pool with a 3' puromycin for mRNA display. Simple libraries are encoded by single randomized sequence (red). Constant regions are depicted in black. The 5' constant region includes the T7 promoter sequence, a translation enhancer sequence, and the initiator methionine codon. The 3' constant region provides sequence for primer annealing and splint hybridization and also encodes a flexible linker for spacing the nucleic acid fusion. B) Cartoon depicting in vitro translation and fusion formation.