7

FN3 Domain Engineering

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1. Introduction

The Fibronectin Type III domain (FN3) is a small globular protein domain of 90-100 amino acids found in thousands of proteins, over all of protein sequence space from bacteria to humans. In fact, sequence analysis estimates that the FN3 domain can be found in approximately 2% of all animal proteins (Bork & Doolittle, 1992). The frequency that these domains are found is even greater when considering the larger family, the Ig-like fold, of which FN3 is a specific member. Despite weak to no sequence identity and greatly divergent functions, FN3 domains fold into a common structure. Over 230 structures of FN3 domains have been deposited to the PDB database as of 2011 (> 990 when considering all Ig-like domains), showing that the fold consists of 7 antiparallel β-strands that pack into two β-sheets (Main et al., 1992) (Figure 1). These β-strands are designated with the nomenclature A-F with the loops connecting each strand designated by the strands it connects (For example, the AB loop connects strands A and B). With a few exceptions, structural variability of these domains is found exclusively in the loops connecting the β-strands while the strands remain structurally conserved.

The small size, simple structure, and robust prokaryotic expression levels of many FN3 domains has led to the development of these proteins as important tools for the understanding of protein folding and stability. To date, two members of the FN3 family, the tenth FN3 domain from human Fibronectin (FnFN10) and the 3rd FN3 domain from human Tenascin-C (TnFN3) have been the most widely studied. Despite low levels of sequence identity (23%), these two FN3 domains fold into very similar structures with the majority of structural deviations occurring in the loops connecting the β-strands (Leahy et al., 1992, Main, et al., 1992). Of great importance to the use of FN3 domains in novel applications is the ability of proteins with very diverse sequences to fold into a common structure, thus allowing for these domains to be heavily mutated in order to incur a desired function. This property has been demonstrated in a series of experiments published from the Clarke lab in which a "fold approach" was taken to characterize the folding and stability of a number of FN3 and Ig domains. The resulting work has lead to the proposal that proteins of the FN3 and related families fold by similar mechanisms involving nucleation from the B, C, E and F strands, even when no sequence similarity is detectable (Clarke et al., 1999, Hamill et al., 2000a, Hamill et al., 2000b). Despite such similar structures and folding pathways, FN3 domains can exhibit markedly different conformational stabilities. For example, the FnFN10 and TnFN3 domains have conformational stabilities of 9.4 and 6.7 kcal mol^{-1} respectively and FnFN9, which resides directly adjacent to FnFN10 in the Fibronectin protein is

approximately 5-fold less stable than FnFN10 (Clarke et al., 1997, Cota & Clarke, 2000, Hamill et al., 1998, Plaxco et al., 1997). Understanding the causes of these differences is important for producing FN3 domains for pharmaceutical or industrial applications. One explanation for the high stability of FnFn10 may be the rapid rate at which this isolated domain folds, despite the presence of 8 proline residues that might be expected to slow down folding due to cis-trans isomerization (Plaxco et al., 1996, Plaxco, et al., 1997). Indeed the data available so far for FN3 domains supports the hypothesis that the refolding rates of β-sheet proteins reflect overall stability (Finkelstein, 1991). Another contribution to the differences in conformational stability of these proteins may be differences in structural plasticity. NMR spectroscopy was used to determine that the side-chains in the hydrophobic core of TnFN3 had a higher degree of mobility than those of FnFN10, while mutational analysis has shown that FnFN10 is more amenable to surface mutation due to greater flexibility in peripheral regions of the protein (Best et al., 2004, Cota et al., 2000). In support of this model, Billings and colleagues produced a chimera of FnFN10 and TnFN3 which was composed of the hydrophobic core of TnFN3 and the surface and loops of FnFN10. This chimeric FN3 domain showed greater stability than that of TnFN3, suggesting that the surface composition and loops can significantly contribute to stability (Billings et al., 2008).

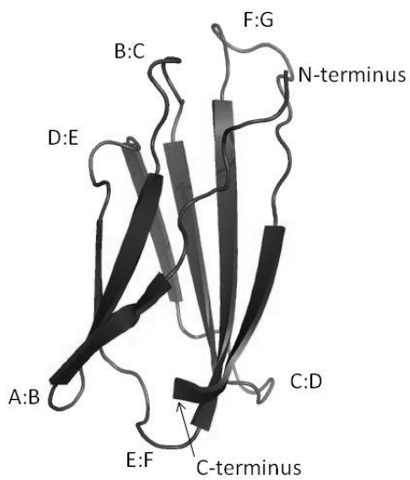

Fig. 1. Solution structure of FN3 domain FnFN10 (PDB 1TTF). Labels indicate the identity of loop regions as well as the N and C-termini.

The contributions of surface residues to FN3 stability and the ability of such residues to tolerate mutation are keys to the development of these domains for biotechnology applications in which various surfaces or loops are mutated to employ a new function (see below). This property was first exploited by Koide et al. who demonstrated that the surface exposed loops of FnFN10 could be randomized and used to select FN3 domains that interact with a novel ligand, ubiquitin, thereby imparting a non-natural function to the FN3 domain (Koide et al., 1998). Importantly, the authors were able to confirm that the FN3 domains produced with such foreign loops were able to maintain the overall FN3 structure, although the resulting binders had reduced conformational stability compared to that of FnFN10. Numerous subsequent biochemical and biotechnological applications of FN3 domains are described in this chapter. The success of these techniques is strongly dependent upon the ability of highly diverse FN3 domains to fold into a common structure while tolerating a

high level of sequence mutation. In the next section, selection systems which have been used to select FN3 domains of novel function are described, followed by descriptions of protein engineering studies designed to increase the overall stability of the FN3 scaffold and to evolve FN3 scaffolds with high affinity and selectivity for a particular target. Finally, a report of novel applications of engineered FN3 domains is provided.

2. Selection technologies

Over the last twenty years, a variety of display technologies have evolved as tools for protein engineering and selection of binding ligands. Such techniques can be broadly separated into phage display, *in vitro* display (including ribosome display, mRNA display, and DNA display) and yeast display strategies (Figure 2). Phage display requires propogation and secretion from bacteria (ie. *E. coli*) using prokaryotic secretion machinery while yeast display relies on the growth and secretion properties of eukaryotic yeast. In contrast, the *in vitro* display technologies are accomplished completely *in vitro* which allows for significant manipulation of the selection conditions and much larger libraries.

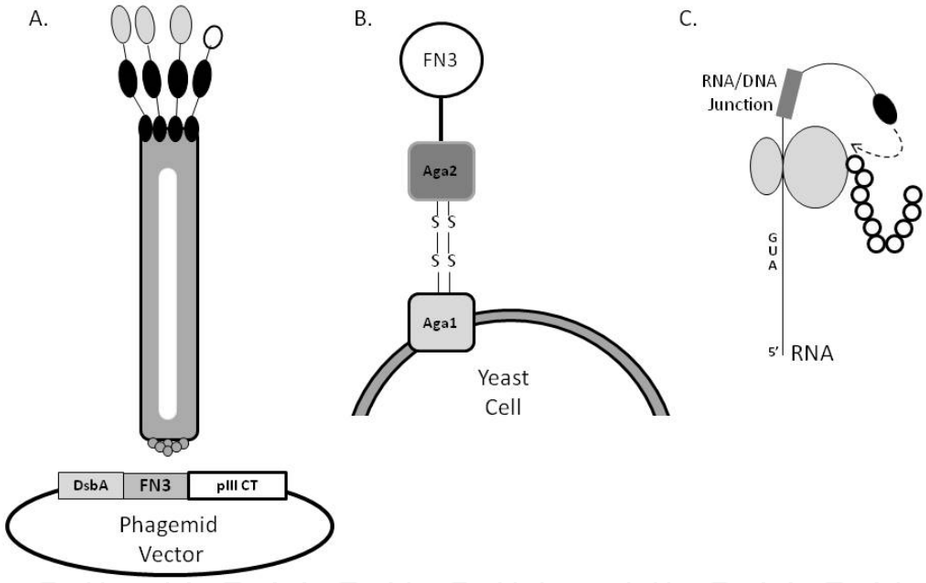

Fig. 2. Display platforms used for engineering and selection of FN3 domains. A) Phage display using a phagemid vector where the FN3 domain is fused to the C-terminal domain of M13 pIII. The signal sequence used to direct secretion is the DsbA sequence. B) Yeast display platform involving fusion of the FN3 domain to the yeast cell membrane protein Aga2p. Aga2p associates with membrane bound Aga1 via disulfide linkages. C) mRNA display involving an RNA/DNA hybrid wherein the RNA encodes the FN3 domain; the ribosome is depicted with the 2 shaded ovals with the transcribed protein illustrated by the hollow circles. Puromycin is depicted with a solid oval with the linkage site to the nascent protein strand shown by a dotted arrow.

Koide and coworkers first demonstrated the utility of the FN3 domain as a scaffold suitable for creation of libraries of variants (Koide, et al., 1998). Based on analysis of FN3 domains involved in protein interaction motifs that suggested loop residues were frequently involved

in binding, a library containing randomized sequences in the FG and BC loops was designed. The FN3 scaffold library was fused to the C-terminal fragment of pIII using a phagemid vector and pIII display was achieved by infection with helper phage. Following five rounds of selection, a ubiquitin specific binder with approximately μM affinity for ubiquitin was isolated. Despite reduced stability and solubility compared to the native parental FN3 domain from fibronectin, this study set the groundwork for future studies expanding the applications of FN3 domains to therapeutic proteins. Richards and coworkers in the Koide lab subsequently demonstrated display and selection of FN3 domains on pVIII using a phagemid display vector (Julie Richards et al., 2003). Because of the relatively low affinity of the parent FnFN10 domain for integrin binding, multivalent pVIII display was used to identify a high affinity, selective binder to αvβ3. The selected FN3 domain inhibits αvβ3 dependent processes *in vitro* in a manner similar to that described for antibodies thereby providing additional support for the utility of FN3 scaffolds as antibody-mimetics.

In 2006, Steiner and coworkers reported that highly stable, highly expressed proteins often demonstrate compromised display from standard phage display vectors (Steiner et al., 2006). For filamentous phages, translated proteins need to traverse the bacterial wall to the periplasm before assembling into the mature phage particle. Transolocation is triggered by the signal sequences that precede the mature gene sequence and most standard phage display vectors rely on Sec-dependent translocation sequences (ie. OmpA). Steiner et al. showed that exchange of the phage-fusion protein Sec-dependent signal sequence to the cotranslational signal recognition particle (SRP) translocation pathway enabled display improvements of up to 1000-fold. As illustrated in Figure 2A, Koide and coworkers applied this strategy to develop an improved phage display vector employing the DsbA signal sequence instead of the OmpA sequence used in earlier work. (Koide et al., 2007)

In vitro display strategies have proved to be a valuable tool for selection of high affinity scFvs, protease resistant peptides, and other scaffold protein libraries (Binz et al., 2005, Eldridge et al., 2009, Hanes & Pluckthun, 1997). Xu and coworkers applied a novel mRNA display strategy to construct a library based on the structural analogy between FN3 domains and antibody V-domains (Xu et al., 2002). The library encoded 21 random residues in the BC, DE, and FG loops of the FnFN10 domain with a theoretical diversity of 20^{21}. While one advantage of *in vitro* display libraries is the size of libraries it is possible to generate, the practical size of such libraries is ~10^{12}. The Xu library was constructed in multiple pieces using PCR and random oligonucleotides to introduce diversity in to the loop regions. To remove non-productive sequences from the library, each segment was fused in frame to a T7 promoter and amino and carboxy terminal tags and the translated product subjected to affinity purification on the appropriate affinity tag ligands. Productive library members were recovered by PCR and joined to prepare the full length library. For mRNA display, a library of dsDNA is transcribed to generate mRNA which is ligated to a puromycin containing oligonucleotide (Takahashi, 2003)(Figure 2B). An *in vitro* translation reaction is performed to yield the RNA/DNA/protein complex that can be selected for binding. The final step in the selection cycle is to recover the mRNA-protein complexes by PCR to regenerate an enriched dsDNA library. Xu et al. selected high affinity, highly selective inhibitors to TNFα thereby confirming that the FnFN10 scaffold had broad utility for protein therapeutics and diagnostics applications. Reports from the Roberts lab have described

mRNA display selections for FN3 binders in a variety of applications involving both intracellular and extracellular targets (Liao et al., 2009, Olson & Roberts, 2007).

Odegrip and coworkers have described the optimization of an *in vitro* display system that addresses the RNA instability challenges seen with mRNA and ribosome display (Odegrip et al., 2004). The method, termed CIS display, makes use of the property of the DNA replication initiator protein (RepA) to bind exclusively to the DNA from which it has been expressed (ie. in cis). Recently, Jacobs and coworkers have applied CIS display for identification of stable, high affinity binders from a consensus sequence based on FN3 domains from human tenascin C (manuscript in preparation).

One of the limitations of phage display systems is their reliance on bacterial expression. Wittrup and coworkers have exploited the eukaryotic secretion machinery in yeast to develop a display platform that can be screened to discriminate between domains of different stability and different affinity (Shusta et al., 1999) (Figure 2C). One advantage of yeast display is the ability to use fluorescence activated cell sorting (FACS) to sort specific clones on the basis of scaffold display level and binding activity. Yeast display has the disadvantage of limiting library diversity to ~10^8 due to the much lower transformation efficiency of yeast compared to bacteria. Despite the limitations on library size dictated by yeast, the studies by Lipovsek et al. demonstrate the utility of yeast display for maturation of high affinity binders (Lipovsek et al., 2007). Randomizing only two loops (14 residues) of the ^{10}Fn3 domain and using libraries several orders of magnitude smaller than those possible with mRNA display, Lipovsek was able to select binders to TNFα with sub-nanomolar affinity, a 340-fold improvement from the parental clone. Hackel and coworkers have extended the application of yeast display with more complex library designs building on the basic principle of improved display correlating with improved stability. Using FACS to sort clones on the basis of display level and binding activity together with a step-wise affinity maturation scheme that relies on the yeast homologous recombination machinery for *in vivo* shuffling, stable, high affinity binders to a variety of targets were identified (Hackel et al., 2010, Hackel et al., 2008a).

3. Engineering for stability and solubility

One of the significant advantages of alternative scaffolds compared to antibodies is the improved biophysical properties for scaffold domains. Indeed, the stability and solubility properties of FN3 domains are an important point of differentiation that may allow for increased tissue penetration, reduced immunogenicity, and development of high concentration formulations. Often, sequence modifications to very small stable protein domains can have a significant impact on protein stability and solubility. Thus, researchers working on protein scaffolds have developed strategies to maximize and screen for domain stability as part of the selection process.

As the originators of the FN3 scaffold design work, Koide and coworkers were early leaders in exploring domain stability. In contrast to the generally accepted premise that surface electrostatic interactions have little role in overall protein stability, the studies by Koide demonstrate that a negatively charged patch on the surface of FnFN10 plays an important role in limiting the stability of the domain (Koide et al., 2001). Mutation of one of the negatively charged residues to a neutral asparagine residue or to a positively charged lysine

residue increased the T_m for the proteins by 7° or 9°C compared to the native domain. These studies suggest the potential for improved stability of selected FnFN10 domains by engineering surface residues.

As researchers began to consider introduction of diversity into a variety of alternative scaffold domains to enable selection of novel therapeutic and diagnostic candidates, Batori and coworkers systematically explored the effects of loop elongation on each of the loops in FnFN10 (Batori et al., 2002). In order to determine which loops of the domain might be best utilized for target binding, they assessed the impact of elongation on the conformational stability of the domain. While it was possible to introduce up to four additional glycine residues in all six loops of FnFN10 while retaining the global fold, EF loop elongation was highly destabilizing. Mutations in the other five loops had only modest destabilizing effects suggesting the potential to use the loops for engineering binding affinity.

Dutta et al. explored the potential to apply yeast two-hybrid fragment complementation to select FN3 domains with improved stability (Dutta et al., 2005). To assess complementation, FN3 fragment pairs were designed wherein the C-terminal fragment of each pair was fused to the LexA DNA binding domain and the N-terminal fragment was fused to the B42 activation domain. A yeast two-hybrid β-galactosidase assay was used to evaluate each of the combinations for the ability to regenerate a highly stable domain. The scientists hypothesized that an increase in the affinity of fragment complementation would increase the stability of the uncut parental protein. Using three previously identified destabilizing mutations, a library of mutants was designed with the intent to select for compensating mutations in the opposite fragment. Mutations obtained from the selection re-introduced into the wild type and mutant domains resulted in ~2 kcal/mol increase in stability and demonstrated the utility of the fragment complementation method for identification of stabilizing mutations.

Stability and solubility properties of candidate therapeutics are of key importance for successful development. From a chemistry, manufacturing and controls (CMC) perspective, the stability properties of a biotherapeutic candidate often play a major role in the design of purification strategies, protease sensitivity and storage conditions. For example, an early step in the purification of bacterially expressed FN3 domains with a high melting temperature can involve heating the bacterial lysate to > 60°C where most host proteins unfold irreversibly. The FN3 domain remains in solution and significant purification is achieved. For storage of final product, high stability products offer the potential advantage of room temperature storage. Parker and coworkers (Parker et al., 2005) reported on a strategy to improve the thermal stability of selected FN3 domains. In previous experiments, two anti-VEGF-R2 binding clones were identified with only six amino acid differences between sequences. Nevertheless, there were significant differences in the biophysical properties of the clones. Clone VR28 had a relatively low affinity of 13 nM for VEGR2 and relatively higher thermal stability of 62°C while clone 159 was higher affinity with a Kd of 0.34 nM for VEGF-R2 and a thermal stability of 32-52°C. In addition, the two clones exhibit significant differences in solubility and aggregation state. Examination of the sequences for the wt and two FnFN3 derivatives enabled design of mutants with high affinity (0.59 nM) and high thermal stability (59°C).

Olson and Roberts took a novel approach to identifying FN3 domain variants with good stability and solubility properties using a green fluorescent protein (GFP) reporter screen

(Garcia-Ibilcieta et al., 2008), SDS-PAGE analysis and chemical denaturation (Olson & Roberts, 2007). A library comprising 7 random residues in the BC loop and 10 random residues in the FG loop was synthesized by overlap PCR and inserted in frame between a T7 promoter and a tobacco mosaic virus translation enhancer for mRNA display. The library was also cloned into a GFP fusion vector for assessment of library quality (ie. in frame sequences, no stop codons) and folding. Approximately 45% of the library sequences appeared to be in frame with no stop codons. Given the large size of the library (> 10^{13} sequences), the functional size remains significantly larger than is possible with other display methods. Overall fluorescence intensities of "functional" clones populated a continuum of fluorescence values from <1%-130% of the parental domain standard. Approximately 20% of the library had fluorescence values between 80%-130%, suggesting that these variants are well folded with an additional 30% of the library exhibiting fluorescence values consistent with potential utility. This study demonstrates the value of GFP screening to evaluate complex FN3 domain libraries for library quality and maintenance of proper folding.

Olson and coworkers applied the GFP screening strategy for identification of FN3 scaffold IkBa binders with improved solubility (Olson et al., 2008). Three rounds of evolution using error-prone PCR on a low solubility binder were performed. Following each round of evolution, approximately 2000 colonies were screened for increased fluorescence using the GFP fusion technology described above. By combining two point mutations correlated with improved solubility, Olson was able identify an FN3 clone with expression improved to ~ 1mg/L culture while maintaining affinity. The improvements in solubility and binding suggest that the overall stability of the domain could be improved with only modest changes to the overall sequence.

Most recently, a report by Jacobs et al. describes the use of consensus design to prepare a highly stable FN3 domain (Jacobs, et al. 2012). Following an alignment of the 15 FN3 domains found in the human protein tenascin C or 15 FN3 domains from human fibronectin, the researchers designed an alignment based on the most conserved amino acid at each position. The resulting consensus domains (Tencon, **Ten**ascin **con**sensus, or Fibcon, **Fib**ronectin **con**sensus) demonstrate excellent stability with T_ms of 78°C and 90°C respectively and adopt the designed fold as confirmed by x-ray crystallography. A series of mutations were designed to increase the stability of Tencon even further by consideration of core packing and secondary structure preferences for amino acids. Several of the mutations which were shown to improve domain stability individually were combined to yield scaffold with significantly improved stability and a T_m ~ 93°C. The authors note that preliminary data suggests that high affinity, high stability Tencon FN3 domain binders have been identified from libraries based on the engineered consensus domain and that the improved stability and biophysical properties of library members can be traced to the increase stability of the parent scaffold.

4. Engineering to improve binding affinity

The compact, simple structure and exceptional stability of the FN3 domain has enabled the development of numerous methods for the engineering of high-affinity binders. The engineering of an FN3 domain to specifically bind to a non-natural target protein was first described by Koide and colleagues, who engineered the FnFN10 to bind specifically to

ubiquitin (Koide, et al., 1998). In this seminal paper, the authors introduced random amino acids into the BC and FG loops of FN3 and selected binders via phage display. The authors chose to randomize these particular loops due to the sequence variations of these loops among FN3 family members as well as the fact that FG and/or BC loops have been shown to be responsible for interactions with integrins and the human growth hormone receptor (de Vos et al., 1992, Main, et al., 1992). One variation found in FN3 structure is the length of the loops connecting the β-strands. Koide et al. randomized replaced the BC loop with 5 random amino acids, the same length as that of the native FnFN10, while the randomized FG loop was shortened from 8 residues to 5. The resulting library was estimated to be 10^8 in complexity. After selection by phage display, one dominant clone was identified that bound specifically to ubiquitin, albeit with low affinity (~ 5 μM). Alanine scanning experiments demonstrated that residues of both the BC and FG loop contribute to ubiquitin binding.

A similar FnFN10 phage display library randomizing 5 residues in each of the BC and FG loops was displayed on M13 phage, this time with a complexity of $2X10^9$ (Karatan et al., 2004). After three rounds of selection against the SH3 domain of human c-Src, 6 variants were found that bound to the target specifically, with affinities ranging from 0.25 to 1.3 μM. Most of the clones discovered contained proline rich loops, reminiscent of the binding of natural proteins to SH3 domains (Olson, et al., 2008). As with the previous example, both the BC and FG loops were found to contribute to SH3 binding.

A more complex library consisting of a mixture of three separately made libraries in which the FG loop, the BC and FG loops, and the BC, FG, and DE loops were randomized was produced for selection using the mRNA display system (Dineen et al., 2008, Xu, et al., 2002). This library design randomized 21 FN3 residues simultaneously, 10 residues from the FG loop, 7 residues from the BC loop, and 4 residues from the DE loop. Because of the use of an *in vitro* display system, a library of very high complexity (10^{12}) was produced. The preselected library was panned against TNFα which was coupled to sepharose beads and screening for binding completed after 9 and 10 rounds of selection. After 10 rounds, a diverse set of TNFα binding sequences were identified with K_D values ranging from 1-24 nM. In order to drive the selection process to higher affinity, 4 additional rounds of selection were completed either with or without the incorporation of error-prone PCR to increase diversity. The tightest binder obtained (K_D 20 pM) was found from the selection strategy incorporating error-prone PCR, indicating that adding diversity to a pool of enriched binders can lead to higher affinity binding. The effect on binding of framework mutations outside of the randomized loops that might arise from error-prone mutagenesis was not investigated in this study. Interestingly the tightest binding clone found had an FG loop that was truncated to 4 residues, highlighting the importance of minimizing entropic factors when producing high affinity interactions.

The same FnFN10 mRNA Display library used to isolate TNFα binders was also utilized to select FN3 domains that bind to the extracellular domain of VEGFR2 (Getmanova et al., 2006). After 6 rounds of selection, clones binding to VEGFR2 that could compete with the binding of VEGF to this receptor were identified. As the affinities of clones from the original rounds of selection showed only modest affinity (~ 10 nM), an affinity maturation strategy employing hypermutagenic PCR to randomize the loops of the best binder from round 6 was employed. Although this technique led to only slight improvements in affinity, 2

serendipitous mutations in the N-terminal A-strand were found to improve the binding to VEGFR2, leading to a second affinity maturation strategy where the FG loop was further diversified in combination with truncation of the N-terminus. This alternative strategy led to the discovery of anti-VEGFR2 FN3 molecules with affinity as high as 320 pM.

The previous examples utilizing mRNA display demonstrate the power of large libraries to generate high affinity binding molecules. However, several studies have shown that sub-nanomolar binding affinities can be obtained even with smaller functional libraries. Lipovsek et al. used yeast surface display (Boder & Wittrup, 1997) to select lysozyme binders from FnFN10 libraries in which 7 BC loop and 7 FG loop residues were randomized simultaneously or individually (Lipovsek, et al., 2007). In this case, triplet codons were used to generate DNA diversity, thus incorporating all 20 amino acids and no stop codons into the functional library. 1 round of magnetic-activated cell sorting and 3 rounds of fluorescence-activated cell sorting were used to select yeast displaying FN3 molecules that bound to lysozyme. No enrichment was obtained from a library with only the BC loop randomized and specific binders were selected from all other libraries. Affinity maturation libraries in which 1 loop of a binder was fixed and the other randomized were produced and subjected to 4 more rounds of FACS sorting. FN3 domains binding to lysozyme with affinities as high as 350 pM were obtained after maturation. Interestingly, the majority of FN3 molecules found after affinity maturation, and all of the tightest binders, were found to have conserved cysteine residues in each of the BC and FG loops, which presumably stabilize the confirmations of the loops by forming a disulfide bond leading to a decrease in the entropic penalty of binding and resulting in higher affinities. Similarly, disulfides bonds between CDR1 and CDR3 have been found to stabilize camelid single domain antibodies (Dong et al., 2011, Sweeney et al., 2008, Tanha et al., 2001).

A series of studies from the Wittrup lab investigated the effect of amino acid composition and loop length on the ability to select high affinity binders by yeast surface display (Hackel, et al., 2010, Hackel et al., 2008b, Hackel & Wittrup, 2010). A library, produced by PCR and recombination in yeast, randomized the BC, DE, and FG loops of FnFN10 simultaneously. Diversity was achieved not only by incorporating random amino acids via NNB diversity, but also by varying the loop lengths within the library from 4-7 residues (DE), 6-9 residues (BC), and 5-10 residues (FG) (Hackel, et al., 2008b). As this library was displayed on yeast and could only be made to a diversity of $2.3X10^7$ clones, additional diversity was incorporated between rounds of sorting using error-prone PCR of the loops in combination with loop shuffling. 3 rounds of selection against lysozyme followed by 4 additional rounds with affinity maturation and decreasing concentrations of target protein were employed to produce FN3 molecules with binding affinities ranging from 1 uM to 2 pM. Sequence analysis of the selected clones suggests that both loop shuffling and error-prone PCR can contribute to increased affinities. Mutagenesis of the highest affinity binder indicated that binding is dominated by the BC and FG loop and revealed that framework mutations derived from error-prone PCR can have a large effect on affinity. The highest affinity binders were all found to have length deviations compared to wild-type FnFN10 in at least one of the loops, highlighting the potential benefits of incorporating loop length diversity into a library. In support of this idea, loop length diversity was also found to be important in selecting FnFN10 variants against MBP, hSUMO4, and ySUMO4 using a minimalist library composed of only Tyr and Ser at diversified positions (Koide, et al., 2007).

It will be interesting to examine if dominant loop lengths emerge as these libraries are used to select binders to additional targets.

A variation of this yeast display library was produced in which the diversity of certain FnFN10 loop positions thought to play a role in protein stability were biased towards that of the WT FnFN10 protein in order to maximize the stability and folding of library members (Hackel, et al., 2010). Three library compositions were compared, the first in which the BC, DE, and FG loops were completely randomized, a second in which certain positions in the DE loop were biased towards WT sequences and the remaining loop sequences randomized as 50% Ser, 50% Tyr, and finally a third design in which structurally important residues in the BC and FG loop were biased towards WT sequences and the diversity of the remaining residues was tailored to the diversity found in CDR3 of antibodies (Zemlin et al., 2003). Yeast surface display levels of random clones from these libraries confirmed that molecules from the 3rd library were generally more stably folded, demonstrating the benefit of incorporating WT sequences into certain loop positions. For analysis, these three individual libraries were pooled and selections carried out against 7 different targets: human A33, mouse A33, epidermal growth factor receptor (EGFR), mouse IgG, human serum albumin, Fcγ receptor IIA and Fcγ receptor IIIA. After selections by yeast surface display, binders were selected against each target, although only one binding sequence was found for several of the targets. 90% of the recovered sequences were derived from the 3rd library design described above, evidence that the improved stability of this library compared to the others leads to easier selection of binders. Due to the complex nature of the designs and the mixing of the libraries for selections, it is not possible to determine if the increased binding efficiency of this library is due to the stabilizing mutations or to the use of diversity tailored to resemble the CDRs of antibodies.

An alternative approach to achieve high affinity relies not on the design of FN3 libraries, but solely on an increase in avidity for target binding (Duan et al., 2007). A FnFN10 variant with an enhanced RGD sequence in the FG loop that has improved affinity for αvβ3 integrin (J. Richards et al., 2003) was produced as a fusion protein to the COMP assembly domain, which forms a pentamer via a five-stranded a-helical bundle (Malashkevich et al., 1996). In this construct, the FN3 domain was extended from the COMP domain with a 20 amino acid helical linker followed by a 25 residue flexible linker. The majority of the fusion protein produced in *E. coli* was found to be pentameric, however monomers, dimers, trimers, and tetramers could also be detected by SDS-PAGE. Biacore analysis demonstrated a > 100-fold decrease in k_{off} for the pentameric FN3 form in comparison to the monomeric form, indicating an avidity effect in binding.

The studies reviewed here collectively show that a wide range of FN3 library designs can lead to the selection of high-affinity binding molecules. The choice of loops to be randomized, amino acid composition of the randomizations, length of randomized loops, specific positions randomized within the loop, and choice of affinity maturation strategy can all have a large effect on the quality and affinity of binders isolated. It has been demonstrated that large *in vitro* display libraries or comparatively smaller phage display and smaller yet yeast surface display libraries can all be utilized effectively. No consensus has yet emerged as to which loop design strategy yields the best binders, although current studies indicate that the FG and BC loops are most important for target binding, while the DE loop may help to stabilize the confirmations of the other loops. Strict comparisons of the

effectiveness of the different library designs described are difficult as the target molecules as well as the display systems and selection methods utilized are often different between studies. However, the pace of published studies describing the selection of FN3 binding reagents has quickened considerably since the first description by Koide *et al*. We anticipate that as more detailed characterizations for FN3 binders are reported, rules governing library design for this scaffold class will begin to emerge.

Targets	Display System	Library	Maturation Strategy	Highest Affinity	Reference
Ubiquitin	P	BC5 + FG5	none	~ 5 μM	Koide, Bailey et al. 1998
Src SH3	P	BC5 + FG5	none	250 nM	Karatan, Merguerian et al. 2004
TNF-α	R	BC7 + DE4 + FG10	error prone PCR	20 pM	Xu, Aha et al. 2002
VEGFR2	R	BC7 + DE4 + FG10	hypermutagenic PCR, N-terminal truncation	320 pM	Getmanova, Chen et al. 2006
MBP, hSUMO4, ySUMO4	P + Y	Tyr/Ser BC(6-10) + DE(4-10) + FG(9-13)	none	5 nM	Koide, Gilbreth et al. 2007
lysozyme	Y	BC7, FG7, BC7 + FG7	re-randomize BC loop	350 pM	Lipovsek, Lippow et al. 2007
lysozyme	Y	BC(6-9) + DE(4-7) + FG(5-10)	error prone PCR + loop shuffling	1.1 pM	Hackel, Kapila et al. 2008
Human A33, mouse A33, EGFR, mIgG, HSA, FcγRIIA and FcγRIIIA.	Y	various loop compositions	error prone PCR + loop shuffling	250 pM	Hackel, Ackerman et al. 2010
rabbit IgG, goat IgG	Y	various loop compositions and lengths	error prone PCR + loop shuffling	51 pM	Hackel and Wittrup 2010
Abl kinase SH2 domain	P		none		Wojcik, Hantschel et al. 2010
Phosphorylated IκBα	R	BC7 + FG10	none	18 nM	Olson, Liao et al. 2008
N Protein of SARS	R	BC7 + FG10	none	1.7 nM	Liao, Olson et al. 2009
Estrogen Receptor α	H	FG7, AB7	none	not reported	Koide, Abbatiello et al. 2002

Table 1. Selected binders from naïve FN3 libraries. Display systems are abbreviated as P for phage display, Y for yeast surface display, R for mRNA display, and H for yeast 2-hybrid. Numbers in parenthesis after loop descriptions refer to the length of randomized loops. Tyr/Ser refers to a loop composition made entirely of tyrosine and serine amino acids.

5. Applications

Over the last decade since the first reports on the utility of FN3 domains as antibody mimics or alternative scaffold proteins, this class of proteins has been engineered for a variety of applications. Affinities of FN3 binders have now been described with values and specificity

similar to those obtained for antibody antigen interactions. The inherent biophysical properties of FN3 domains and the development of strategies for stability and affinity evolution have extended the utility of the scaffold. On the basis of promising preclinical tumor xenograft studies for an anti VEGFR2- FN3 domain (Dineen, et al., 2008), the first FN3-based therapeutic has been evaluated in human patients in a Phase 1 study (Molckovsky & Siu, 2008, Sweeney, et al., 2008). Initial pharmacokinetic studies for a PEGylated anti-VEGFR2 FN3 domain dosed intravenously have demonstrated that the scaffold has a terminal half-life of ~69 hours with a maximum tolerated dose of 2 mg/kg weekly. No clinically significant immunogenicity was observed and a biomarker for VEGFR2 demonstrated target engagement. These studies provide the foundation for advancement of additional FN3 domain scaffolds into the clinic.

One of the many attractive features of FN3 domain scaffolds is the ability to link domains with different specificities together to form multi-specific therapeutics. As many inflammatory and oncologic diseases have been demonstrated to be dependent on activities in multiple pathways, the ability to create multi-specific inhibitors has significant potential for biotherapeutics. Emanuel and coworkers recently provided the first report of a bispecific FN3-domain inhibitor (Emanuel et al., 2011). Adnectins™ with high affinity to epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 receptor (IGF-1R) were selected using mRNA display. Given that both receptors mediate proliferative and survival cell signaling in cancer, bispecific constructs with Adnectins™ fused in tandem via a flexible glycine-serine linker were evaluated *in vitro.* In *in vivo* studies, pegylated bispecific constructs inhibited growth of EGFR and IGF-1R driven tumor xenografts, induced degradation of EGFR and reduced EGFR phosphorylation. These studies demonstrate that engineering of multi-specific FN3 domains as anti-tumor agents may have the potential to improve efficacy over mono-specific biologic therapies.

Active sites of proteins are often located at domain interfaces in multi-domain proteins. Huang et al. explored the potential to engineer a novel interdomain "active site" by linking a low-affinity peptide binding domain (the primary domain) with an unrelated FN3 domain (the enhancer domain) and evolving for high affinity, high specificity binding (Huang et al., 2008). Evolution was initiated from the Erbin PDZ domain that has micromolar affinity for the C-termini of p-120-related catenins. An FN3 library was linked to the PDZ domain to create a two-domain protein interface not naturally observed. The strategy, termed "directed domain interface evolution" was used to select a novel protein that bound to the target peptide with an affinity enhancement of nearly 500 fold significantly greater than that attainable by evolution of the PDZ domain alone. The x-ray crystal structure showed a clamshell structure quite similar to the intended design. The novel "affinity-clamp" protein demonstrates the utility of FN3 domains as affinity reagents that may be useful in a variety of applications. Huang and Koide (Huang & Koide, 210) demonstrated the utility of the affinity-clamp strategy to monitor a peptide biomarker using FRET-optimized fluorescent proteins. Fusion of one fluorescent protein to the clamp PDZ domain and a second fluorescent protein to the FN3 domain allowed for strong FRET activity for the unbound affinity clamp. In the presence of target peptide, the affinity clamp undergoes a significant conformational change and a large decrease in FRET. The researchers demonstrated that peptide concentration in crude cell lysates could be readily quantified over 3 orders of magnitude using FRET measurements thus providing a general strategy for monitoring

peptide motifs via such label-free sensors. Olson and coworkers took a similar approach using mRNA display to select high-affinity phosphorylation-specific IκBα-binding FN3 domains (Olson, et al., 2008). The selected domain specifically recognized endogenous phosphorylated IκBα from mammalian cell extracts. The utililty of such specific reagents for monitoring kinase activity was demonstrated by creation of n IκB kinase (IKK) FRET biosensor. To engineer the biosensor, cyan fluorescent protein (CFP) was fused at one end of the FN3 domain and yellow fluorescent protein (YFP) at the other end. The IκBα peptide sequence, an IKK substrate, was inserted between the YFP and the FN3 domain where it is readily phosphorylated in the presence of IKK. Binding of the phospho-peptide to the FN3 domain induces a conformational change bringing the two fluorescent domains close enough to enable FRET.

The absence of disulfide bonds in FN3 domains is an attractive feature that suggests the potential for these domains to function intracellularly. Liao and coworkers demonstrated this potential with selection of FN3 domains that bind to the nucleocapsid protein (N) from several acute respiratory syndrome (SARS) coronavirus (Liao, et al., 2009). The researchers demonstrated that intrabodies are well expressed in mammalian cells and that they co-localize with the N-protein. Most significantly, they were able to demonstrate that selected intrabodies inhibited virus replication and that intrabodies binding two unique sites on N-protein can synergize to inhibit virus replication. This work represents the first demonstration of intracellularly functional FN3 domains and provides the framework for expanding the utility of FN3 scaffolds beyond extracellular targets. Ishikawa and coworkers from the same lab used the selected SARS N-protein binders to prepare nanowire/nanotube biosensors (Ishikawa et al., 2009). In2O3 nanowire based biosensors were configured with the SARS N-protein FN3 domain binder to enable detection of subnanolar concentrations of N-protein in the presence of high concentrations of background protein. The concentration dependent binding of N-protein demonstrates the utility of biosensors modified with engineered FN3 domains for selective and sensitive detection of biomarker proteins.

A particularly elegant application of FN3 domain binders as fluorescent biosensors was recently reported by Gulyani et al. (Gulyani et al., 2011) A Src-family kinase (SFK) biosensor was prepared using a FN3 domain specific for activated SFKs. Binding of the FN3 domain to its target was monitored by attachment of a bright, environmentally sensitive fluorescent dye to the scaffold protein where target protein binding was shown to increase fluorescence. Here again, the lack of disulfide bonds in FN3 domains provides the ability for the biosensor to function in living cells where SRC activation dynamics can be monitored. Using automated image analysis, the biosensor showed specific activation of SFKs during protrusion with a level of activity proportional to the velocity of the extending edge. Such studies may be extended to other cellular activities via the combination of high throughput FN3 domain selections, intracellular functionality, optimization of fluorescent dye attachment and biosensor engineering.

6. Conclusion

The applications described above represent an evolving landscape for FN3 scaffold domains with strategies designed to take advantage of the novel features of such domains. The

advent of multiple display platforms together with the development of strategies for improving stability, solubility and affinity of FN3 domain binders has laid the foundation for novel applications of the platform. We anticipate that bispecific FN3 domain scaffolds will become an important therapeutic modality with the potential for increasing specificity and efficacy. In addition, the chemical properties of FN3 domains will likely extend their utility to intracellular targets. Increasingly, diagnostics and biosensors based on FN3 domains are expected to play a role in monitoring biomarkers, intracellular signaling networks and providing high sensitivity images of intracellular activities. Finally, we expect that the biophysical properties of the FN3 scaffolds will play an important role in novel targeting and delivery technologies.

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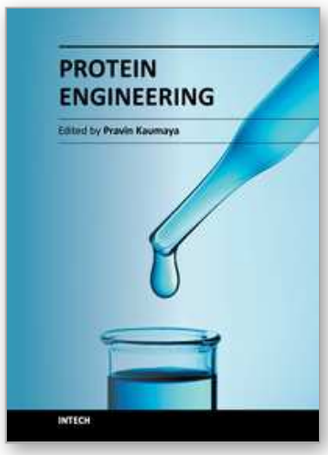

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