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Directed evolution of the forkhead-associated domain to generate anti-phosphospecific reagents by phage-display

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Abstract

While affinity reagents are valuable tools for monitoring protein phosphorylation and studying signaling events in cells, generating them through immunization of animals with phosphopeptides is expensive, laborious and time consuming. An attractive alternative is to use protein evolution techniques and isolate new anti-phosphopeptide binding specificities from a library of variants of a phosphopeptide-binding domain. To explore this strategy, we attempted to display on the surface of bacteriophage M13, the N-terminal Forkhead-associated domain (FHA1) of yeast Rad53p, which is a naturally occurring phosphothreonine (pT)-binding domain, and found it to be non-functional due to misfolding in the bacterial periplasm. To overcome this limitation, a library of FHA1 variants was constructed by mutagenic PCR and functional variants were isolated after three rounds of affinity selection with its pT peptide ligand. A hydrophobic residue at position 34 in the β 1-strand was discovered to be essential for phage-display of a functional FHA1 domain. Additionally, by heating the phage library to 50°C prior to affinity selection with its cognate pT peptide, we identified a variant (G2) that was ~8°C more thermal stable than the wild-type domain. Using G2 as a scaffold, we constructed phage-displayed libraries of FHA1 variants and affinity selected for variants that bound selectively to five pT peptides. These reagents are renewable and have high protein yields (~20–25 mg/L), when expressed in *Escherichia coli*. Thus, we have changed the specificity of the FHA1 domain and demonstrated that engineering phosphopeptide-binding domains is an attractive avenue for generating new anti-phosphopeptide binding specificities *in vitro* by phage-display.

Keywords

alanine-scanning; FHA1 libraries; phosphothreonine peptides; isothermal calorimetry; affinity selection

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Introduction

A cascade of signaling events, involving many protein-protein interactions, are initiated within the cell in response to an external stimulus, such as binding of a ligand to its receptor. The signal is translocated to downstream effectors by the reversible action of protein kinases, phosphatases and phosphopeptide-binding domains. The human genome encodes for ~500 protein kinases and a third of that number for protein phosphatases¹, and defective expression of kinases or phosphatases is the cause for various types of diseases². Radioisotopic labeling studies have shown that a third of the total proteins in the cell are phosphorylated at any given time³. Phosphorylation of serine/threonine residues on proteins can lead not only to conformational changes in proteins but also create binding sites for phosphopeptide-binding domains, which play a critical role in the formation of multiprotein signaling complexes for relaying the signal to downstream signaling proteins⁴. Similar to the recognition of phosphotyrosine (pY) residues by Src homology-2 (SH2) domains and phosphotyrosine binding (PTB) domains, phosphorylation of serine/threonine residues creates binding sites for proteins containing phosphoserine/threonine (pS/T)-binding domains, such as the 14-3-3 proteins, tryptophan-tryptophan (WW) domain of Pin1 protein⁹, FHA domain found in prokaryotic and eukaryotic signaling proteins, and WD40 repeats of F-box proteins¹³. These phosphoprotein-binding domains play a critical role in the formation of signaling complexes that eventually relay the extracellular signal downstream in the pathway. Therefore, it is evident that protein phosphorylation is a very important post-translational modification, which is responsible for regulating proteins, translocating them to their proper subcellular location, and facilitating the formation of multiprotein complexes via protein interaction domains, for transducing signals to downstream effectors and regulating processes such as gene expression, cytoskeletal rearrangements, cell cycle progression, DNA repair and apoptosis.

Among the pS/T-binding domains, the FHA domains are unique in that they recognize only pT-containing peptides and do not show binding to either non-phosphorylated or pS-containing peptides. The optimal binding motifs for various FHA domains, from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Arabidopsis thaliana* and *Mycobacterium tuberculosis* were determined by using oriented phosphopeptide libraries that contain a fixed pT residue, which is flanked by four degenerate residues on either side of it¹⁷. From these screens, the pT +3 residue was found to be one of the major determinants of binding specificity. For example, the N-terminal FHA1 domain from *S. cerevisiae* Rad53 protein kinase prefers Asp at the pT +3 position¹⁸, the C-terminal FHA2 domain from the same protein prefers Leu/Iso at the pT +3 position¹⁹ and Met/Leu/Phe at the pY +3 position²⁰, and the FHA domain of the human Chk2 DNA damage check point kinase prefers Iso/Leu at the pT +3 position²¹. The specificity of FHA domains ranges from recognizing singly or doubly phosphorylated sequences²² to binding to an extended binding surface²³. From alanine-scanning experiments of the pT peptide, it was determined that only the pT +3 residue contributed significantly to binding to the FHA1 domain¹⁶. Interestingly, the nonconserved residues (G133 and G135) contribute to the pT +3 residue specificity²⁴. The tightest FHA domain: pT peptide interaction reported²⁵ has a K_d value of 100 nM. This article provides structural insights for the specific pT vs. pS recognition by FHA domains.

Immunizing animals with synthetic phosphopeptides has been previously the standard route chosen for generating antibodies that recognize phosphorylated residues in proteins. These antibodies are valuable tools for studying the phosphorylation of proteins upon cellular stimulation, for instance by epidermal growth factor (EGF) or insulin, and for unraveling biologically important signal transduction pathways. With the existence of thousands of phosphorylation sites in the human proteome, the typical method for generating anti-phosphopeptide antibodies requires immunization with a specific phosphopeptide, making

this process time consuming, expensive, and laborious. An ideal alternative would be to bypass the immunization step and use recombinant methods to generate antibodies in less time. For example, antibody fragments and various engineered proteins²⁸ have been exploited as scaffolds for generating useful affinity reagents. In order to generate affinity reagents against phosphopeptides, an ancillary route would be to use a naturally occurring phosphopeptide-binding domain as a scaffold, generate variants of this domain by Kunkel mutagenesis²⁹ and isolate variants that bind specifically to phosphopeptides of interest. Towards this end, we displayed the N-terminal domain of *S. cerevisiae* Rad53 protein, the FHA1 domain, and two of its engineered variants (3C-3S and 4C-4S) on the surface of bacteriophage M13. All of them were initially found to be functionally inactive when phage-displayed. This bottleneck was overcome with the discovery of mutations in the 3C-3S variant that restored binding to the pT peptide ligand, when phage-displayed. We then went on to improve the thermal stability of one of the functional variants (D2), by ~8°C compared to the WT FHA1 domain ($T_m = 66.7^\circ\text{C}$). Next, by alanine scanning, we identified residues in the most thermal stable variant, named G2 ($T_m = 74.9^\circ\text{C}$), that were critical for binding to the pT peptide ligand. We constructed phage-displayed libraries of the G2 variant and isolated variants that specifically recognize five different pT peptide sequences from transcription factor jun-B, Activating transcription factor 2 (ATF2), Mitogen-activated protein kinase 1 (MAPK1), Mitogen-activated protein kinase 3 (MAPK3) and transcription factor jun-D. By this approach, we have optimized this domain for use as a scaffold from which novel anti-phosphospecific affinity reagents have been generated.

Results and Discussion

Enabling phage-display of a functional FHA1 domain

When the wild-type (WT) FHA1 domain was displayed as an N-terminal fusion to capsid protein III of M13 bacteriophage, binding to its cognate pT peptide (Rad9-pT: SLEVpTEADATFYAKK) was not detected with phage particles. We considered the possibility that loss of activity of the phage-displayed domain was a consequence of disulfide bonds forming incorrectly, upon folding in the oxidizing environment of the periplasm. The FHA1 domain has four cysteine residues (C34, C38, C74 and C154), but they do not participate in disulfide bond formation, as shown in its three-dimensional structure¹⁷. To rule out the likelihood that the cysteines were interfering with phage-display of a functional domain, three or all four cysteines were mutated to serines, in the 3C-3S and 4C-4S variants, respectively. Nevertheless, both of these variants still remained non-functional when examined by phage ELISA. The D2 variant was functional when displayed on the surface of bacteriophage M13 (Fig. 1a). Affinity selection for isolating the D2 variant is described below.

As we could detect the presence of the Flag-epitope (by using anti-flag antibody) at the N-terminus of the phage-displayed WT FHA1 domain and its 3C-3S and 4C-4S variants, we hypothesized that while the FHA1 domains were being displayed, they lacked the proper conformation for ligand recognition. To confirm our hypothesis, we generated a single-chain antibody fragment (scFv) that recognizes the FHA1 domain when it is folded, but not denatured (Kritika Pershad and Brian K. Kay, in preparation). It was observed that the scFv bound only to the D2 variant, and not to the WT or the other two variants, which confirms that they are misfolded in the bacterial periplasm and henceforth non-functional when displayed on the surface of bacteriophage (Fig. S1). This was a surprise since the WT FHA1 domain, and its 3C-3S and 4C-4S variants, when expressed in the bacterial cytoplasm as Glutathione S-transferase (GST) fusions could bind its cognate peptide ligand (Fig. 1b). The D2 variant is functional, both as a GST fusion and when displayed on the surface of bacteriophage M13. The three-dimensional structure of the WT FHA1 domain¹⁷ represented

in cartoon fashion with PyMol software (<http://www.pymol.org>) is shown in Figure 1c, along with the hypothetical structures of the 3C-3S, 4C-4S and D2 variants.

To investigate whether the WT FHA1 domain could be properly phage-displayed with overexpression of chaperone proteins, we co-expressed five chaperones that have been shown to improve folding and yields of single-chain variable fragments (scFv)³⁰ of antibodies. This, however, did not restore the activity of the phage-displayed WT FHA1 domain (Fig. S2). The WT FHA1 was transported to the bacterial periplasm via the DsbA signal sequence³¹, which transports proteins to the periplasm as they are being translated. When the WT FHA1 domain was transported to the periplasm with a different signal sequence, TorA (a member of the twin-arginine translocation (Tat) pathway), which transports only fully folded proteins to the periplasm, the activity of the phage-displayed WT FHA1 domain was only partially restored (Fig. S3). This finding indicates that the WT FHA1 domain is not able to fold correctly in the bacterial periplasm.

Accordingly, we wondered if we could rescue binding by applying directed evolution techniques. A mutagenic library comprising of 2×10^4 variants was constructed by mutagenic PCR, using the 3C-3S version of the domain as the starting template. (We were interested in leaving one cysteine in the domain, which was away from the binding surface, so that it could be derivatized through maleimide coupling chemistry for future immobilization to resin). Mutagenic PCR was performed to amplify and generate mutations randomly across the coding region of the 3C-3S variant. This method³⁵ generates an error rate of 0.66% per position and the estimated mutants in the library are ~4% wild-type, 12%, 20%, 22%, 18%, and 12%, with 1 to 5 mutations, respectively, and 12% with 6 or more mutations. From sequencing 30 clones chosen at random from the library, it was observed that the numerical distribution of mutations in the library matched the predictions. Affinity selection of the library of variants with the cognate pT peptide, captured on plastic wells via Neutravidin coating, yielded six clones that specifically bound to the phosphorylated, but not the non-phosphorylated, peptide ligand (Fig. S4). Mutations observed in the six binders are listed in Table S1. The D2 variant, which is the tightest binder, carried one mutation (S34F), suggesting that this position, in the β 1 strand, facilitates proper folding of the phage-displayed FHA1 domain.

To survey what amino acids at position 34 permit proper folding, the D2 coding sequence was mutated one at a time to each of the other 18 natural amino acids by Kunkel mutagenesis²⁹. Phage ELISA showed that the FHA1 mutants bound to the pT peptide only when a hydrophobic amino acid (F, A, M, I, L, Y, and V) was present at this position, with a few exceptions (W, C, and P). On the other hand, hydrophilic residues at this position rendered the FHA1 variants non-functional with respect to display of a functional FHA1 domain (Fig. 2). Of all the 20 amino acids, the residue at position 34 that gave the highest level of binding was phenylalanine (i.e., the D2 variant).

Thermal stability of the D2 variant and selection of more stable variants

To evaluate the thermal stability of the D2 variant, phage particles displaying the D2 variant were heated at various temperatures (30°C, 37°C, 50°C, 60°C, 70°C, 80°C and 95°C) prior to incubation with the cognate pT peptide (Rad9-pT) and the non-phosphorylated form of the same peptide (Rad9-T). Phage ELISA showed that the D2 variant was functional when heated at 30°C and 37°C, but there was a severe drop in binding (~60%) upon heating at 50°C (Fig. 3).

The D2 variant (purified six-his tagged protein) was mixed with SYPRO orange dye with or without the Rad9-pT-containing peptide and heated from 25°C to 95°C in a real-time PCR experiment known as the Fluorescence-based thermal shift (FTS) assay³⁶. In this assay,

initially, the fluorescence of SYPRO orange dye is quenched in the aqueous environment of a folded protein, but as the protein starts to unfold upon heating, the dye interacts with the hydrophobic core of the protein and its fluorescence increases. Therefore, an increase in fluorescence of the dye is directly proportional to protein unfolding, until a temperature (referred to as T_m here) is reached at which the dye fluorescence decreases due to aggregation and precipitation of the protein. Since the melting temperature (T_m) of the D2 variant was determined to be $\sim 5^\circ\text{C}$ lower than the T_m of the WT FHA1 domain, to improve its thermal stability, affinity selection was carried out by heating the phage library displaying D2 variants at 50°C prior to incubation with the pT peptide ligand to favor the isolation of variants that remained folded and functional above 50°C ³⁷. A similar approach was employed to improve the thermal stability, expression and affinity of a recombinant antibody fragment³⁸. A mutagenic library (6.5×10^7 variants) was constructed using the D2 coding sequence as the starting template by following the protocol for mutagenic PCR³⁵. Random mutations were generated in the coding region of the D2 variant. The expected number of mutations is the same as described for the 3C-3S mutagenic library. Sequencing 60 library members revealed that the D2 library contains $\sim 8\%$ wild type, 16%, 20%, 23%, 15%, and 10%, with 1 to 5 mutations, respectively, and 8% had 6 or more mutations. Three rounds of affinity selection with the D2 phage library, which was heated to 50°C , yielded five FHA1 variants (A12, H7, G7, A1 and G2) that were more thermally stable than the starting D2 domain. The mutations observed in each one of the thermally stable mutants are listed (Table S2). Mutations were observed in 4 out of 11 β -strands, and in 4 loops of which two loops ($\beta 3$ - $\beta 4$ and $\beta 4$ - $\beta 5$) are involved in interaction with the pT peptide ligand and the other two loops ($\beta 1$ - $\beta 2$ and $\beta 8$ - $\beta 9$) do not interact with the pT peptide ligand. Several mutations were also observed at the N-terminus of the FHA domain, before the $\beta 1$ -strand, indicating that this region may be critical for structural and thermal stability.

The T_m values of FHA1 variants were determined by FTS assay³⁶. A representative T_m curve for the G2 variant at $1 \mu\text{M}$ concentration is shown (solid squares), alone and in the presence of the pT peptide (Fig. 4). No shift in the T_m was observed in the presence of the non-phosphorylated form of the same peptide (data not shown). The T_m values of the thermally stable variants (along with WT and D2 variant) at $1 \mu\text{M}$ and $4 \mu\text{M}$ concentration, along with the T_m shift in the presence of two different concentrations ($50 \mu\text{M}$ and $250 \mu\text{M}$) of the pT peptide, are listed in Table S3. Thus, using high temperature during affinity selection enabled us to isolate variants with favorable mutations that improved the thermal stability, as well as their expression in *E. coli*. The SDS-PAGE gel of all the purified FHA1 variants is shown (Fig. S5) and the yields per liter shake flask for the thermally stable variants, A12, H7, G7, A1 and G2 are 36, 37, 43, 56, and 63 mg, respectively and their SDS-PAGE ge

Determining the dissociation equilibrium constants (K_d) by isothermal calorimetry (ITC)

The affinity of the G2 variant and the WT FHA1 domain to the pT peptide (SLEVpTEADATFYAKK) was determined by ITC (Fig. 5). The sample cell contained the purified FHA1 domains ($35 \mu\text{M}$) and the injection syringe was filled with the pT peptide ($350 \mu\text{M}$). As seen in Figure 5, the G2 variant bound with a K_d of $\sim 0.89 \mu\text{M}$, which was similar to the K_d ($\sim 1 \mu\text{M}$) of WT FHA1 domain. The FHA1 domains bound to the pT peptide with a stoichiometry of 1:1, and a single-site binding model was used to fit the ITC data. The ITC results revealed that the thermally stable G2 variant and the WT FHA1 domain bound to the pT peptide with similar affinities. The previously reported K_d for WT FHA1 domain by ITC is $0.53 \mu\text{M}$ ¹⁷.

Alanine-scanning to determine the FHA1 residues important for interaction with the pT peptide

From previous structural studies on the FHA1-pT complex from various species, it has been shown that residues from four loops (i.e., β 3- β 4, β 4- β 5, β 6- β 7 and β 10- β 11) contact the pT peptide. Interestingly, the β 4- β 5 loop varies in sequence, structure and length among FHA domains⁴⁰, which may account for different specificities among FHA domains: for instance, the β 4- β 5 loop in FHA1 domain comprises of 11 amino acid residues, and on the other hand, the β 4- β 5 loop in Chk2 FHA domain is longer and contains 19 residues with a helical insertion in the loop²¹. The length and structure of this loop determines its positioning either close to or away from the pT +3 residue, which is an important determinant of binding specificity, therefore, resulting in specific binding to phosphopeptides with either charged (for FHA1 domain) or hydrophobic residues (for Chk2 FHA domain) in the pT +3 position. Mutating residues in the β 10- β 11 loop has been shown to alter the binding specificity of the FHA1 domain to be more like FHA2²⁴ and the residues in this loop may play an important role in the binding of the FHA1 domain to a pT peptide (from Mdt1 protein) containing a hydrophobic residue at the pT +3 position⁴⁰. Residues from β 6- β 7 loop are known to be responsible for conferring preference for binding to pT- and not pS-containing peptides³⁹.

To establish which residues in the G2 variant are important for interaction with the pT peptide (SLEVpTEADATFYAKK), alanine-scanning of each of the residues from the three loops (11 from β 4- β 5 loop, 5 from β 6- β 7 and 8 from β 10- β 11) was performed and binding of the mutants to the cognate pT peptide was tested by phage ELISA (Fig. 6a-c). Five mutants (L78A, R83A, L84A, S85A and H88A) from the β 4- β 5 loop, four mutants (S105A, T106A, N107A and G108A) from the β 6- β 7 loop and five mutants (G133A, V134A, G135A, V136A and D139A) from the β 10- β 11 loop had reduced, or no, binding to the pT peptide when mutated to alanine indicating that the interaction of these residues with the pT peptide is important for binding. Four out of these 14 mutations (S85A, H88A, N107A and G108A) destroyed folding or denatured the FHA1 domain, as determined with a recombinant antibody that recognized folded, but not denatured, FHA1 domain (Fig. S6). Therefore, 10 residues from the three loops, 3 from β 4- β 5 (green spheres), 5 from β 10- β 11 loop (red spheres) and 2 from β 6- β 7 (orange spheres) were identified to be critical for pT peptide recognition (Fig. 6d), and not for folding of the FHA1 domain. These residues are good candidates for oligonucleotide-directed mutagenesis and generation of a phage-displayed library of FHA1 variants that can be screened against various pT peptides. According to our alanine-scanning experiments, the G133A and G135A mutants showed reduced binding to the pT peptide (SLEVpTEADATFYAKK), indicating that these two positions in the FHA1 domain may play an important role in interaction with the pT peptide, as previously described²⁴.

A library of FHA1 variants, generated by mutating residues important for interaction with the pT peptide, will be a useful resource for isolating affinity reagents with new anti-phosphopeptide binding specificities different from that of the original FHA1 domain scaffold. Previously, it has been shown that variants of Erbin PDZ domain, generated by mutating ten residues known to be important for interaction with peptide ligands from structural studies, had different binding specificities compared to the wild-type PDZ domain⁴¹. Ernst et al. have demonstrated that it is possible to change the specificity of a protein interaction domain so that it binds a ligand for which an interaction was not previously detected.

To test the feasibility of generating novel phosphopeptide-binding specificities, we constructed two different phage-displayed libraries of FHA1 variants. The first library (FHA1G2 library) had 8 residues randomized (from the β 4- β 5 and β 10- β 11 loops combined) which were found to be important to bind to the pT peptide ligand from our

alanine-scanning results. In this library, only 60% clones had mutations in both the loops. To overcome this limitation, a second library was constructed (G2-*Xma*I library; 10 residues were randomized from the β 4- β 5 and β 10- β 11 loops combined) adopting strategies that resulted in 99% of the clones having mutations in both the loops.

Characterization of the oligonucleotide-directed phage-displayed libraries

Sequencing the DNA of 30 individual clones, randomly chosen from the FHA1G2 library, revealed that 61% clones had both the loops mutated, 17% had mutations in one loop, 11% were wild-type sequences, and 11% contained stop codons. The library's size was determined to be 5×10^9 , of which 3×10^9 clones have mutations in both loops.

The G2-*Xma*I libraries were constructed using the pKP700 phagemid vector containing two *Xma*I restriction sites, one in β 4- β 5 loop and the other in β 10- β 11 loop. Colony PCR, followed by *Xma*I digestion of 93 colonies (randomly chosen from the four G2-*Xma*I libraries combined), revealed that 99% of the clones had both the loops mutated. Thus, the efficiency for library construction increased from ~60% to 99%. In this method, we have combined two strategies for efficiently cleaving the wild-type phagemid vector; one is to use the intrinsic ability of TG1 cells to cleave the uracil-containing wild-type DNA (50–60% efficient from our experience) and the second is eliminating the remaining wild-type DNA by digestion with *Xma*I.

Affinity selection of FHA1 variants with novel binding specificity

Two rounds of affinity selection were performed against five different pT peptide sequences from transcription factor jun-B, Activating transcription factor 2 (ATF2), Mitogen-activated protein kinase 1 (MAPK1), Mitogen-activated protein kinase 3 (MAPK3) and transcription factor jun-D. Affinity reagents isolated from phage-displayed libraries of FHA1 variants, were tested by phage-ELISA (Fig. 7) for binding to the cognate pT peptide, the non-phosphorylated form of the same peptide and the cognate peptide for the original FHA1 domain (Rad9-pT). All of the FHA1 variants bind to their cognate pT peptide, with little or no binding to the original Rad9-pT peptide. All the interactions are pT dependent and no binding was detected for the non-phosphorylated forms of any of the peptides. This result demonstrates that the FHA1 domain can be engineered to bind to a new pT peptide and that the residues we randomized to make the library contribute to binding specificity. Our success rate with isolating affinity reagents for various pT containing peptides from these phage-displayed libraries of FHA1 variants is ~60%.

Binding specificity of the MAPK3 affinity reagent and examining the amino acids in the MAPK3 peptide ligand contributing to binding

ELISA with the purified FHA1 variant (B1), which we selected against the MAPK3-pT peptide, revealed that specific binding was observed for its cognate pT peptide with little or no binding detected for 10 other phosphopeptide sequences and to the non-phosphorylated form of the same peptide (Fig. 8a). Cross-reactivity was observed with Myc-pT peptide, corresponding to a phosphopeptide in Myc transcription factor, which was not surprising because both phosphopeptides contain Leu in the pT (+3) position and this position has been shown from mutational analysis and from screening combinatorial phosphopeptide libraries to be a major determinant of binding specificity for the FHA1 domain. Furthermore, the same FHA1 domain has been previously observed¹⁸ to cross-react with various pT peptides that contain the same pT (+3) residue, with dissociation constants ranging from 0.36 to 71 μ M, suggesting that residues on either side of the pT moiety contribute to and fine tune the binding specificity of the FHA1 domain.

Alanine replacements of the MAPK3-pT peptide (ADPEHDH**p**TGFLTEYKKK), corresponding to the MAPK3 protein, revealed that the pT (−1, +2 and +3) residues are critical for binding to the FHA1 domain, as replacing these residues leads to >90% loss of binding (Fig. 8b). There was 60–70% loss of binding when pT (+1, +4, −2 or −3) residues were replaced with alanine, indicating that these residues also contribute to binding. Mutating pT (−4) residue had no effect on binding, revealing that this residue is not important. No binding was detected for either the non-phosphorylated form of the same peptide or for a pT-containing peptide in which the residues (−4 to +4) were replaced with alanine.

From previous studies¹⁶ it has been shown that the pT (+3) residue is a key determinant for binding specificity for FHA domains. Therefore, a pT peptide was synthesized in which the +3 residue was fixed and residues from −4 to +4 were replaced with alanine. To our surprise there was only 15% residual binding, which suggests that the other residues surrounding the pT also contribute to the binding specificity in addition to the pT (+3) residue. All these results combined show that the pT (−3 to +4) residues form the binding epitope for the FHA1 domain. Alanine scanning of the FHA1 variant (B1) binding to the MAPK3-pT peptide revealed that 5 out of the 8 residues contribute to binding (not shown). Binding is completely eliminated when I84 and L78 are mutated to alanine, whereas the R133A mutant retains 40% binding and the binding of L134A and D135A mutants are each reduced by 20%. Therefore, all the residues randomized during library construction may not be important for binding to a specific pT peptide, but a unique combination of these residues will contribute to the binding specificity.

In conclusion, we have generated functional and thermally stable FHA1 variants. We have engineered the specificity of the FHA1 domain to recognize various phosphothreonine peptides, for which binding was not previously detected. The phage-displayed library of FHA1 variants is a useful resource to isolate renewable anti-phosphospecific reagents, *in vitro*, by phage-display. These anti-phosphospecific reagents will serve as valuable tools for monitoring phosphorylation of proteins inside cells, for instance upon stimulation with a growth factor or upon DNA damage and as detection reagents for Western blotting, immunoprecipitation and cell-staining experiments. For the first time, a pT-binding domain has been engineered for displaying its functional variant on the surface of bacteriophage M13 and using it as a potential scaffold for generating affinity reagents to various pT peptides. This strategy serves as a platform for exploiting other phosphopeptide-binding domains as scaffolds for generating recombinant phosphospecific affinity reagents. These reagents will be an attractive alternative for antibodies, because they are renewable, specific, have excellent expression in *E. coli* (20–25 mg/L), can be generated in a short time (<2 weeks), and are cost effective compared to immunizing animals.

Materials & Methods

Gene synthesis and subcloning into phage and expression vectors

A variant of the FHA1 domain from the *S. cerevisiae* Rad53 protein, named the 3C-3S variant, which has three cysteine residues mutated to serine (C34S, C38S and C154S), was commercially synthesized (Blue Heron Biotechnology) with codons optimized for expression in *E. coli* and the DNA was provided after subcloning into the a derivative of the pUC 119 plasmid. The 3C-3S coding sequence was amplified by polymerase chain reaction (PCR), using primers FHA1-NcoI-Fw and FHA1-NotI-Rv and the AccuPrime™ Pfx DNA polymerase (Invitrogen), for creating flanking *Nco*I/*Not*I sites for subcloning into the phagemid vector (pKP600) in-frame with the gene III coding sequence. The phagemid vector⁴² used is a modified version of the pKP300 vector except that it has a DsbA signal sequence and lacks the alkaline phosphatase coding sequence. All the primers were ordered

from Integrated DNA Technologies and their sequences are listed in Table S4. Following the protocol for Kunkel mutagenesis²⁹, the WT FHA1 domain (containing four cysteine residues at positions 34, 38, 74 and 154) was generated using the 3C-3S coding sequence as the template and two oligonucleotides; the first oligonucleotide (KM-S34C+S38C-FHA1) mutated S34 and S38 to cysteine, and the second oligonucleotide (KM-S154C-FHA1) converted position S154 serine to cysteine. Another FHA1 variant, 4C-4S (all four cysteines mutated to serines) was generated from the 3C-3S variant using one oligonucleotide, KM-C74S-FHA1, which mutated position 74 cysteine to serine. All the phagemid vectors were sequenced using the primer DsbA-Fw. For generating GST fusions of the FHA1 domains for cytoplasmic expression, their coding sequence was amplified by PCR creating *Bam* HI/*Eco* RI flanking sites using the primers-FHA1-BamHI-Fw and FHA1-EcoRI-Rv and AccuPrime™ *Pfx* DNA polymerase. The pGEX-2T GST fusion vector (GE Healthcare) was cleaved with the same two restriction endonucleases and the FHA1 domains were subcloned in-frame with the GST coding sequence. The final construct was sequenced using the primer Seq-pGEX-Fw. (All restriction enzymes were purchased from New England BioLabs.)

For expression on a large scale, the FHA domains were subcloned into a modified version of the pET29b expression vector (gift from Dr. Brian Kuhlman, University of North Carolina) in-frame with a C-terminal six-histidine tag for protein purification, by immobilized metal affinity chromatography (IMAC). The FHA1 domain coding sequences were amplified by PCR using the AccuPrime™ *Pfx* DNA polymerase and FHA1-NdeI-Fw and FHA1-XhoI-Rv primers, which created flanking *Nde* I/*Xho* I recognition sites for subcloning into the pET29b expression vector (for cytoplasmic expression). All constructs were DNA sequenced.

Protein and phage enzyme-linked immunosorbent assay (ELISA)

To amplify the phage particles displaying the recombinant FHA1 variants, TG1 bacterial cells (5 mL; Stratagene) harboring the phagemid DNA were infected at mid-log ($OD_{600nm}=0.5-0.6$) with M13K07 helper phage (New England Biolabs) at a multiplicity of infection (MOI) of 20 for 1 h at 37°C at 150 rpm. Infected cells were centrifuged, and the pellet was resuspended in fresh Luria Bertani medium (LB: 10 g Tryptone, 5 g Yeast extract, and 10 g NaCl per liter) supplemented with 50 µg/mL Carbenicillin (CB) and 50 µg/mL Kanamycin (Kan), and phage were amplified overnight at 30°C at 250 rpm.

To set up the ELISA, biotinylated peptides (100 µL, 5 µg/mL) were immobilized on Nunc MaxiSorp flat-bottom 96 well plates (Thermo Fisher Scientific) via NeutrAvidin™ Biotin binding protein (100 µL, 10 µg/mL; Thermo Fisher Scientific) and blocked with 2% skim milk in Phosphate Buffered Saline (PBS: 0.14 M Sodium Chloride, 0.003 M Potassium Chloride, 0.002 M Potassium Phosphate, and 0.01 M Sodium Phosphate; pH 7.4). After the wells were washed with PBS, the wells were filled with phage particles (100 µL of culture supernatant, diluted 1:2 with PBS containing 0.1% Tween 20; PBST) for 1 h and washed three times with PBST. The binding phage particles were detected using anti-M13 antibody conjugated to Horseradish Peroxidase (HRP) (GE Healthcare), diluted 1:5000 with PBST. After washing away the unbound antibody, the chromogenic substrate, 2,2'-Azinobis (3-ethylbenzothiazoline-6-Sulfonic Acid) diammonium salt (Thermo Fisher Scientific), supplemented with hydrogen peroxide, was added (100 µL per well), and the absorbance of the green colored complex was measured at 405 nm on a POLARstar OPTIMA microtiter plate reader (BMG Labtech).

For production of the FHA1-GST fusion protein, BL21 DE3 cells (10 mL; Stratagene) harboring the expression vector was grown overnight at 30°C using the Overnight Express™ Autoinduction System 1 (Novagen). The next day, cells were lysed using BugBuster® 10X Protein Extraction Reagent (Novagen) following the manufacturer's instructions. Cell lysate

(100 μ L diluted 1:5 with PBST) was incubated with the biotinylated peptides as described above and detected using anti-GST antibody conjugated to HRP (diluted 1:10,000 with PBST; GE Healthcare).

Peptides

Peptides were synthesized at the Research Resource Center, University of Illinois at Chicago and were >90% purity. All the peptides, except for the one used for ITC, were biotinylated at their N-terminus, and amidated at their C-terminus. The peptide used for ITC is SLEVpTEADATFYAKK¹⁷. It was purified by HPLC and does not contain a linker and N- or C- terminal modifications. In several experiments, the peptides contained a tripeptide spacer, SerGlySer (SGS), between the N-terminal biotin and the peptide sequence, and often required the addition of two or three lysine residues at the C-terminus to increase their solubility. The peptides used for affinity selection experiments did not contain the tripeptide spacer because we did not want it to potentially be part of the FHA1 domain binding 'epitope'. However, the peptides used to confirm binding in ELISA experiments did have the SGS linker, between the N-terminal biotin and the target peptide sequence. The phosphopeptide sequences, with their SGS linkers, are as follows: Rad9-pT: SGS-SLEVpTEADATFYAKK¹⁷; Rad9-pS: SGS-SLEVpSEADATFYAKK; Rad9-pY: SGS-SLEVpYEADATFYAKK; Plk1-pT: SGS-AGPMQSpTPLNGAKK⁴³, BRCT-pS: SGS-AYDIpSQVFPFAKKK⁴⁴. All the other phosphopeptides, whose sequences are obtained from phosida.com (posttranslational modification database) website, are listed in Table S5 and they do not contain the SGS linker.

Fluorescence-based thermal shift (FTS) assay

The FTS assay was performed on a MxPro-Mx3005P instrument (Stratagene), following a published protocol³⁶. It is a real-time assay using the SYBR[®] Green (with dissociation curve) to measure the increase in fluorescence, as the protein unfolds upon heating and binds the dye, making it fluorescent. The default FRROX filter set was used with an excitation wavelength of 492 nm and emission wavelength of 610 nm. FHA1 domains (2 μ M and 8 μ M, 2 $\times$ of final concentration) were mixed with 10 $\times$ SYPRO[®] Orange protein gel stain (Invitrogen; 5000X concentration in DMSO) to give a final dye concentration of 10X. In the protein only wells, the dye+protein mixture (10 μ L) was diluted 1:1 with PBS. In the other wells, 10 μ L of either the pT peptide or its non-phosphorylated form (2 $\times$ the final concentration) was added to 10 μ L of dye+protein mixture. The final reaction volume was 20 μ L, with final concentrations of 1 μ M or 4 μ M FHA1 domains, 50 μ M or 250 μ M of pT and non-phosphorylated peptides, and 5X final concentration of the SYPRO Orange dye. The assay was performed in duplicates in 96 well white PCR plates (Bio-Rad) covered with optically clear Microseal[®] 'B' Film (Bio-Rad) and heated from 25°C to 95°C. The melting curve was obtained with fluorescence (R) values plotted on Y-axis and increasing temperature (°C) on X-axis. The mid-point of the curve is considered the melting temperature inflection point.

Construction of libraries of phage-displayed FHA variants by random mutagenesis

To generate the FHA1 library, from which functional phage-displayed variants can be isolated by affinity selection, mutagenic PCR³⁵ was performed (primers: MP-FHA1-Fw and MP-FHA1-Rv), to amplify the coding sequence of the 3C-3S variant. The PCR product (~0.34 μ g) was digested with *Nco* I and *Not* I, and subcloned into a phagemid vector (pKP600, ~1 μ g), generating in-frame fusions with the gene III coding sequence at the C-terminus and a FLAG epitope tag at the N-terminus. The recombinant DNA was concentrated using a phenol:chloroform:isoamyl alcohol mixture (Sigma), and electroporated into TG1 bacterial cells. The cells were allowed to recover for 40 minutes, with shaking at 250 rpm at 37°C, of which various dilutions (10 μ L and 100 μ L of 10⁻¹ and

10^{-2}) were plated on 10 cm LB/CB agar plates. The remaining cells were plated on three 15 cm LB/CB (50 μ g/mL) agar plates. The next day, colonies were counted on the titration plates and the library diversity was determined to be 2×10^4 clones. The bacterial lawn on 15 cm plates was scraped, 30 mL of LB/CB media was inoculated with $\sim 1 \times 10^8$ cells and grown to mid-log ($OD_{600nm} = 0.5$) followed by infection with M13KO7 helper phage (MOI=20) for 1 h at 37°C at low speed (150 rpm). Infected cells were collected by centrifugation, resuspended in 30 mL of fresh LB/CB/Kan (50 μ g/mL) media and incubated overnight at 30°C with shaking at 250 rpm. The following day, phage particles were precipitated using 1/5 volume of 24% polyethylene glycol (PEG) and 3 M NaCl, and the phage pellet was resuspended in PBS (1 mL), and stored in aliquots at -80°C with 16% final glycerol concentration. Similarly, another mutagenic library (FHA1D2 library) was constructed, with a final diversity of 6×10^7 variants, using the pKP600 vector with the D2 variant coding sequence as the starting template. The schematic representation of library construction is shown in Figure 1. All the restriction enzymes were purchased from New England Biolabs. The concentration of carbenicillin (CB) and kanamycin (kan) antibiotics is 50 μ g/mL.

Affinity selection for isolating functional phage-displayed FHA1 variants

To isolate functional, phage-displayed FHA1 variants, three rounds of affinity selection were performed with its cognate pT peptide (SLEVpTEADATFYAKK) and the FHA1 library. All the selection steps were performed at room temperature. The biotinylated peptide (200 μ L, 10 μ g/mL) was immobilized on Nunc polystyrene tube (Thermo Fisher Scientific) via NeutrAvidin™ Biotin binding protein (200 μ L, 20 μ g/mL; Thermo Fisher Scientific) and blocked with 2% skim milk in PBS. Then, the phage library⁴⁵ ($\sim 1 \times 10^{10}$ phage particles) was incubated with the blocked target for 1 h, followed by six washes with PBST and six washes with PBS. Phage particles bound to the target were eluted using 100 mM glycine-HCl (100 μ L; pH 2.0), neutralized with 2 M Tris-base (6 μ L; pH 10) and used to infect 800 μ L of TG1 cells at mid-log ($OD_{600nm}=0.5$) for 40 min at 37°C. The cells were plated after infection, scraped the next day; phage was amplified and precipitated as described above. The second and third rounds of affinity selection were conducted in the same manner, except that only 1/2 the volume of the eluted phage were used to infect bacterial cells after round 2 and 1/4 of the volume was used in round 3. After the third round of affinity selection, 96 individual clones were propagated as phage, followed by phage-ELISA to identify functional clones that recognize the pT peptide ligand. Positive binding clones were sequenced, and further specificity tests were performed.

Construction of phage-displayed libraries by oligonucleotide-directed mutagenesis

1. FHA1G2 library—For constructing site-directed libraries of FHA1 variants, the pKP700 vector with the FHA1G2 coding sequence was used as the starting template. 8 residues (L78, R83, L84 from the $\beta 4$ - $\beta 5$ loop and G133, V134, G135, V136, D139 from the $\beta 10$ - $\beta 11$), in the FHA1G2 coding sequence were randomized based on our alanine-scanning results. Following Kunkel mutagenesis protocol²⁹, two oligonucleotides ($\beta 4$ - $\beta 5$ NNK and $\beta 10$ - $\beta 11$ NNK, 5' phosphorylated) with NNK codons (N = A, G, C, or T; K = G or T) at these 8 positions were annealed to the single-stranded uracilated phagemid DNA (pKP700) at a molar ratio of 1:5 (single-stranded DNA:oligonucleotides), extended using T7 DNA polymerase and the covalently closed circular DNA was sealed by T4 DNA ligase (both from New England BioLabs). A total of 15 transformations were done into electrocompetent TG1 bacterial cells (Lucigen Corporation). After recovery, the cells were pooled, plated on three 15 cm 2 $\times$ YT/CB agar plates, and incubated overnight at 30°C. The lawn of colonies were scraped with a total of 6 mL of freezing media (2 $\times$ YT/CB/16% glycerol) media and the library cells were stored at -80°C .

2. G2-XmaI library—A second library (G2-XmaI) was constructed, using two oligonucleotides (Phos β 4- β 5 Lib and Phos β 10- β 11 Lib, 5' phosphorylated) randomized at a total of 10 positions (S82, R83, L84 from the β 4- β 5 loop, and G133, V134, G135, V136, E137, S138, D139 from the β 10- β 11) with NNK codons. S82 was randomized in this library, because it was shown to make contacts with the pT peptide from previously structural studies¹⁷. L78 was excluded because preliminary affinity selection results revealed that many binding clones retained Leu at this position. E137 and S138 were randomized to facilitate efficient annealing of the oligonucleotide to the template DNA, however, according to alanine-scanning results, these residues were not important for binding to the pT peptide. The pKP700 phagemid DNA used for constructing this library has two *Xma*I restriction sites, one in the β 4- β 5 loop and the second one in the β 10- β 11 loop, introduced by Kunkel mutagenesis, using primers pKP700-G2-XmaI#1 and pKP700-G2-XmaI#2. The G2-XmaI library consisted of four sub-libraries, each with a diversity of $\sim 5\text{--}7 \times 10^9$ members. Each sub-library was constructed by performing 25 transformations into electrocompetent TG1 cells (total of $\sim 2.5 \times 10^9$ transformants). The recovered cells were pooled, grown to an $\text{OD}_{600\text{nm}}=1.0$ (10^9 cells/mL), and phagemid DNA was purified from half the number of cells (3×10^{11} cells) using the PureLink™ HiPure Plasmid Filter Maxiprep Kit (Invitrogen). The DNA (10 μg) was cut with *Xma*I restriction enzyme (5 units/ μg of DNA) for 16 h/37°C. The cut DNA was purified using one QIAquick® PCR Purification Kit (Qiagen), 10 transformations were done into electrocompetent TG1 cells (total of $\sim 5\text{--}7 \times 10^9$ transformants), and after recovery, the cells were plated on ten 15 cm 2×YT/CB agar plates. The next day, colonies were scraped with a total of 40 mL of freezing media and the library cells were stored at -80°C .

Amplification of the FHA1G2 and G2-XmaI libraries

For amplifying the library phage, 2×YT/CB media was inoculated with sufficient number of scraped cells (to cover $10\times$ the library diversity), grown to mid-log phase (infect $10\times$ the library diversity number of cells; 4×10^8 cells/mL at mid-log), and infected with the trypsin cleavable helper phage (TM13KO7, 10^{10} pfu/mL) for 1 h at $37^\circ\text{C}/150$ rpm. Infected cells, recovered after centrifugation, were resuspended in fresh 2×YT/CB/Kan medium (10 times the initial volume) and phage were amplified for 18–19 h at 30°C , with 250 rpm shaking. Phage were concentrated 100 fold by PEG/NaCl precipitation, filtered through 0.45 μm syringe filters, glycerol was added to a final concentration of 16%, and the phage library was stored at -80°C . For the four sub-libraries, phage was amplified from each library separately and pooled before performing affinity selections with various pT-containing peptides.

Affinity selection against various phosphothreonine peptides

Dynabeads® MyOne™ Streptavidin T1 magnetic beads (Invitrogen Dynal AS, 100 μL) were incubated with the biotinylated pT peptide (1.2 μg ; 1.5 μM concentration) for 30 min. All the selection steps were performed at room temperature. The unbound target was removed and the beads were blocked for 1 h with blocking buffer (2% skim milk in PBS with 1 μM free biotin; 1 mL). The phage library (3×10^{12} phage) was incubated for 15 min with equal volume of 4% skim milk in PBS, and then added to the blocked beads. Washing the beads three times with PBST and twice with PBS minimized non-specific binding of phage particles to the beads. Phage bound to the target were eluted using TPCK treated trypsin (Sigma-Aldrich, 400 μL at 100 $\mu\text{g}/\text{mL}$ concentration) and used to infect 800 μL of TG1 cells at mid-log growth phase ($\text{OD}_{600\text{nm}}=0.5$) for 40 min at 37°C . The cells were then plated on one 15 cm 2×YT/CB agar plate and the colonies were scraped the next day with 8 mL of freezing media. For amplifying the phage for the second round of selection, $\sim 10^8\text{--}10^9$ cells were inoculated into 40 mL of 2×YT/CB media, grown to mid-log, and 5 mL was infected with trypsin cleavable helper phage (TM13KO7; 10^{10} pfu/mL). The infected cells, after centrifugation, were resuspended in 30 mL of 2×YT/CB/Kan media, phage were

amplified overnight at 30°C/250 rpm and precipitated (30 fold) with PEG/NaCl mixture. The second round of affinity selection was conducted in the same manner, except that more number of washes were done before eluting the bound phage (five times with PBST and three times with PBS) and after infecting TG1 cells at mid-log with eluted phage, 10 µL and 100 µL of 10⁻² and 10⁻⁴ dilutions were plated on 2×YT/CB agar plates (10 cm). After the second round of affinity selection, 96 individual clones were propagated as phage, followed by phage ELISA to identify functional clones that recognize the pT peptide ligand, and positive binding clones were sequenced.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

pT	phosphothreonine
pS	phosphoserine
pY	phosphotyrosine
pS/T	phosphoserine/threonine
FHA	Forkhead-associated domain
GST	Glutathione S-transferase
PCR	polymerase chain reaction
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
T_m	melting temperature
ITC	isothermal calorimetry
k_d	dissociation constant
IMAC	immobilized metal affinity chromatography
HRP	horse radish peroxidase
PBS	phosphate buffered saline
PBST	PBS with 0.1% Tween 20
FTS	fluorescent thermal shift
LB	Luria Bertani
WT	wild-type
PEG	polyethylene glycol
CB	Carbenicillin
Kan	Kanamycin

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Highlights

1. Generating affinity reagents to phosphoepitopes by phage-display.
2. Engineered the FHA1 domain for functional phage-display.
3. Thermally stable variants of the FHA1 domain were generated.
4. FHA1 residues important for interaction with the pT peptide were identified.
5. Anti-phosphospecific reagents were isolated by screening FHA1 libraries.

Figure 1. ELISA for detecting interaction between the FHA1 domain and the immobilized pT peptide ligand

(a) Phage ELISA demonstrates that the WT FHA1 domain and two of its variants, 3C-3S and 4C-4S, are non-functional and do not bind to the cognate pT peptide (Rad9-pT), when displayed on the surface of bacteriophage M13 as protein III fusions, whereas the D2 variant is functionally active when phage displayed. Binding of phage particles was detected using anti-M13 antibody conjugated to HRP. (b) GST fusions of the WT FHA1 domain and three of its variants, 3C-3S, 4C-4S, and D2, bind specifically to the cognate pT peptide (Rad9-pT; shown as solid histograms) immobilized on plastic microtiter plate wells via Neutravidin. Binding was detected using anti-GST antibody conjugated to HRP. (c) The structures of the WT FHA1 domain¹⁷ (PDB code: 1G6G) and three variants- 4C-4S, 3C-3S and D2 prepared using PYMOL (<http://www.pymol.org/>) are shown as a cartoon representation with the cysteine (C), serine (S), and phenylalanine (F) residues shown as red, pink and green spheres respectively. The β -strands are labeled from 1 to 11 in the WT FHA1 domain structure and the N- and C- termini are noted.

Figure 2. Hydrophobic residues are preferred at position 34 for activity of phage-displayed FHA1 variants

Through Kunkel mutagenesis⁶, position 34 in the β 1 strand of the D2 FHA1 domain was replaced one at a time with each amino acid and binding to the immobilized Rad9-pT peptide was monitored by phage ELISA. Phage binding was detected using anti-M13 antibody conjugated to HRP. The phage-displayed FHA1 variants that contain a hydrophobic residue at position 34 (with exceptions of W, C, and P) retain binding to the Rad9-pT. Presence of polar or hydrophilic amino acids at this position fails to rescue binding of the phage to the Rad9-pT peptide. The binding signal for all the variants was normalized for Flag epitope display.

Figure 3. Thermal stability of bacteriophage particles displaying the D2 variant

Phage particles displaying the D2 variant were heated at various temperatures (30°C, 37°C, 50°C, 60°C, 70°C, 80°C and 95°C) for 3 h and allowed to cool to room temperature. The treated phage particles were then incubated with the cognate pT peptide (Rad-pT; squares) and the negative control peptide (Rad9-T; circles) immobilized on plastic via neutravidin. Phage binding was detected using an anti-M13 antibody conjugated to HRP.

Figure 4. Determining the T_m of FHA1 variants by FTS Assay

The FHA1 domain variant was mixed with SYPRO orange dye, and heated from 25°C to 95°C on an Mx3000P Real-time thermal cycler instrument. When the protein domain is in its native conformation, the SYPRO orange dye signal is quenched in the aqueous environment, but when the dye's fluorescence increases upon exposure to the protein's hydrophobic core⁸. The melting curve for the G2 variant (without any peptide) is shown as boxes. The shift in T_m in the presence of 50 μ M and 250 μ M of the pT peptide are shown as diamonds and circles, respectively.

Figure 5. Determining the K_d in solution by ITC

A fixed amount (10 μL) of the pT peptide (SLEVpTEADATFYAKK; 500 μM) was injected from the syringe into the sample cell containing the FHA1 domain (35 μM). The K_d values of the (a) WT FHA1 domain, (b) D2 variant and (c) G2 variant, were determined to be $1 \pm 0.17 \mu\text{M}$, $0.97 \pm 0.15 \mu\text{M}$ and $0.89 \pm 0.1 \mu\text{M}$, respectively. A stoichiometry of 1:1 was observed for all the three FHA1 domains with their pT peptide complexes. Each measurement was repeated three times.

Figure 6. Alanine-scanning of residues from three loops in the FHA1 domain

Residues 78–88 in the β 4- β 5 loop, 104–108 in the β 6- β 7 loop, and 133–139 in the β 10- β 11 loop were mutated to alanine one at a time and the mutants were tested for binding to the Rad9-pT peptide by phage ELISA. Binding was detected using an anti-M13 antibody conjugated to HRP. Phage ELISA for (a) β 4- β 5 loop alanine-scan mutants, (b) β 6- β 7 loop alanine-scan mutants, and (c) β 10- β 11 loop alanine-scan mutants. (d) Residues from the β 4- β 5 loop (green spheres), β 6- β 7 loop (orange spheres), and β 10- β 11 loop (red spheres) which are important for interaction with the Rad9-pT peptide, but not for folding of the FHA1 domain, are shown. Binding of all the variants was normalized for the display of the Flag epitope. The binding signal of the G2 mutant for the Rad9-pT peptide was taken as 100% and the percentage binding of all the twenty-four alanine-scan mutants was calculated accordingly.

Figure 7. FHA1 variants with novel specificities

Phage-ELISA was performed after two rounds of affinity selection against five different pT-containing peptides. The peptides screened are from a) Transcription factor jun-B, b) Activating transcription factor 2 (ATF2) c) Mitogen-activated protein kinase 3 (MAPK3), d) Transcription factor jun-D, and e) Mitogen-activated protein kinase 1 (MAPK1). Binding to the cognate pT peptide (black histograms), the non-phosphorylated form of the same peptide (grey histograms) and the original pT peptide from yeast Rad9 protein (white histograms) are included in the five panels. Error bars represent standard deviation of duplicate measurements.

Figure 8. Characterizing the specificity of an anti-MAPK3 affinity reagent, and determining the peptide residues important for interaction

a) The binding specificity of an anti-MAPK3 affinity reagent (B1) was monitored with 11 different phosphopeptide sequences and block (2% skim milk in 1X PBS), along with its cognate pT peptide (MAPK3-pT) and the non-phosphorylated form of the same peptide (MAPK3-T). b) Binding of the anti-MAPK3 affinity reagent (B1) to its cognate MAPK3-pT peptide is set to 100% and the various alanine-scanned peptide variants are compared to it.