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A comparison of phosphospecific affinity reagents reveals the utility of recombinant Forkhead-associated domains in recognizing phosphothreonine-containing peptides

Research Paper

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Phosphorylation is an important post-translational event that has a wide array of functional consequences. With advances in the ability of various technologies in revealing and mapping new phosphosites in proteins, it is equally important to develop affinity reagents that can monitor such post-translational modifications in eukaryotic cells. While monoclonal and polyclonal antibodies have been shown to be useful in assessing the phosphoproteome, we have expanded our efforts to exploit the Forkhead-associated 1 (FHA1) domain as scaffold for generating recombinant affinity reagents that recognize phosphothreonine-containing peptides. A phage display library of FHA1 variants was screened by affinity selection with 15 phosphothreonine-containing peptides corresponding to various human transcription factors and kinases, including human Myc, calmodulin-dependent protein kinase II (CaMKII), and extracellular-signal regulated kinases 1 and 2 (ERK1/2). The library yielded binding variants against 10 targets (66% success rate); success was largely determined by what residue occurred at the +3 position (C-terminal) to the pThr moiety (i.e., pT+3). The FHA domains binding Myc, CaMKII, and ERK1/2 were characterized and compared against commercially available antibodies. All FHA domains were shown to be phosphorylation-dependent and phosphothreonine-specific in their binding, unlike several commercial monoclonal and polyclonal antibodies. Both the pThr and the residue at the pT+3 position were major factors in defining the specificity of the FHA domains.

Introduction

Protein phosphorylation is an important post-translational modification that principally occurs on serine (89%), threonine (10%), and tyrosine (<1%) residues [1–4]. With over 100,000 phosphosites reported to date [5], there is a tremendous need for highly sensitive and specific probes to monitor the phosphorylation of particular residues in proteins during cell growth, differentiation, and disease [6]. One such class of reagents are antibodies, which can be generated by immunizing animals with phosphopeptides; such antibodies have allowed the identification of physiologically important phosphosites, changes in phosphorylation states, and subcellular translocation of particular proteins upon phosphorylation [7–10].

While monoclonal and polyclonal antibodies have been historically invaluable to the field of eukaryotic cell signaling, drawbacks include production cost, renewability [11], and limited control over specificity, which can result in cross-reactive reagents [12–16]. One strategy to overcome these limitations is to use recombinant affinity reagents, as they eliminate the need for animals, there is more control in epitope recognition, they are sequenced and renewable reagents and they are amenable to protein engineering [17,18]. To this extent, several engineered phosphate-binding domains, such as the Src Homology 2 (SH2) domain [19], a recombinant phosphospecific antibody fragment [20], the 10th fibronectin type III domain (10FnIII) [21], and the Forkhead-associated 1 (FHA) domain [22], have all been used successfully for generating recombinant affinity reagents to phosphopeptides.

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A major advantage of the FHA domain, compared to other engineered scaffolds, is its natural ability to recognize a phosphothreonine (pThr, pT) residue in a post-translationally modified protein [23]. Within the FHA domain, there is a pocket that interacts with the γ-methyl group and phosphate of pThr, which allows the domain to discriminate between phosphoserine (pSer) and pThr [24]. Utilizing the domain's natural ability to discriminate between pSer and pThr, the specificity of one particular FHA domain, the FHA1 domain of yeast Rad53 protein, was reengineered through phage display [22]. In this report, we demonstrate that the engineered FHA domains are exquisitely selective in binding pThr-, and not pSer- or phosphotyrosine (pTyr)-containing peptides, unlike several polyclonal and monoclonal antibodies tested. Furthermore, we also show that our library is capable of producing a variant that recognizes a doubly-phosphorylated peptide. In this regard, the FHA domain offers great promise in generating highly specific pThr-binding reagents, a feat not readily achievable through traditional immunological means.

Materials and methods

Reagents

Peptides were synthesized at University of Illinois at Chicago's Research Resource Center, with >90% purity. All peptides were biotinylated at their N-terminus and amidated at their C-terminus, and included lysine and tyrosine residues to increase peptide solubility and for measuring absorbance, respectively. The cognate targets for the Myc, ERK1/2, and CaMKII FHA domain affinity reagents are FELLPpTPPLSPS (Myc-pT58), HTGFLpTEpYVATRW (ERK1-pT202/pY204 + ERK2-pT185/pY187), and LKGAILpTTMLATRN (CaMKII-pT305), respectively. The following peptides were used in a pThr substitution study: FELLPpTPPLSPS (pT58), FELLPpSPPLSPS (pT58pS), FELLPpYPPLSPS (pT58pY), FELLPTPPLSPS (T58), HTGFLpTEpYVATRW (pT202), HTGFLpSEpYVATRW (pT202pS), HTGFLpYEpYVATRW (pT202pY), HTGFLTEYVATRW (T202), LKGAILpTTMLATRN (pT305), LKGAILpSTMLATRN (pT305pS), LKGAILpYTMLATRN (pT305pY), LKGAILTTMLATRN (T305).

Three commercial anti-phosphopeptide antibodies were compared to the recombinant FHA domains generated in this report. Two were polyclonal antibodies (pAb), pAbαMyc (Abnova, catalog# PAB0541) and pAbαCaMKII (Thermo Scientific, catalog# PA5-35521), and one was a monoclonal antibody (mAb) mAbαERK1-pT202/pY204 + ERK2-pT185/pY187 (mAbαERK1/2) (Abcam, catalog# ab136926). As all three are rabbit antibodies, a goat anti-rabbit immunoglobulin G (IgG), conjugated to Horseradish peroxidase (HRP; Abcam, catalog# ab97051), served as the common secondary reagent. Another secondary reagent was the anti-Flag epitope mAb, M2, which was conjugated to HRP (Sigma–Aldrich, catalog# A8592).

DNA constructs

The coding sequences for individual FHA domains were amplified from virions by the polymerase chain reaction (PCR). The double-stranded DNA product was digested with *Nco* I and *Not* I restriction endonucleases and subcloned into the pET29b expression vector. These constructs included a 3XFlag®-tag sequence (DYKDHDGDYKDHDIDYKDDDDK), followed by a His_6-tag, at the C-terminus of the fusion proteins. All constructs were verified by DNA sequencing.

Protein purification

Overexpression of the constructs and their purification was carried out using standard methods [25]. Briefly, BL21DE3 cells containing the expression vector was grown at 30°C for 24 hours using the Overnight Express™ Autoinduction System 1 (Novagen). Bacterial cells were lysed using a Sonic Dismembrator (Branson Model 500). The lysate was mixed with Clontech His-60 Ni Superflow resin (Clontech Laboratories), and the His_6-tagged proteins eluted with 50 mM sodium phosphate, 300 mM sodium chloride, 250 mM imidazole (pH 8.0).

Enzyme-linked immunosorbent assays (ELISA)

ELISAs were performed using an established protocol [25], except that non-specific binding in microtiter plate wells was blocked with 1% casein in phosphate buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4). The absorbance was read at 405 nm wavelength in 10-min intervals, for a total of 40 min. All experiments were performed in triplicate, and repeated at least three times to confirm reproducibility of the data.

Results and discussion

Production of FHA domains by recombinant phage display

Phage display is a powerful technique that allows for the rapid and efficient production of affinity reagents, such as antibodies [26], without the need to immunize animals [27]. To generate recombinant affinity reagents that are phosphothreonine-specific, a phage display library was constructed by randomizing residues in the β4-β5 and β10-β11 loop regions of a thermostable variant (FHA1G2) of the FHA1 domain of the yeast Rad53 protein [22,28] (Fig. 1a). The library was incubated separately with a variety of phosphothreonine-containing peptides, which were chosen based on the physiological importance of the pThr residue in a eukaryotic signaling pathway, and included protein kinases and transcription factors. After three rounds of affinity selection, individual clones were tested by an enzyme-linked immunosorbent assay (ELISA), and unique clones were identified by DNA sequencing (Fig. 1d). With biotinylated, phosphorylated forms of the peptides as targets, we were able to produce recombinant affinity reagents in less than two weeks for 10 out of 15 peptide attempted, reflecting a 66% success rate (Table 1).

Biochemical and structural studies [29] have revealed that a major determinant of specificity for FHA domains is the +3 position (C-terminal) to the pThr moiety. To date, FHA domains can be categorized into three groups based on their recognition of the pT+3 position – pTxxD, pTxx(I/L), and pTxx(A/S) – with the yeast Rad53 protein FHA1 domain falling into the first category. We also confirmed (see below) this position to be important for binding to our FHA domains. As seen in Table 1, we isolated FHA domain variants to peptides with D, L, V, P, S, and W, in the +3 position. We have yet to test phosphothreonine-containing peptides with A, C, Q, E, H, M, F, N, T, and Y at the +3 position.

The five peptides that failed to yield binders included pThr-containing phosphopeptides corresponding to nucleolin (NCL), histone H1, polo-like kinase 1 (PLK1), mitogen-activated protein kinase kinase 2 (MAP2K2), and isoform 1 of epidermal growth factor receptor precursor (EGFR). The inability to isolate FHA1 domains that bound to these particular phosphopeptides was reproducible; their sequences either contained K, R, and G at

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